American Institute of Integrative Oncology
RESEARCH & EDUCATION

Foundations of Integrative Oncology
COURSE

GRAND ROUNDS CALL
With Dr. Nalini Chilkov
July 10th, 2019
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

Agenda

- Clinical Pearl:
  - Why Liquid Biopsies? When To Assay Circulating Tumor Cells and Cell Free Tumor DNA
- Clinical Questions:
  - Does Resveratrol Fuel her2neu breast cancer?
    - Review of Resveratrol and Cancer
  - How to safely manage hypercoagulation and risk of thrombus formation
    - Interactions with anti coagulant pharmaceuticals
    - Nutriceutical and Botanical Interventions
    - Basic Biomarkers for Monitoring
    - Is there lecture material available on this topic?
  - Should Girls Be Getting the HPV Vaccine?
    - Overview of HPV infections
    - Overview of 10 years of vaccination in first world countries vs no vaccination in 3rd world countries
    - Naturopathic Approach to HPV infection
    - Melatonin Enhances HPV vaccine efficacy in mice
  - Research
    - The Importance of assessing PERCEIVED STRESS in cancer patients

Clinical Pearl: **Why Liquid Biopsies? When to Assay Circulating Tumor Cells and Cell Free Tumor DNA**
Slides and References are found in Resource Library

Case Study:
Submitted by: No Case Study Submitted
Overview:
Core Questions:
Recommendations:

Questions & Answers

Jaclyn Tolentino, D.O.: **Looking for a better understanding, your reflections and opinion on the article *Resveratrol fuels HER2 and ERα-positive breast cancer behaving as proteasome inhibitor* (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5361678/) 2016**

IN VIVO *MURINE and IN VITRO CELL CULTURE*
Our results demonstrate that resveratrol, acting as a proteasome inhibitor, leads to Δ16HER2 accumulation which favors the formation of Δ16HER2/HER3 heterodimers. The consequential activation of downstream mTORC1/p70S6K/4EBP1 pathway triggers cancer growth and proliferation. This study provides evidence that resveratrol mechanism of action (and hence its effects) depends on the intrinsic molecular properties of the cancer model under investigation, exerting a tumor-promoting effect in luminal B breast cancer subtype models.

***Curr Protein Pept Sci.* 2018;19(3):311-322. doi: 10.2174/1389203718666170111115914.**

*******"might resveratrol be protective against breast cancer or does it rather fuel it?".******

***Walking a Tightrope: A Perspective of Resveratrol Effects on Breast Cancer.* Bartolacci C1, Andreani C1, Amici A1, Marchini C1.**

Abstract

It is an acknowledged fact that health benefits are derived from fruit- and vegetables-enriched diets. In particular, polyphenols, compounds bearing one or more hydroxyl groups attached to an aromatic ring, are ascribed for most of such beneficial effects. Among them, resveratrol, a phytoalexin found in numerous plant species, and more notably in grapes, has widely piqued the interest of the scientific community by virtue of its anti-aging, anti-inflammatory and anti-oxidant properties. Moreover, evidence claiming resveratrol ability to hinder processes underlying all the three steps of carcinogenesis (tumor initiation, progression and metastasization) has propelled an incredibly massive number of studies aimed at enquiring its eventual clinical potential in the fight against cancer. However, despite a large body of data pointing to the advantages of dietary resveratrol intake in respect of certain disease conditions, and cancer inter alia, its real position still remains quite ambiguous. In this uncertain scenario, the present review focuses its attention on the highly entangled relationship between resveratrol and breast cancer, attempting to shape the plethora of controversial results stemming from studies carried out on several in vitro and in vivo breast cancer models. Coping with such a tricky matter, there are so many variabilities concerning both resveratrol

itself (dosage, administration, bioavailabilty, among others) and the unique molecular traits of each specific breast cancer subtype that must be taken into account when facing the dilemma: "might resveratrol be protective against breast cancer or does it rather fuel it?".

Breast Cancer Res Treat. ***2013 Aug;141(1):55-65.***

Immunoliposome encapsulation increases cytotoxic activity and selectivity of curcumin and resveratrol against HER2 overexpressing human breast cancer cells.

Catania A1, Barrajón-Catalán E, Nicolosi S, Cicirata F, Micol V.

Abstract

Natural compounds have been studied as a source of countless bioactive compounds with diverse activities. Among them, many dietary phytochemicals have been thoroughly studied for their cytotoxic or apoptotic effects in several cellular models in order to explain their anticancer capacity. Curcumin and resveratrol are two natural compounds with a large body of evidence showing their cytotoxic activity against a wide variety of cancer cells; however, their poor absorption, bioavailability, and low selectivity have limited their clinical use. With the aim of improving bioavailability and selectivity, the antiproliferative effects of free-, liposomed-, and immunoliposomed-curcumin and/or resveratrol formulations have been compared in two human breast cancer cell lines with different HER2 expression levels. **The results demonstrate that when HER2-targeted immunoliposomes are coupled to trastuzumab there is a dramatic increase in the antiproliferative effects of curcumin and resveratrol in HER2 positive human breast cancer cells in comparison to regular liposomed or free forms, indicating an increase of its therapeutic effect.** The enhancement of the cytotoxic effects was also correlated to the uptake of curcumin at intracellular level, as shown by using ImageStream technique. The striking efficacy of the immunoliposomed formulation containing both resveratrol and curcumin suggests a multitargeted mechanism of action that deserves further study. These findings show the potential of HER2-targeted nanovesicles to develop new drug delivery systems for cancer therapy based on these compounds and justify further preclinical trials. ***PMID: 23959397***

DOI 10.1007/s10549-013-2667-y

Cell Death Discov. 2015; 1: 15061.

Published online 2015 Dec 7. doi: 10.1038/cddiscovery.2015.61

Resveratrol chemosensitizes HER-2-overexpressing breast cancer cells to docetaxel chemoresistance by inhibiting docetaxel-mediated activation of HER-2–Akt axis

B S Vinod,1,2 H H Nair,1,2 V Vijayakurup,1,2 A Shabna,1 S Shah,1 A Krishna,1 K S Pillai,1 S Thankachan,1 and R J Anto1,*

Molecules. 2017 Jun; 22(6): 1014.

Published online 2017 Jun 18. doi: 10.3390/molecules22061014 PMID: 28629161

Cancer Chemoprevention by Resveratrol: The p53 Tumor Suppressor Protein as a Promising Molecular Target

Danielly C. Ferraz da Costa,1 Eliane Fialho,2,* and Jerson L. Silva3,*

Norbert Latruffe, Academic Editor, Ole Vang, Academic Editor, and Dominique Vervandier-Fasseur, Academic Editor

Resveratrol regulates a variety of processes and signaling pathways that involves procarcinogen bioactivation and carcinogen detoxification [11,12]; reduction of oxidative stress [13] and inflammation [14]; apoptosis induction through the activation of both extrinsic and intrinsic pathways [15,16,17,18,19,20]; and other anticancer effects [21,22]. Resveratrol affects the three phases of carcinogenesis, tumor initiation, promotion and progression, and it also suppresses the final steps of carcinogenesis, e.g., angiogenesis and metastasis [11,23]. Resveratrol also impacts mitochondrial functions (the respiratory chain, oncoproteins, gene expression, among others), including those directly involving the p53 protein tumor suppressor protein [24]. Finally, in various cancer types, resveratrol behaves as a chemosensitizer that lowers the threshold of cell death induction by classical anticancer agents and counteracts tumor cell chemoresistance [25,26].

In vitro and in vivo studies have shown that resveratrol can act as a tumor-initiation suppressor by modulating phase I and phase II cytochrome P450 enzymes (CYPs) [5,12,27,28,29,30]. Resveratrol blocks the transcriptional activation of CYPs and inhibits the activity of CYP1A1, CYP1B1 and CYP1A2, which are phase I enzymes responsible for activating xenobiotics, thus halting the transformation of procarcinogen

agents into potential carcinogens [12,27,28]. Additionally, resveratrol enhances the expression and/or activity of phase II enzymes, including glutathione peroxidase, glutathione S-transferase, UDP-glucuronyltransferase, NAD(P)H:quinone oxidoreductase, and heme oxygenase, among others [5,29,31], subsequently stimulating carcinogen detoxification processes.

The role of resveratrol in the intracellular redox status has been described in many studies. Like other polyphenols, this microcomponent acts as an important cellular antioxidant, and its effects are dependent on the concentration tested and the cell type used. However, it has been proposed that resveratrol may also act as a pro-oxidant agent, representing one of the antineoplastic mechanisms of action involved in tumor cell death. Likewise, resveratrol has been shown to decrease the mitochondrial membrane potential and increase reactive oxygen species (ROS) generation, thus promoting apoptosis [32,33].

Some effects of resveratrol have been attributed to its estrogenic activity resulting from its structural similarity to diethylstilbestrol, a synthetic estrogen. Resveratrol can bind to estrogen receptors and may function as an agonist or antagonist, leading to opposite responses depending on the concentration, competition and expression of estrogen receptors in the cells. In MCF-7 human breast cancer cells, for example, which express these receptors, resveratrol may act as a superagonist [34].

Resveratrol is recognized as a potent tumor growth inhibitor in diverse experimental models. Previous studies have established its anticancer effects in a variety of cultured tumor cell lines, including colon [35,36,37], breast [19,38,39], prostate [40,41], pancreatic [42,43,44], lung [19,45,46], melanoma [47,48], glioma [49,50], and leukemia cells [51,52], among others. It has been suggested that the anti-proliferative properties of this microcomponent are related to its capacity to block DNA synthesis and to interfere with various stages of cell progression by regulating the machinery of proteins involved in cell cycle control. Hsieh and colleagues [53] first reported the ability of resveratrol to control growth and cell cycle transitions in human breast carcinoma cell lines with different metastatic potentials: highly invasive MDA-MB-435 and the minimally invasive MCF-7 cells. Resveratrol exerts a greater inhibitory effect on MDA-MB-435 cells, in which a reduction of the fraction of cells in the G1 phase and a corresponding accumulation of cells in S phase was observed. Since then, many other studies have reported that resveratrol at micromolar concentrations arrests the cell cycle of a variety of human cancer cell lines at the G1/S transition, in S phase or in the G2/M phase [5,54,55]. Although several studies have found that the induction of cell cycle arrest by resveratrol is a reversible process and does not trigger apoptosis, many other authors have reported that this process is often followed by apoptotic cell death [56].

Investigations regarding the effects of resveratrol on apoptosis induction in tumor cells have revealed that this compound can stimulate cell death by modulating proteins involved in both the intrinsic and extrinsic apoptotic pathways [56]. Numerous lines of evidence suggest that resveratrol affects the activation of pro-apoptotic and the inhibition of anti-apoptotic molecules. These mechanisms have been described in culture cells and in vivo in tumor models that have been chemically induced in animals and xenographic models in nude mice [55]. Studies suggest that resveratrol-mediated apoptosis includes the activation of death receptors such as Fas and TRAIL; the activation of Bax, Bak, PUMA, Noxa and Bim; and the inhibition of Bcl-2 and Bcl-XL. Some of these responses are mediated by the activation of the p53 tumor suppressor protein by resveratrol, as will be later discussed.

In addition, resveratrol demonstrates important anti-angiogenic effects, thus contributing to the reduction of the metastatic potential of tumor cells. The following mechanisms are involved in this process: the inhibition of extracellular matrix metalloproteinases gene expression, such as MMP-2 and MMP-9, which are involved in tumor invasiveness; and the inhibition of the expression of HIF-1α and VEGF, factors that are directly related to new blood vessel formation [54,55].

Although some of the anti-carcinogenic effects of resveratrol have already been shown to be triggered by the activation of extracellular receptors [57], there is evidence that its internalization by cells is necessary for the activation of some specific intracellular targets. It has been demonstrated by multiphoton microscopy

(two-photon excitation) that resveratrol, but not its glycosylated or sulfated metabolites, is efficiently captured by neuroblastoma cells, allowing this molecule to exert its antitumor effects, unlike metabolites [58]. Additionally, the importance of cell membrane microdomains in the early biochemical events triggered by resveratrol resulting in cancer cell death, as well as in its absorption and distribution, has been previously described. Resveratrol accumulates in lipid rafts and is then taken up by cells through raft-dependent endocytosis. These events allow for the activation of kinase pathways and redistribution of cell death receptors within lipid microdomains, ultimately leading to apoptotic cell death [17].

Int J Mol Sci. 2018 Mar; 19(3): 652.

Published online 2018 Feb 26. doi: 10.3390/ijms19030652

PMCID: PMC5877513

PMID: 29495357

Regulation of Cell Signaling Pathways and miRNAs by Resveratrol in Different Cancers

Ammad Ahmad Farooqi,1 Sumbul Khalid,2 and Aamir Ahmad3,*

Resveratrol has been shown to modulate key regulators of oncogenic signaling pathways. It strongly inhibits the TGF/SMAD pathway and reduces phosphorylated SMADs, resulting in the inhibition of cancer cell proliferation and metastasis. Resveratrol has shown experimentally verified activity against WNT signaling and can inhibit the translocation of β-catenin into the nucleus. Resveratrol, context-dependently, regulates the NOTCH pathway in cancer cells, all of which underlines its flexible and potent anticancer activity.

Reduced apoptosis is a hallmark of aggressive cancer cells, and resveratrol re-balances pro- and anti-apoptotic proteins to improve the efficacy of TRAIL-based therapeutics.

Resveratrol has been shown to efficiently upregulate various tumor suppressor miRNAs in different cancers, and its suppressive effects on oncogenic miRNAs have also been well documented.

***Dr. Chilkov*:**
Few human studies. Most studies are murine or cell culture.

Susie Thomson:

- **Please provide guidelines for a patient who is currently on anticoagulant medication who would like to take nutraceuticals and Phytochemicals as well.**
  - *More information is required including the age and medical hx of the patient, CVD history, Oncology hx, Diabetes?, Obesity? Levels of Inflammation?*
  - *Multiple factors can be co-morbid for risk of thrombus formation*
    Name of anticoagulant medication is not given
    Other medications patient is taking is not given

PLEASE SUBMIT A FORMAL CASE STUDY NEXT MONTH --Our approach is HIGHLY INDIVIDUALIZED

- **Please address interactions and safety guidelines for Tumeric, resveratrol, omega 3 FA.**
  - *Most oral Rx anticoagulants are targeting larger fibrin clots. While phytochemical and nutriceuticals primarily inhibit platelet aggregation, a different mechanism and influencing smaller clot formation in capillaries not major blood vessels. There is generally not a lot of adverse interactions at conservative dosing (2 grams per day or less) Our non-pharmaceutical interventions are mild compared to pharmaceuticals*
- **Is this information within the course materials?**
  - *Yes there is a Clinical Pearl 06-14-2017*

Dr. Chilkov

Increased Risk of Hypercoagulation in Cancer Patients

Cancer patients overall have a 7 fold higher risk of venous thrombosis
Patients with hematologic malignancies have a 28 fold higher risk of venous thrombosis
Patients with distant metastases have a 20 fold higher risk of venous thrombosis

"the large proportion of terminal events in neoplastic diseases are thrombotic"

Risk Factors Include:
- ***Age, Race, Gender***
- ***Advanced Disease, Angiogenesis and Metastasis***
- ***Recent Diagnosis or Recent Recurrence***
- ***Surgery, Chemotherapy, Radiotherapy, Hormonal and Biological Therapies***
- ***Immobility, Sedentary, Dehydration***

Primary BioMarkers
- ***CBC + platelets + diff***
- ***D-dimer < 0.4***
- ***Fibrinogen < 375 (ideal 275-300)***
- ***HS CRP < 1.0***
- ***Homocysteine < 8.0***
- ***TGFb1 (varies)***

Selected Interventions
Proteolytic Enzymes
- ***Lumbrokinase & Nattokinase***

PhytoChemicals & Botanicals
- ***Curucminoids from Curcuma longa,***
- ***Resveratrol from Vitis vin. and Polygonum cuspidatum,***
- ***Berberine from Scutellaria baicalensis,***
- ***Salvianolic Acids, Tanshinones, Cryptotanshinones from Salivia miltiorrhiza,***
- ***Ginsenosides from Panax Ginseng, ,***
- ***Polysaccharides from Angelica sinensis and Astragalus,***
- ***Quercitin from red onions and red apples***

Nutraceuticals
- ***Omega 3 Fatty Acids, Ascorbic Acid. Nicotinic Acid,***

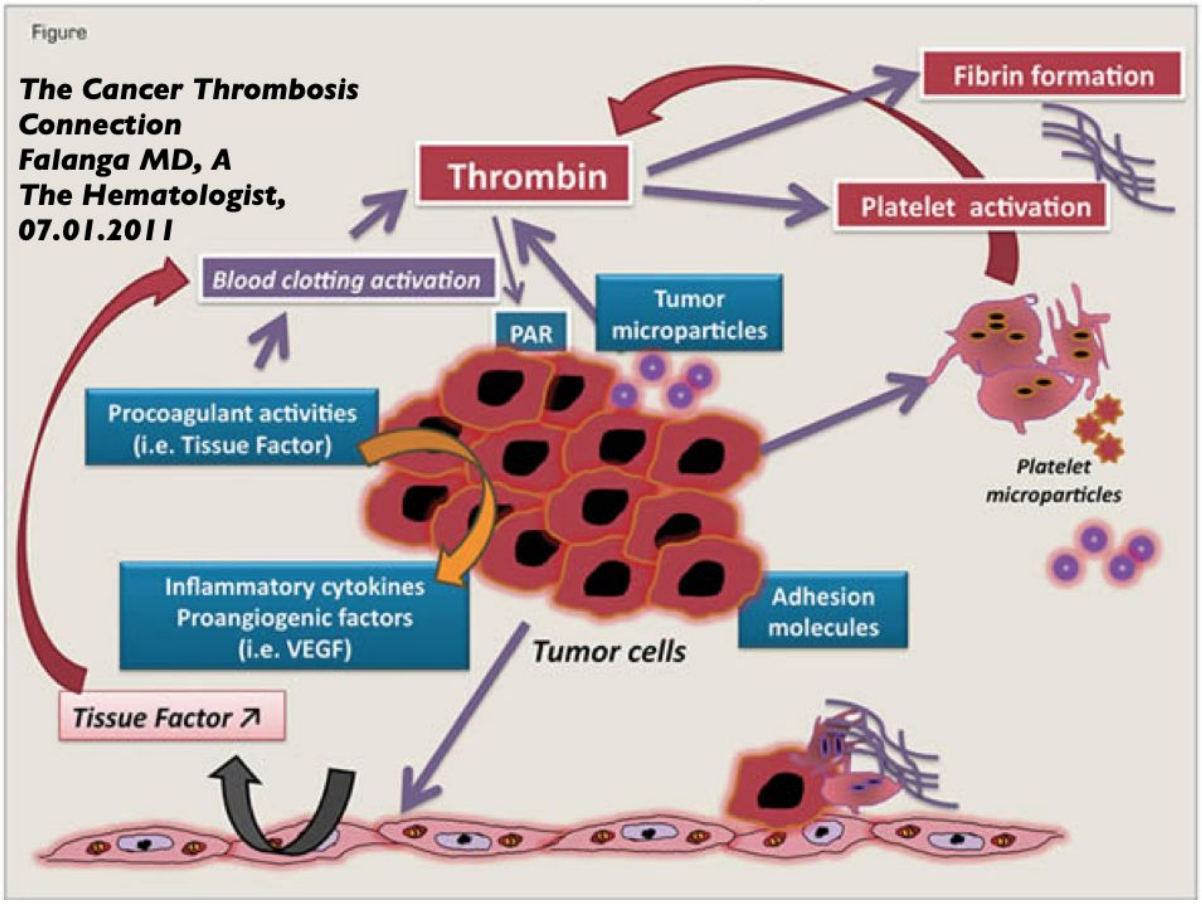
Pharmaceuticals
- ***ORAL: ASA, Apixaban (Eliquis), Dabigatran etexilate (Pradaxa)***
- ***Low Molecular Weight Heparin (injection)***

Anti-Inflammatory Diet
Lifestyle Factors: Hydration, Movement

Treatment Goals
MONITOR COAGULATION FACTORS and BIOMARKERS
PREVENT THROMBOTIC EVENT

- ***Inhibit Tissue Factor***
- ***Inhibit Platelet Aggregation, Thrombin and Fibrin Formation***
- ***Promote Fibrinolysis***
- ***Inhibit Angiogenesis and Metastasis***
- ***Manage Inflammation and Oxidative Stress***
- ***Manage CoMorbidities***

Mechanisms of Hemostasis by Tumor Cells



Susie Thomson: **Should girls be getting the HPV vaccine?**

Dr. Chilkov
Are vaccines inherently good or bad?.....
What about cancer vaccines?

Clin Infect Dis. 2016 Aug 15; 63(4): 519–527.

Published online 2016 May 26. doi: 10.1093/cid/ciw354

PMCID: PMC4967609
PMID: 27230391

REVIEW

Impact and Effectiveness of the Quadrivalent Human Papillomavirus Vaccine: A Systematic Review of 10 Years of Real-world Experience

Suzanne M. Garland,1 Susanne K. Kjaer,2 Nubia Muñoz,3 Stan L. Block,4 Darron R. Brown,5 Mark J. DiNubile,6 Brianna R. Lindsay,6 Barbara J. Kuter,6 Gonzalo Perez,6,7 Geraldine Dominiak-Felden,8 Alfred J. Saah,6 Rosybel Drury,8 Rituparna Das,6 and Christine Velicer

Prophylactic human papillomavirus (HPV) vaccination programs constitute major public health initiatives worldwide. We assessed the global effect of quadrivalent HPV (4vHPV) vaccination on HPV infection and disease. PubMed and Embase were systematically searched for peer-reviewed articles from January 2007 through February 2016 to identify observational studies reporting the impact or effectiveness of 4vHPV vaccination on infection, anogenital warts, and cervical cancer or precancerous lesions. **Over the last decade, the impact of HPV vaccination in real-world settings has become increasingly evident, especially among girls vaccinated before HPV exposure in countries with high vaccine uptake. Maximal reductions of approximately 90% for HPV 6/11/16/18 infection, approximately 90% for genital warts, approximately 45% for low-grade cytological cervical abnormalities, and approximately 85% for high-grade histologically proven cervical abnormalities have been reported. The full public health potential of HPV vaccination is not yet realized. HPV-related disease remains a significant source of morbidity and mortality in developing and developed nations, underscoring the need for HPV vaccination programs with high population coverage.**

The review and meta-analysis included data from 6**5 articles from 14 high-income countries (most of which had girls/women-only programs)**, encompassing follow-up **data from more than 60 million individuals over 8 years.**

During that time, the prevalence of HPV 16 and 18 decreased by 83% among girls age 13 to 19 years old, and by 66% among women age 20 to 24 years. In addition, cross protection occurred, with the prevalence of HPV 31, 33, and 45 decreasing by 54% among girls age 13 to 19 years.
There was also evidence of herd protection from the vaccine. Specifically, anogenital warts decreased by 67% among girls age 15 to 19 and by 54% among women age 20 to 24, but also by 31% among women age 25 to 29, 48% among boys age 15 to 19, and 32% in men age 20 to 24.

“Our results provide strong evidence of HPV vaccination working to prevent cervical cancer in real-world settings, as both the cause (high-risk HPV infection) and proximal disease endpoint (CIN2+) are significantly declining,” wrote Mélanie Drolet, PhD, of Laval University in Quebec, and colleagues from the HPV Vaccination Impact Study Group. “In terms of global policy implications, these results reinforce the recently revised position of WHO recommending HPV vaccination of multiple age cohorts of girls, and provide **promising early signs that the WHO call for action on cervical cancer elimination might be possible if sufficient population-level vaccination coverage can be reached.”**
https://www.cancernetwork.com/cervical-cancer/meta-analysis-shows-importance-hpv-vaccines

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Front Oncol. 2019; 9: 355.

Published online 2019 May 8. doi: 10.3389/fonc.2019.00355 PMCID: PMC6517478 PMID: 31134154

Mucosal and Cutaneous Human Papillomavirus Infections and Cancer Biology

Tarik Gheit*

Mucosal HPV infections occur during the first sexual exposures in early adulthood (30), although non-sexual infection may also be possible (31).

Although a majority of sexually active women will acquire a genital HPV infection, most (>90%) cervical HPV infections are resolved by the host immune system within 1–2 years (32, 33) and give rise only to asymptomatic infections.

However, a minority of HPV infections become persistent. The risk of developing epithelial cell abnormalities and cancer is then increased (32).

Persistent infection may be explained by several factors.

- Alcohol consumption and the HR HPV load synergistically increased the risk of persistent HR HPV infection in women (34).
- Cigarette smoking alone also plays an important role in the acquisition of persistent HPV infections in women, by decreasing the probability of clearing oncogenic infections (35). Smoking is also a risk factor for a persistent oral HPV infection (36).
- Host genetic risk factors may also predispose an individual to persistent HPV infection and developing cervical cancer, as supported by the high rate of heritability of cervical cancer (37–39).
- Defective immune response due to genetic variations (e.g., inflammasome genetics) has been associated with virus persistence and progression to cervical cancer (39–41).
- Human leukocyte antigen (HLA) genes also play a key role in the persistence of HPV infections and progression to cervical cancer, depending on their ability to bind to HPV antigens (39, 42).

The bacterial microbiome composition may also play a role in the outcome of mucosal HPV infection.

A chronic mycoplasma infection was shown to promote cervical dysplasia induced by HPV (302). Therefore, identifying the bacterial microbiome could allow the identification of patients at high risk for developing cervical cancer.

Prophylactic vaccines show a good efficiency in covering targeted HPVs, but they are inefficient in existing infections. Therefore, therapeutic vaccines are needed to fill this gap.

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Arch Virol. 2018 Mar;163(3):587-597. doi: 10.1007/s00705-017-3647-z. Epub 2017 Nov 17.

Enhancement of therapeutic DNA vaccine potency by melatonin through inhibiting VEGF expression and induction of antitumor immunity mediated by CD8+ T cells.

Baghban Rahimi S1, Mohebbi A1,2, Vakilzadeh G3,4, Biglari P2, Razeghi Jahromi S5, Mohebi SR6, Shirian S7, Gorji A4,8, Ghaemi A9,10.

Abstract

To be effective, therapeutic cancer vaccines should stimulate both an effective cell-mediated and a robust cytotoxic CD8+ T-cell response against human papillomavirus (HPV)-infected cells to treat the

pre-existing tumors and prevent potential future tumors. In this study, the therapeutic experiments were designed in order to evaluate antitumor effect against the syngeneic TC-1 tumor model. The anti-tumor efficacy of a **HPV-16** E7 DNA vaccine adjuvanted with melatonin (MLT) was evaluated in a C57BL/6 mouse tumor model by measuring tumor growth post vaccination and the survival rate of tumor-bearing mice, analyzing the specific lymphocyte proliferation responses in control and vaccinated mice by MTT assay. The E7-specific cytotoxic T cells (CTL) were analyzed by lymphocyte proliferation and lactate dehydrogenates (LDH) release assays. IFN-γ, IL-4 and TNF-α secretion in splenocyte cultures as well as vascular endothelial growth factor (VEGF) and IL-10 in the tumor microenvironment were assayed by ELISA. **Our results demonstrated that subcutaneous administration of C57BL/6 mice with a DNA vaccine adjuvanted with MLT dose-dependently and significantly induced strong HPV16 E7-specific CD8+ cytotoxicity and IFN-γ and TNF-α responses capable of reducing HPV-16 E7-expressing tumor volume. A significantly higher level of E7-specific T-cell proliferation was also found in the adjuvanted vaccine group.** Furthermore, **tumor growth was significantly inhibited when the DNA vaccine was combined with MLT and the survival time of TC-1 tumor bearing mice was also significantly prolonged. In vivo studies further demonstrated that MLT decreased the accumulation of IL-10 and VEGF in the tumor microenvironment of vaccinated mice. These data indicate that melatonin as an adjuvant augmented the cancer vaccine efficiency against HPV-associated tumors in a dose dependent manner.**

PMID: 29149434 DOI: 10.1007/s00705-017-3647-z

Naturopathic Approaches to HPV infection

CONSIDER

Indole 3 Carbinol (I3C), Di Indole Methane (DIM), EGCG, Curcumin, Resveratrol, Lomatium, L-MTHF, VItamin A (not beta carotene), Zinc, Homeopathic Thuja

Escharotic Topical Cervical Treatment

Vaginal Suppositories; VITAMIN A, EGCG, Lomatium

Wise Woman Herbals VAG-PAK Suppositories

Each Vag Pack suppository contains:

- Magnesium sulfate
- Iron subsulfate
- Hydrastis
- Vitamin A
- Tea Tree Essential Oil
- Bitter orange essential oil
- Thuja essential oil
- In a base of cocoa butter

Immune Enhancement, Inflammation Control, Lifestyle factors, Acupuncture

Dr Tori Hudson ND Oral Protocol

1500 mg coriolus versicolor (make sure it is hot water extract) 2x daily

5 mg LMTHF methylated folate

200 mg selenium

200 mg DIM

Reference

TriCurin, a synergistic formulation of curcumin, resveratrol, and epicatechin gallate, repolarizes tumor-associated macrophages and triggers an immune response to cause suppression of HPV+ tumors.

Mukherjee S, Hussaini R, White R, Atwi D, Fried A, Sampat S, Piao L, Pan Q, Banerjee P.

Cancer Immunol Immunother. 2018 May;67(5):761-774. doi: 10.1007/s00262-018-2130-3. Epub 2018 Feb 16.

PMID: 29453519

Research:

PERCEIVED STRESS SCALE:

Assessment. 2004 Sep; 11(3): 216–223.

doi: 10.1177/1073191104267398 PMCID: PMC2746492 NIHMSID: NIHMS122193 PMID: 15358877

Assessing Stress in Cancer Patients A Second-Order Factor Analysis Model for the Perceived Stress Scale

The Perceived Stress Scale was developed to measure the degree to which situations in one's life are appraised as stressful.

Psychological stress has been defined as the extent to which persons perceive (appraise) that their demands exceed their ability to cope.

Even though perceived stress may decline with time, data suggest that clinical interventions remain necessary and beneficial (see Andersen, 2002, for a discussion).

For example, if quality of life outcomes are to be improved, **the best time to offer psychological/behavioral interventions should be when stress is high—at the time of initial diagnosis and treatment**.

Despite any decline that occurs with time, it is the **magnitude of initial stress that predicts later quality of life outcomes (increased negative affect**: Varni & Katz, 1997; lower self-esteem/self-efficacy: Koopman et al., 2002; Varni, Katz, Colgrove, & Dolgin, 1994; and poorer physical health and sleep quality: Jacobsen et al., 1998).

The PSS can provide clinical information regarding the degree to which cancer patients appraise

their lives, in general, as stressful.

When used within the context of a stress model, the measure has the potential to identify the role of perceived stress in important cancer outcomes, such as patients' quality of life and adherence to treatment.

Cohen, S., Kamarck, T., Mermelstein, R. (1983). A global measure of perceived stress. Journal of Health and Social Behavior, 24, 385-396. Link to full-text (pdf)

Cohen, S., & Williamson, G. (1988). Perceived stress in a probability sample of the United States. In S. Spacapam & S. Oskamp (Eds.), The social psychology of health: Claremont Symposium on applied social psychology. Newbury Park, CA: Sage

References:

1. Andreani, C., Bartolacci, C., Wijnant, K., Crinelli, R., Bianchi, M., Magnani, M., . . . Marchini, C. (2017). **Resveratrol fuels HER2 and ERα-positive breast cancer behaving as proteasome inhibitor**. *Aging,9*(2), 508-523. doi:10.18632/aging.101175
2. Bartolacci, C., Andreani, C., Amici, A., & Marchini, C. (2018). **Walking a Tightrope: A Perspective of Resveratrol Effects on Breast Cancer**. *Current Protein & Peptide Science,19*(3). doi:10.2174/1389203718666170111115914
3. Catania, A., Barrajón-Catalán, E., Nicolosi, S., Cicirata, F., & Micol, V. (2013). **Immunoliposome encapsulation increases cytotoxic activity and selectivity of curcumin and resveratrol against HER2 overexpressing human breast cancer cells.** *Breast Cancer Research and Treatment,141*(1), 55-65. doi:10.1007/s10549-013-2667-y
4. Costa, D. F., Fialho, E., & Silva, J. (2017). **Cancer Chemoprevention by Resveratrol: The p53 Tumor Suppressor Protein as a Promising Molecular Target**. *Molecules,22*(6), 1014. doi:10.3390/molecules22061014
5. Farhan, M., Ullah, M., Faisal, M., Farooqi, A., Sabitaliyevich, U., Biersack, B., & Ahmad, A. (2019). **Differential Methylation and Acetylation as the Epigenetic Basis of Resveratrol's Anticancer Activity**. *Medicines,6*(1), 24. doi:10.3390/medicines6010024
6. Farooqi, A., Khalid, S., & Ahmad, A. (2018). **Regulation of Cell Signaling Pathways and miRNAs by Resveratrol in Different Cancers**. *International Journal of Molecular Sciences,19*(3), 652. doi:10.3390/ijms19030652
7. Garland, S. M., Kjaer, S. K., Muñoz, N., Block, S. L., Brown, D. R., Dinubile, M. J., . . . Velicer, C. (2016). **Impact and Effectiveness of the Quadrivalent Human Papillomavirus Vaccine: A Systematic Review of 10 Years of Real-world Experience**. *Clinical Infectious Diseases,63*(4), 519-527. doi:10.1093/cid/ciw354
8. Gheit, T. (2019). **Mucosal and Cutaneous Human Papillomavirus Infections and Cancer Biology.** *Frontiers in Oncology,9*. doi:10.3389/fonc.2019.00355
9. Golden-Kreutz, D. M., Browne, M. W., Frierson, G. M., & Andersen, B. L. (2004). **Assessing Stress in Cancer Patients.** *Assessment,11*(3), 216-223. doi:10.1177/1073191104267398
10. He, L., Zhang, E., Shi, J., Li, X., Zhou, K., Zhang, Q., . . . Tang, X. (2013). **(−)-Epigallocatechin-3-gallate inhibits human papillomavirus (HPV)-16 oncoprotein-induced**

angiogenesis in non-small cell lung cancer cells by targeting HIF-1α. *Cancer Chemotherapy and Pharmacology,71*(3), 713-725. doi:10.1007/s00280-012-2063-z

11. Liao, T., & Yang, M. (2017). **Revisiting epithelial-mesenchymal transition in cancer metastasis: The connection between epithelial plasticity and stemness**. *Molecular Oncology,11*(7), 792-804. doi:10.1002/1878-0261.12096
12. Mukherjee, S., Hussaini, R., White, R., Atwi, D., Fried, A., Sampat, S., . . . Banerjee, P. (2018). **TriCurin, a synergistic formulation of curcumin, resveratrol, and epicatechin gallate, repolarizes tumor-associated macrophages and triggers an immune response to cause suppression of HPV tumors.** *Cancer Immunology, Immunotherapy,67*(5), 761-774. doi:10.1007/s00262-018-2130-3
13. Pachmann, K., & Schuster, S. (2018). **The Value of Monitoring the Behavior of Circulating Tumor Cells at the End of Endocrine Therapy in Breast Cancer Patients**. *Cancers,10*(11), 407. doi:10.3390/cancers10110407
14. Rahimi, S. B., Mohebbi, A., Vakilzadeh, G., Biglari, P., Jahromi, S. R., Mohebi, S. R., . . . Ghaemi, A. (2017). **Enhancement of therapeutic DNA vaccine potency by melatonin through inhibiting VEGF expression and induction of antitumor immunity mediated by CD8 T cells**. *Archives of Virology,163*(3), 587-597. doi:10.1007/s00705-017-3647-z
15. Vinod, B. S., Nair, H. H., Vijayakurup, V., Shabna, A., Shah, S., Krishna, A., . . . Anto, R. J. (2015). **Resveratrol chemosensitizes HER-2-overexpressing breast cancer cells to docetaxel chemoresistance by inhibiting docetaxel-mediated activation of HER-2–Akt axis**. *Cell Death Discovery,1*(1). doi:10.1038/cddiscovery.2015.61

Introduction to Liquid Biopsies

Rationale and Indications
Circulating Tumor Cells and Cell Free Tumor DNA

Dr. Nalini Chilkov, Founder

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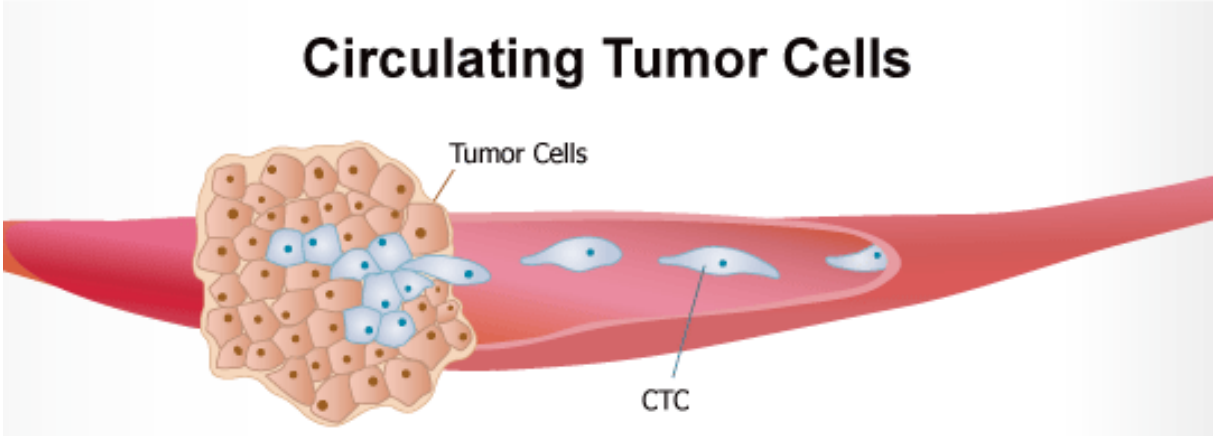
Circulating Tumor Cells



Circulating Tumor Cells (CTCs) are tumor cells that detach from the primary tumor and travel in the bloodstream, spreading from the original tumor to other locations, leading to cancer metastasis. These cells exist in peripheral blood of cancer patients and detection of CTCs can help to determine the process of metastasis. In contrast with other blood cells, the number of CTCs is very rare in blood which makes them difficult to detect.

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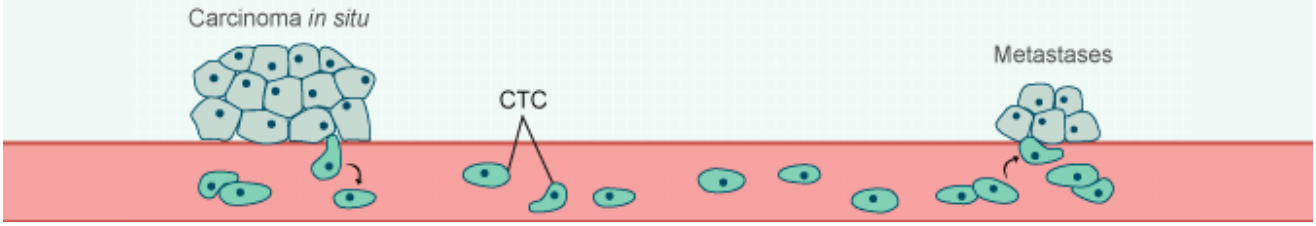
Circulating Tumor Cells

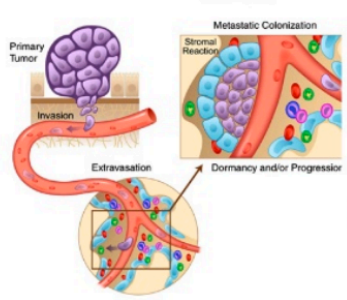


Circulating Tumor Cells (CTCs) are cancer cells that undergo epithelial mesenchymal transition and circulate in the bloodstream.

After detachment from primary tumor site, CTCs metastasize and invades other organs to form metastatic tumor.

These cells exist in peripheral blood of cancer patients and detection of CTCs can help determine the process of metastasis and predict the prognosis of treatment.

However, in contrast with other blood cells, the number of CTCs is extremely rare andthereby very difficult to detect.



- Carcinomas are from **epithelial origin**
- Carcinomas **dissiminate** epithelial cells

⇒ **CETCs** (circulating epithelial tumor cells)

Y. Shiozawa, A M Havens, K J Pienta and R S Taichman, Leukemia (2008) 22, 941–950

TZB

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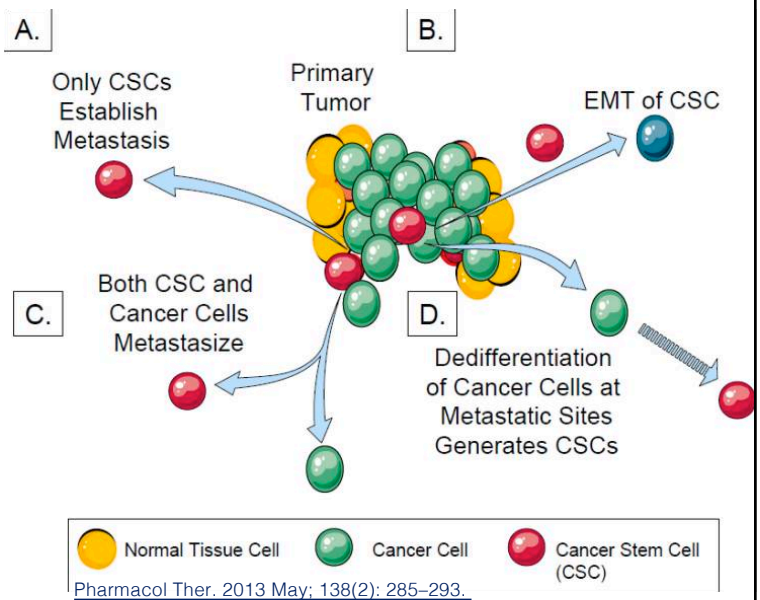
Multiple hypotheses examining the origin of CSCs and metastases

A. Only the CSCs in primary tumors can home and metastasize to distant tissues directly.

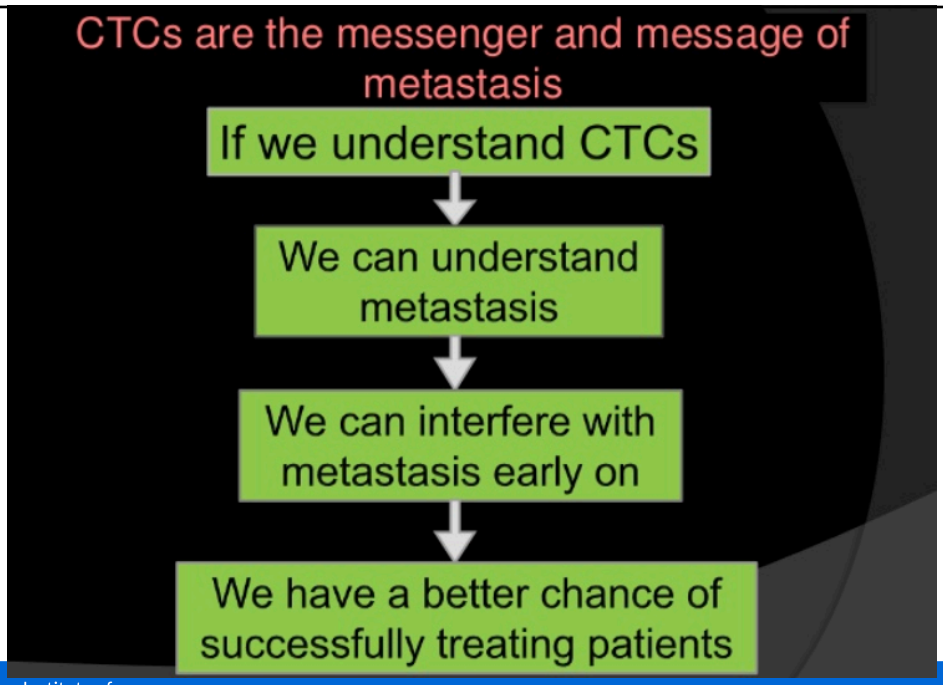
B. CSCs are generated in primary tumors that ultimately undergo an EMT to generate CTCs. The CTCs home to distant tissues and undergo mesenchymal to epithelial transition establishing the disseminated CSCs.

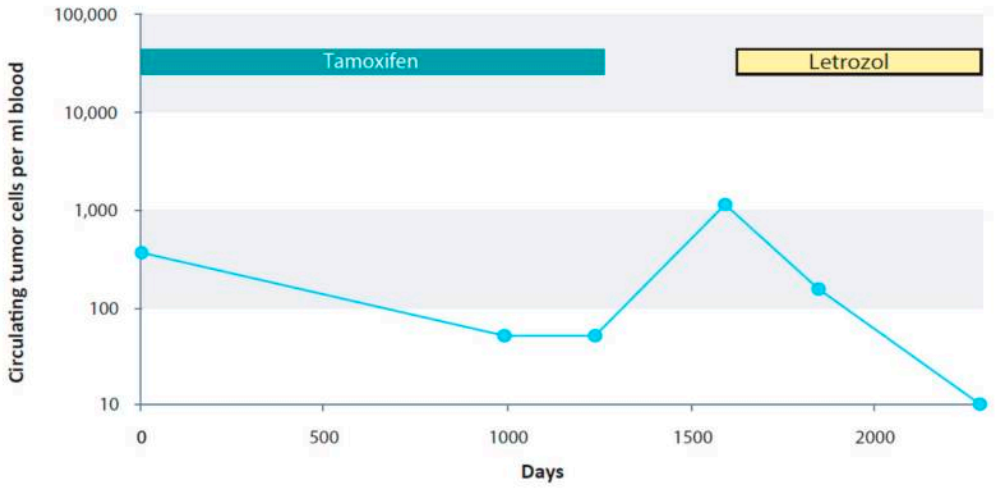
C. In some specific tumor types, all the cancer cells including CSCs demonstrate no significant differences in the ability to generate tumors or establish metastases distant sites in animal models.

D. Non-stem-like cancer cells shed as CTCs from primary tumors circulate and home to distant tissues. Once in the distant site, the cancer cells became CSCs through de-differentiation.



Pharmacol Ther. 2013 May; 138(2): 285–293.
Cancer Stem Cells and their Role in Metastasis. Shiozawa,Y



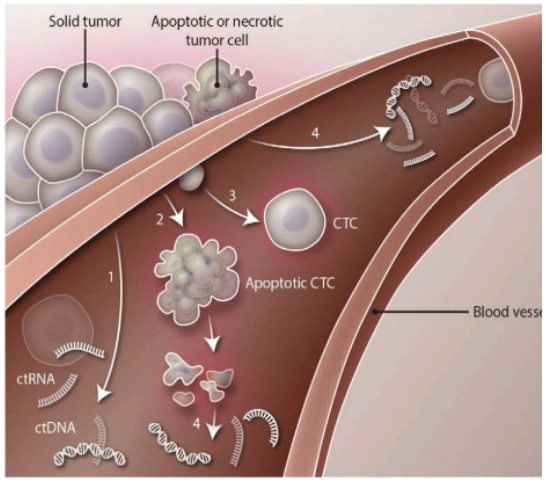


The Value of Monitoring the Behavior of Circulating Tumor Cells at the End of Endocrine Therapy in Breast Cancer Patients. Pachmann K, Schuster S. Cancers (Basel). 2018 Oct 29;10(11)

CETC may provide valuable insights in the tumor activity to track relapses early and also to distinguish between effective and less effective therapies.

CTCs and ctDNA

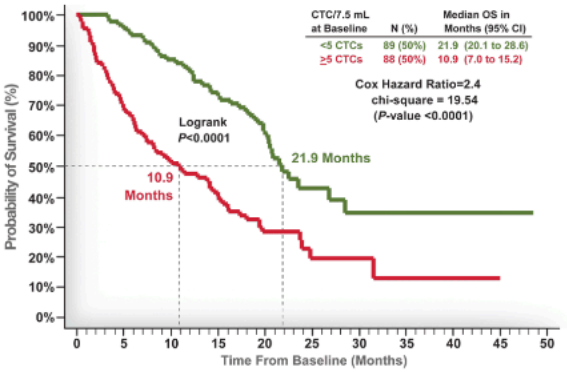
- **Circulating Tumor Cells (CTCs):** whole cells shed from tumors and containing intact genomic material including DNA, RNA, protein
- **Circulating tumor DNA (ctDNA):** fragmented DNA shed into blood as cells die
- Capture and analysis of CTCs and ctDNA in blood, help guide treatment decisions and patient management



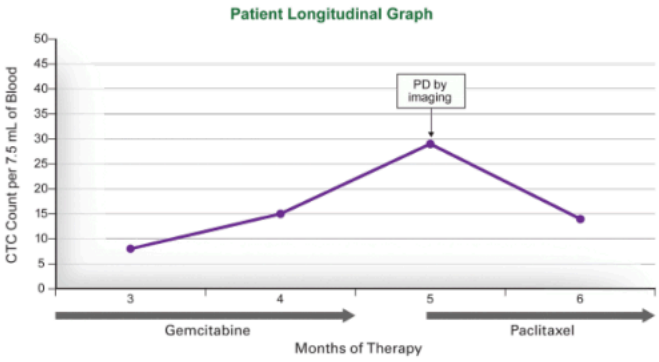
Credit: H. McDonald / Science Translational Medicine

10+ Years Ago, First Generation Circulating Tumor Cell (CTC) Testing Provided Prognosis and Helped Guide Treatment Decisions for Cancer Patients

CTC Count ≥5 Predictive of Shorter Survival



Doctor changed when patient's CTC increased and disease progression was confirmed by imaging



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Challenges of First Generation CTC Testing

- Only captures a single subset of CTCs
  - Only suitable for cells of epithelial origin and expressing cytokeratin (CK)
  - Cannot capture non-epithelial cells that are believed to be undergoing mesenchymal transition (EMT), more worrisome cells
  - more common to find 0 CTCs
- does not provide any biomarker status of the CTCs
  - Thus more challenging for treatment guidance

Fast Forward to Today

Next Generation CTC Testing

- Simple, non invasive blood test
  - Practical, affordable way to serial monitor patients for
    - recurrence, progression, and treatment efficacy
  - Cost Effective
- Superior CTC capture rates compared to first generation (68% vs 89%)*
- Captures multiple subpopulations (CK+ and CK-) CTCs
- Determines biomarker status (e.g. ER, PR, HER2) to help further guide patient management

*Mayer et al, Cancer Genetics, 2011

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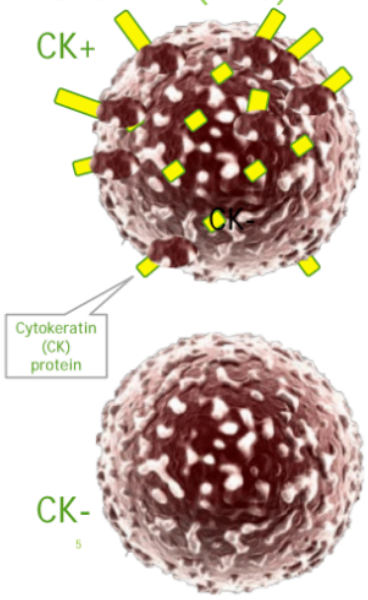
Introduction: CTCs and Epithelial-Mesenchymal Transition (EMT)

- CTCs are a heterogeneous population (display multiple phenotypic states)
- First Generation CTC testing only captures/identifies epithelial phenotype CTCs (EpCAM and CK+)
- First Generation systems do not identify CTCs without cytokeratin (CK-)
- CK- cells may be in epithelial to mesenchymal transition (EMT)
- EMT cells have prognostic importance in multiple tumor types



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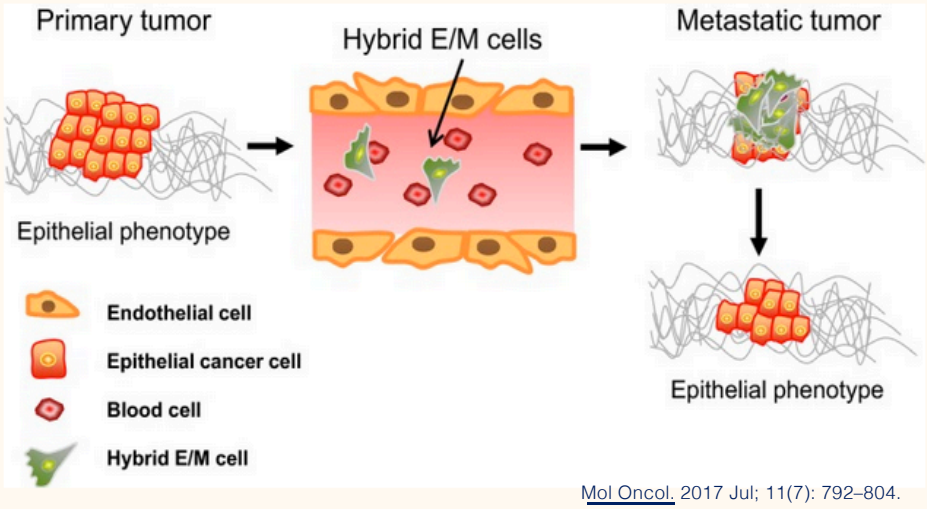
CYTOKERATIN (-) CTCs

Cellular Plasticity

EPITHELIAL MESENCHYMAL TRANSITION and Cancer Metastasis



Mol Oncol. 2017 Jul; 11(7): 792–804.
Revisiting epithelial-mesenchymal transition in cancer metastasis: the connection between epithelial plasticity and stemness. Tsai-Tsen Liao

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Personalized Cancer Diagnostics

When tissue biopsy isn't an option, obtain actionable molecular information for your patient's cancer

When cancer metastasizes or spreads, it sheds tumor cells and cancer-causing DNA fragments into the blood.

Technologies have been developed with advanced methods for detecting and assessing tumor cells and cancer-causing DNA fragments in the blood.

You get the same information you would get from a tissue sample—from a simple blood test.

And because liquid biopsies use blood, you are informed about what's happening with your patients' cancer NOW—not weeks, months, or years ago.

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When Should You Consider A Liquid Biopsy?

- Not enough tissue was obtained from an initial biopsy.
- Cancer starts in a place that is difficult to access for surgical biopsy.
- Cancer recurs after the initial tumor tissue has been removed.
- The patient is not responding to treatment as expected.
- The patient wishes to avoid additional biopsies.
- Monitoring for recurrence or progression

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Circulating Tumor Cells	Cell Free Tumor DNA
CTC PLATFORM	**ctDNA PLATFORM**
Translocations	Single Nucleotide Variants (SNVs)
DNA Amplifications	Insertions
Protein Expression	Deletions

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BIOCEPT TARGET SELECTOR TEST MENU

TEST MENU - TARGET SELECTOR™

Cancer Profiles

- ☐ **Non-Small Cell Lung Cancer Profile**
  • ALK • BRAF • EGFR Mutations • KRAS • PD-L1 • ROS1
- ☐ **Non-Small Cell Lung Cancer Expanded Profile**
  • ALK • BRAF • EGFR Mutations • KRAS • MET • PD-L1 • RET • ROS1
- ☐ **Squamous Cell Lung Cancer Profile**
  • ALK • BRAF • EGFR • FGFR1 • PD-L1 • ROS1
- ☐ **Breast Cancer Profile**
  • AR • EGFR Amp. • ER • FGFR1 • HER2 • PD-L1 • PR • PTEN
- ☐ **Colorectal Cancer Profile**
  • BRAF • KRAS
- ☐ **Gastric Cancer Profile**
  • HER2 • MET
- ☐ **Melanoma Cancer Profile**
  • BRAF
- ☐ **Prostate Cancer Profile**
  • AR • AR-V7 • EGFR Amp. • MET • MYC • PTEN

CTC Enumeration

☐ **CTC Count***

Individual Markers (Please check all that apply)

CTC
(Circulating Tumor Cells)*

• Expression	• FISH	
☐ AR	☐ ALK	☐ MYC
☐ AR-V7	☐ EGFR	☐ PTEN
☐ ER	☐ FGFR1	☐ RET
☐ PD-L1	☐ HER2	☐ ROS1
☐ PR	☐ MET	

Validated tumor types: NSCLC, SCC, Breast, Colorectal, Prostate, Gastric, Ovarian, Pancreatic

ctDNA
(Circulating Tumor DNA)

- **• Molecular**
- ☐ EGFR (Mutations: L858R, Del 19, T790M)
- ☐ KRAS
- ☐ BRAF

Repeat every 3-6 months. Covered by Medicare and most insurance plans
Pathology Report and documentation of diagnosis required

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Example Test for Receptor Expression

Targeted Therapies: Trastuzumab (Herceptin®), pertuzumab (Perjeta®), emtansine (Kadcyla®), and lapatinib (Tykerb®).

HER2

- The human epidermal growth factor receptor 2 (HER2, also called ERBB2 or NEU) is associated with several forms of cancer.
- Approximately 15–30% of breast cancer patients have tumors that overexpress *HER2*, and 7–34% of gastric cancers are *HER2* positive.
- *HER2* overexpression is also associated with salivary duct carcinomas.

Example Test for Receptor Expression. PD_L1

Targeted Therapies: Pembrolizumab (Keytruda®), nivolumab (Opdivo®), and atezolizumab (Tecentriq®)

- Programmed death-1 *(PD-1)* is an immune checkpoint receptor expressed by T cells.
- Expression of *PD-L1* has been described for many tumor types, including melanoma, both squamous and non-squamous NSCLC, breast, ovarian, pancreatic, esophageal, kidney, bladder, and hematopoietic malignancies.
- Upregulation of *PD-1* plays a key role in T-cell exhaustion, which hampers the ability of the immune system to destroy cancer cells.
- When P*D-1* binds its ligand PD-L1 (programmed death ligand-1), a signal is transduced, telling the T-cell to leave other cells alone.
- This checkpoint is in place to protect normal cells from immune attack, but some cancer cells co-opted this system, expressing high levels of *PD-L1* to evade the immune system.
- Detecting PD-L1 in tumor cells identifies cancer patients that may benefit from treatment with immunotherapy agents. These agents, called *PD-1* or *PD-L1* inhibitors (or checkpoint inhibitors) have been shown to increase immune responses to cancers and improve patient survival.

EXAMPLE: CHECK for GENE LOSS or DELETION

PTEN

- Phosphatase with tensin homology **(PTEN) is a frequently inactivated tumor suppressor gene in human cancers.**
- *PTEN* gene loss is found in up to 20% of localized prostate tumors and more than 40% for metastatic castrate-resistant prostate cancer.
- ***PTEN* gene loss may drive progression through activation of the PI3K/AKT pathway**
- ***PTEN* loss/deletion has been shown to be associated with poorer outcomes, including more advanced disease at surgery and shorter time to biochemical recurrence.**
- **Overall survival decreased in patients with *PTEN* loss versus those with no *PTEN* deletion** (7 mo. vs. 12.1 mo.)

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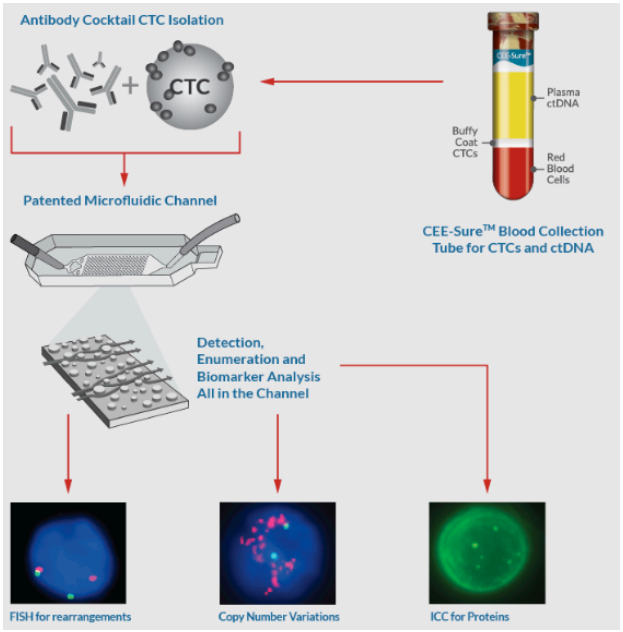
Circulating Tumor Cells obtained through simple venipuncture
can be used to molecularly characterize tumor cells and evaluate
changes in protein expression with the same techniques used in
tissue samples

The same specimen can also be microscopically analyzed for DNA and protein targets

All CTC Test are enumerated can be used for monitoring



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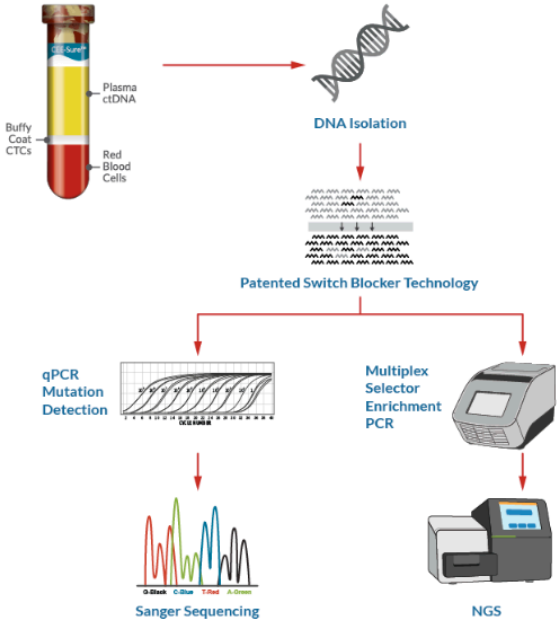
ctDNA Tests

Performing PCR upfront and then sequencing delivers added confidence to final result.

Switch-Blocker technology enriches oncogene mutations and suppresses wild type DNA resulting in ultra-high sensitivity and specificity.

All ctDNA tests are quantitative and can be used to monitor mutation load.

NGS technology allows multiplexing capabilities and future panel development.



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Research Paper

Resveratrol fuels HER2 and ERα-positive breast cancer behaving as proteasome inhibitor

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[3]Aging Research Centre, G. d'Annunzio University, Chieti, 66100, Italy
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Keywords: breast cancer, Δ16HER2 mice, resveratrol, estrogen receptor, proteasome
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ABSTRACT

The phytoestrogen resveratrol has been reported to possess cancer chemo-preventive activity on the basis of its effects on tumor cell lines and xenograft or carcinogen-inducible *in vivo* models. Here we investigated the effects of resveratrol on spontaneous mammary carcinogenesis using Δ16HER2 mice as HER2+/ERα+ breast cancer model. Instead of inhibiting tumor growth, resveratrol treatment (0.0001% in drinking water; daily intake of 4μg/mouse) shortened tumor latency and enhanced tumor multiplicity in Δ16HER2 mice. This *in vivo* tumor-promoting effect of resveratrol was associated with up-regulation of Δ16HER2 and down-regulation of ERα protein levels and was recapitulated *in vitro* by murine (CAM6) and human (BT474) tumor cell lines. Our results demonstrate that resveratrol, acting as a proteasome inhibitor, leads to Δ16HER2 accumulation which favors the formation of Δ16HER2/HER3 heterodimers. The consequential activation of downstream mTORC1/p70S6K/4EBP1 pathway triggers cancer growth and proliferation. This study provides evidence that resveratrol mechanism of action (and hence its effects) depends on the intrinsic molecular properties of the cancer model under investigation, exerting a tumor-promoting effect in luminal B breast cancer subtype models.

INTRODUCTION

Several natural compounds have recently gathered renewed interest as potential anti-tumor agents. Resveratrol (*trans* -3,4',5-trihydroxystilbene), a natural polyphenol found in grapes, peanuts, cocoa, berries, and red wine, has been described as a putative cancer chemo-preventive compound able to counteract breast cancer initiation, promotion and progression [1]. Although several *in vitro* studies have proposed resveratrol to exert an anti-tumor activity by inducing apoptosis and/or cell cycle arrest in various mammary cancer cell lines [2-4], investigations attempting to dissect the *in vivo* effects of resveratrol upon cancer onset and progression have led to contradictory results [5-7]. Such a controversial scenario can be explained recalling that resveratrol, as a phytoestrogen, possesses both estrogenic and anti-estrogenic activities on ERα-positive breast cancer [8-10]. The devised experimental conditions -dose, timing, way of administration- influence the outcome of resveratrol-based studies as well [11]. Moreover, the experimental model taken into consideration is determinant: the majority of available data has been obtained using transplantable or carcinogen-inducible tumor models that, even if informative, fail to reproduce the complexity of spontaneous tumorigenesis [11]. To our knowledge, the solely *in vivo* study on a spontaneous mammary tumor model was reported by Provinciali et al. who claimed how resveratrol supplementation delays tumor onset, growth and metastases in HER2/neu transgenic female mice [12]. This research was performed *in vivo* on transgenic

mice carrying the rat HER2/neu oncogene under the transcriptional control of MMTV promoter, and *in vitro* on human SKBR3 cell line, both models representing ER-negative (-) and HER2-positive (+) breast cancers [13]. They ascribed such protective effects to a decrease in HER2 gene expression and a promotion of *in situ* apoptosis observed inside mammary tumor tissues [12].

Even though HER2 over-expression is considered a hallmark of aggressive breast cancer subtypes, increasing evidence has pointed toward a major contribution of Δ16HER2 splice variant (lacking exon-16), which is commonly co-expressed with wild-type HER2, in promoting cancer progression, metastasis and resistance to Trastuzumab [14, 15]. The relevance of Δ16HER2 and the availability of Δ16HER2 mice, transgenic for the human Δ16HER2 oncogenic isoform, drove us to evaluate the effects of resveratrol intake on Δ16HER2-driven mammary carcinogenesis [16]. Unexpectedly, we found resveratrol to promote Δ16HER2 carcinogenesis by up-regulating Δ16HER2 protein levels in both primary tumors and metastases and down-regulating ERα expression in tumors, thus recapitulating endocrine therapy resistance. Mechanistically, resveratrol partially inhibits proteasome activity and leads to Δ16HER2 protein accumulation. Increased Δ16HER2 levels, in turn, trigger the formation of Δ16HER2/HER3 heterodimers and the consequent HER3 phosphorylation. The resulting activation of mTORC1/p70S6K pathway finally fuels cell proliferation and mammary tumor development in Δ16HER2 mice.

RESULTS

Resveratrol supplementation promotes mammary tumor development in Δ16HER2 mice

In Δ16HER2 mice, the MMTV-driven expression of only five copies of the human Δ16HER2 transgene triggers neoplastic transformation of mammary epithelial cells with a short latency time. Since all transgenic females develop multiple asynchronous mammary tumors on average at the 15th week of age, resveratrol treatment started when the animals were 8 week-old and proceeded up to 23 weeks of age (Figure

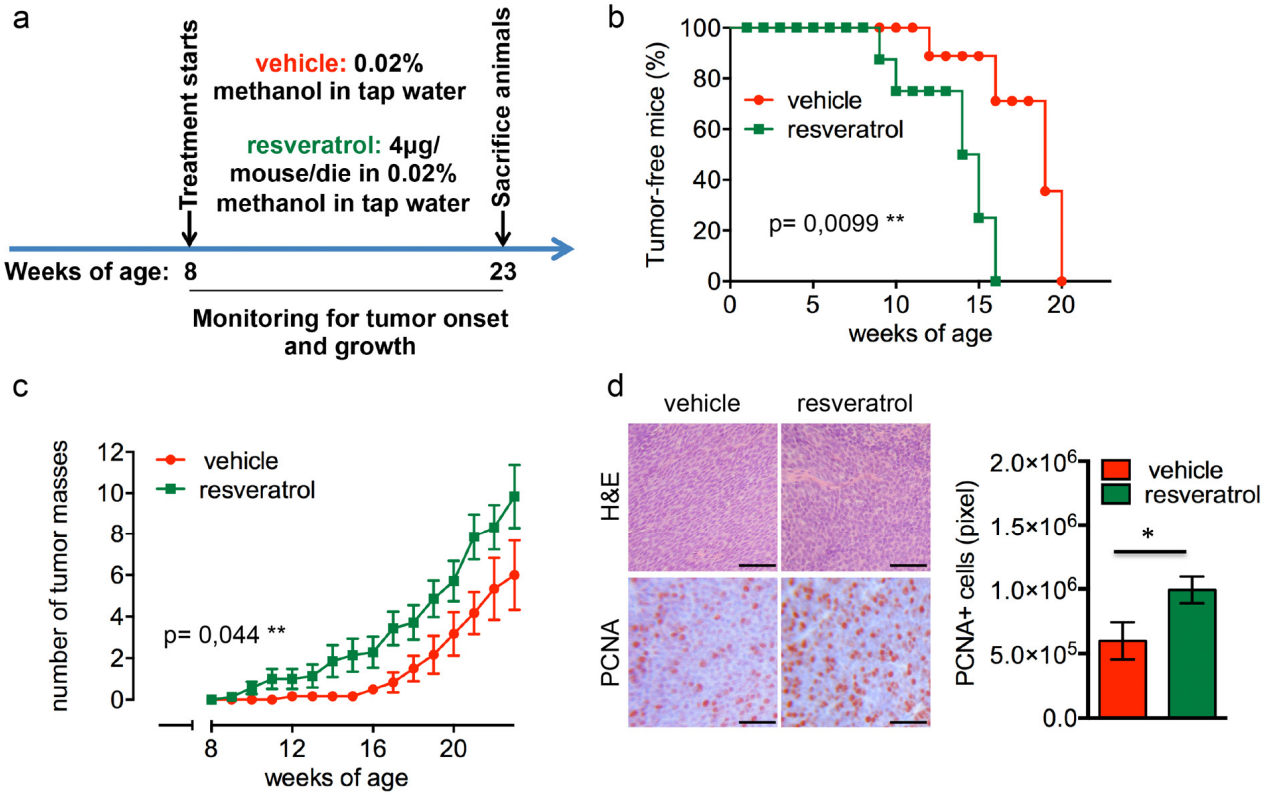

Figure 1. Resveratrol fuels mammary tumor formation in a luminal B breast cancer *in vivo* model. (**a**) Schematic representation of the experimental workflow used for the treatment of Δ16HER2 mice with vehicle or resveratrol. (**b**) Kaplan-Meier disease-free survival plot for vehicle- (*n*= 9) and resveratrol-treated (*n*= 9) Δ16HER2 mice. **p ≤ 0.01, Log Rank test. (**c**) Tumor multiplicity in resveratrol-treated *vs* control mice; the number of palpable mammary tumors per mouse is represented as mean ± SD. Statistical significance was assessed by two-way ANOVA test. (**d**) Left panel: Representative Hematoxyilin-Eosin (H&E) and PCNA stained sections of tumors from resveratrol and vehicle treated mice. Magnification 400 X. Right panel: Quantification of PCNA staining in tumors from resveratrol and vehicle treated mice. Data are expressed as mean ± SEM. *p < 0.05; unpaired two-tailed student t test.

1a). As shown in Figure 1b, resveratrol-receiving mice displayed a significantly anticipated tumor onset developing the first tumor mass at only 9 weeks of age (p=0.0099), while the first tumor appeared at 12 weeks of age in untreated females. In particular, 50% of resveratrol-treated mice presented at least one mammary

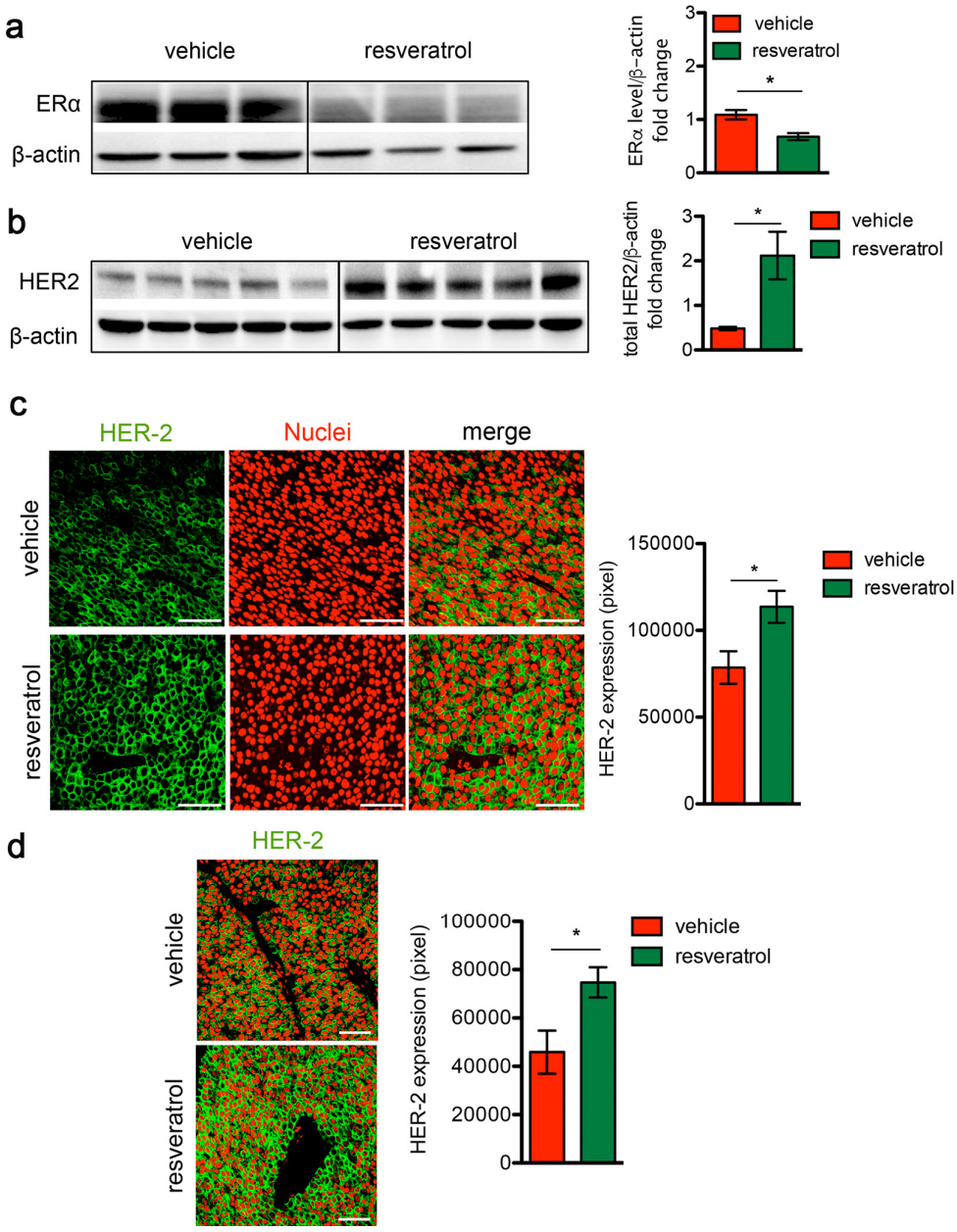

Figure 2. Resveratrol treatment induces HER2 over-expression and ERα down-regulation in HER2+/ERα+ mammary carcinomas. (**a**) Representative western blot analysis of ERα and β-actin (loading control) in spontaneous mammary tumors from Δ16HER2 mice supplemented or not with resveratrol (left panel), and relative densitometry quantification from three independent experiments (right panel). The significance was determined by unpaired two-tailed student t test, *p < 0.05. (**b**) Representative western blot analysis of HER2 and β-actin (loading control) in spontaneous mammary tumors from Δ16HER2 mice, supplemented or not with resveratrol (left panel), and relative densitometry quantification from three independent experiments (right panel). The significance was determined by unpaired two-tailed student t test, *p < 0.05. (**c**) Left panel: representative immunofluorescence images of tumor sections from control and resveratrol supplemented mice stained with an antibody anti-HER2 (green) and DRAQ5 dye (red) for nuclei staining. Magnification 400 X. Right panel: quantification of HER2 staining in tumors from resveratrol and vehicle treated mice. Data are expressed as mean ± SEM. *p < 0.05; unpaired two-tailed student t test. (**d**) Resveratrol treatment induces HER2 over-expression in lung metastases. Left panel: representative immunofluorescence images of lung metastasis sections from control and resveratrol supplemented mice stained with an antibody anti-HER2 (green) and DRAQ5 (red). Magnification 400 X. Right panel: quantification of HER2 staining in tumors from resveratrol and vehicle treated mice. Data are expressed as mean ± SEM. *p < 0.05; unpaired two-tailed student t test.

mammary tumor at 15 weeks of age, when 90% of the control littermates were still tumor-free, although all the animals eventually developed tumors within 20 weeks of age. Moreover, as shown in Figure 1c, mice supplemented with resveratrol suffered from a higher number of tumor masses than controls, with an average of 9 tumors/mouse and 6 tumors/mouse, respectively (p=0.044). The enhanced proliferation rate triggered by resveratrol correlates with an increased expression of proliferating cell nuclear antigen (PCNA) in tumors from treated animals as demonstrated by immunohistochemical (IHC) analysis (Figure 1d).

Resveratrol triggers Δ16HER2 over-expression and ERα down-regulation

To investigate the molecular mechanisms underlying the boosted cancer growth induced by resveratrol, ERα and Δ16HER2 protein levels were analyzed by western blot in tumors explanted from resveratrol-treated and control mice. We found that *in vivo* administration of resveratrol caused a dramatic drop in the ERα expression (Figure 2a, $p<0.05$) and a concomitant increase in Δ16HER2 protein level (Figure 2b, $p<0.05$) in tumors. Confocal microscopy analysis confirmed the significant higher abundance of Δ16HER2 in both primary tumors (Figure 2c) and lung metastatic lesions (Figure 2d, $p<0.05$) from resveratrol-treated mice compared to controls. These data suggest that the effect of resveratrol on Δ16HER2 protein accumulation is similar in primary tumors and metastases, although resveratrol supplementation did not seem to influence the number of lung metastases (3 out of 6 mice were found with pulmonary metastases in the vehicle group, 4 out of 7 mice in resveratrol-group, Fig. S1).

Resveratrol *in vitro* treatment of Δ16HER2+/ERα+ breast cancer cell lines (luminal B subtype) recapitulates the *in vivo* outcomes

To confirm the pro-tumor effect of resveratrol in human Δ16HER2+/ERα+ breast cancer, we tested resveratrol *in vitro* on human BT474 and murine CAM6 cell lines.

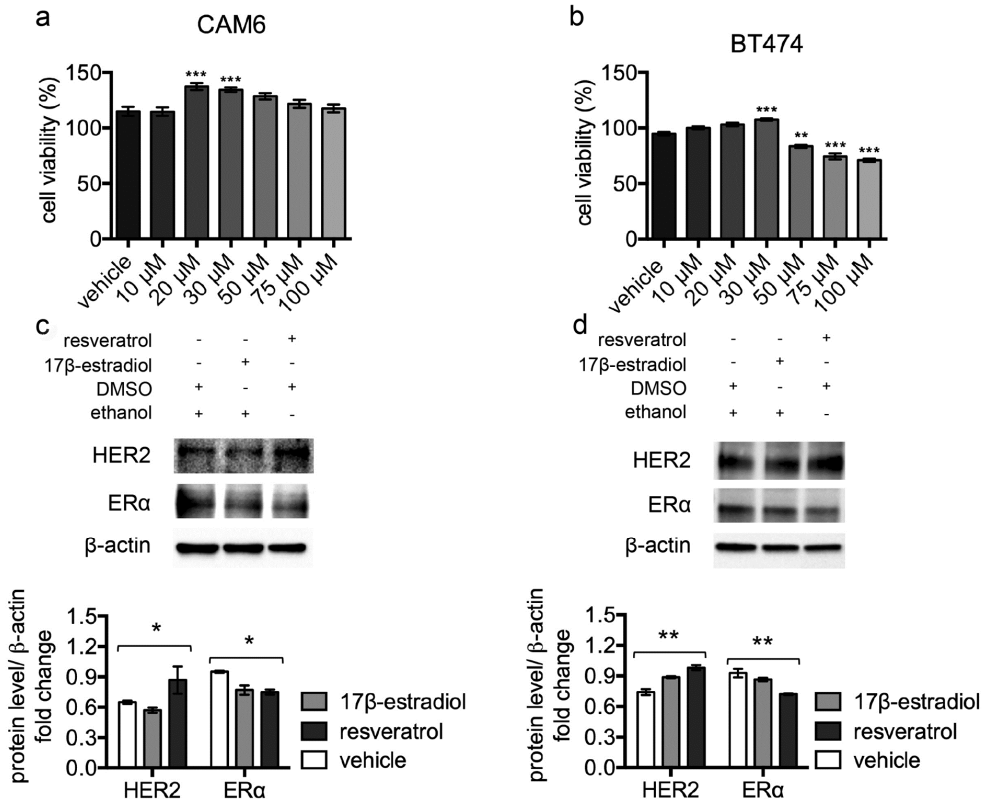

Figure 3. Resveratrol triggers HER2 over-expression and ERα down-regulation in luminal B breast cancer cell lines. (**a**) CAM6 and (**b**) BT474 cells were incubated for 24 hours in the presence of vehicle or increasing concentrations of resveratrol and cell viability was determined by MTT assay. Results (including vehicle group treated with 0.02 % DMSO) are expressed as percentage (%) of cell viability relative to untreated controls. Columns, mean of three separate experiments wherein each treatment was repeated in 16 wells; bars, SE. **p ≤ 0.01, ***p ≤ 0.001, one-way ANOVA followed by Bonferroni's multiple comparison test. Representative western blot analysis of HER2, ERα and β-actin (loading control) in murine CAM6 cells (**c**) or human BT474 cells (**d**), treated with resveratrol or 17β-estradiol or vehicle for 24 hours (upper panel), and relative densitometry quantification (lower panel). The significance was determined by one-way ANOVA (*p < 0.05, **p ≤ 0.01).

CAM6 cells have been established from Δ16HER2 mice and can be considered the *in vitro* counterpart of Δ16HER2 mammary tumors [17]. After 24 hours' incubation, despite high resveratrol concentrations resulted in reduced cell viability, low-range resveratrol concentrations (20-30 μM) promoted cell proliferation of both CAM6 and BT474 cells (Figure 3a and 3b), mimicking the *in vivo* resveratrol effect. Consistently, western blot analysis revealed that treatment with 40 μM resveratrol for 24 hours induced HER2 up-regulation and a concomitant ERα down-regulation in both CAM6 and BT474 cells, recapitulating the molecular events observed inside Δ16HER2 tumors (Figure 3c and 3d). Since 10 nM 17β-estradiol treatment only partially reproduced the resveratrol effect in CAM6 cells, inducing reduction of ERα without enhancing HER2 protein levels, we suppose that resveratrol exerts a more complex action on Δ16HER2+ cancer cells than the mere estrogen-like effect.

Resveratrol causes Δ16HER2 protein accumulation by partial inhibition of proteasome activity

To evaluate whether the resveratrol-induced Δ16HER2 over-expression was due to transcriptional or post-transcriptional mechanisms, Δ16HER2 mRNA levels were analyzed by qRT-PCR in mammary tumors from resveratrol-treated and control mice. Despite the significantly higher level of Δ16HER2 protein detected in resveratrol-treated tumors in comparison with controls (Figure 2b and 2c), no differences in Δ16HER2 mRNA levels were found between the two experimental groups (Figure 4a and 4b). By contrast, the activity of

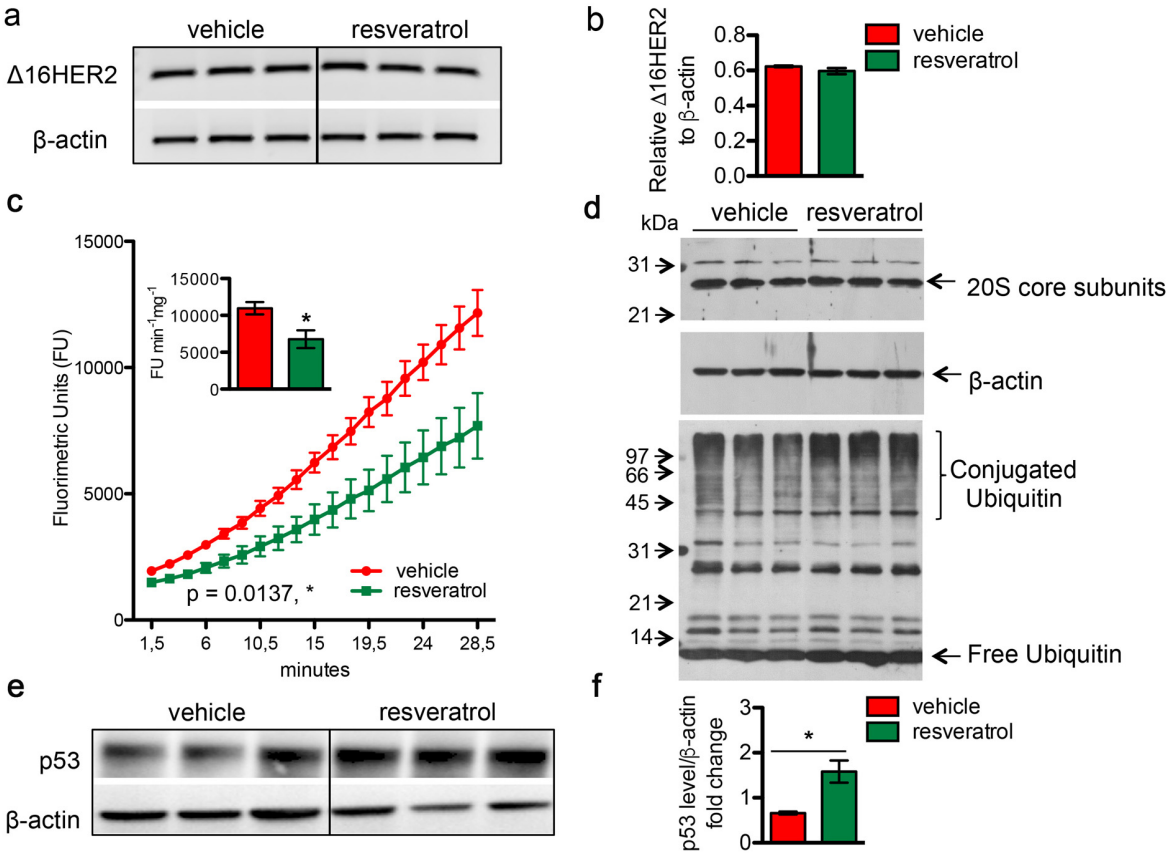

Figure 4. Resveratrol inhibits the chymotrypsin-like activity of 20S proteasome and resulted in an increased accumulation of protein-ubiquitin conjugates. Δ16HER2 and β-actin (internal control) mRNA levels were measured by semi-quantitative RT-PCR (**a**) and qRT-PCR (**b**) analyses in spontaneous mammary tumors from Δ16HER2 mice supplemented or not with resveratrol. (**c**) The chymotrypsin-like activity of the 20S proteasome was measured in tumor samples, from Δ16HER2 mice treated or not with resveratrol, as described in Materials and Methods, and expressed as fluorimetric units (FU) min^{-1} mg^{-1}. The significance was determined by unpaired two-tailed student t test, *p < 0.05. (**d**) Western blot analysis of 20S proteasome subunit content (upper panel) and ubiquitin-protein conjugates and free ubiquitin levels (lower panel) in tumor samples from Δ16HER2 mice treated or not with resveratrol. β-actin was used as loading control. (**e**) Representative western blot analysis of p53 and β-actin (loading control) in spontaneous mammary tumors from Δ16HER2 mice, supplemented or not with resveratrol, and (**f**) relative densitometric quantification from three independent experiments. The significance was determined by unpaired two-tailed student t test, *p < 0.05.

the 20S proteasome, which is responsible for the degradation of the bulk of the cellular proteins, was significantly affected by *in vivo* supplementation of resveratrol, as determined by the 20S chymotrypsin-like activity assay in tumor extracts (Figure 4c). Noteworthy, no difference was found in the cellular levels of the 20S subunits, suggesting that resveratrol may exert a direct inhibitory effect on the proteasome enzymatic activity (Figure 4d, upper panel). In agreement with a partial inhibition of the proteasome, tumor masses from resveratrol-treated mice displayed a higher extent of ubiquitin-conjugated proteins with respect to those obtained from mice receiving placebo (Figure 4d, lower panel). Consistently, western blot analysis also revealed a significant increase in the levels of p53 protein in mammary tumors from resveratrol-

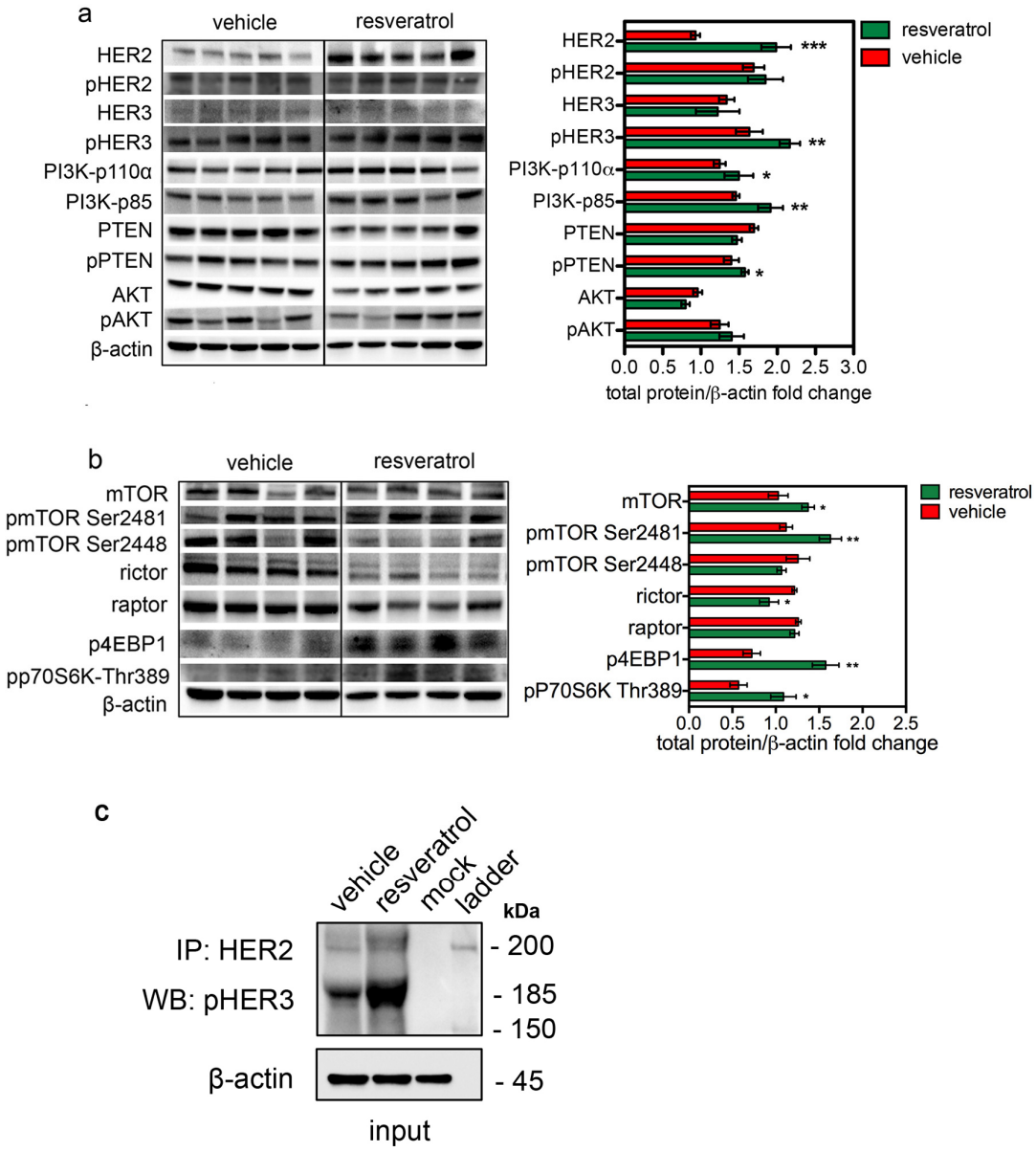

Figure 5. Increased HER2 level induced by resveratrol results in a preferential activation of mTORC1/p70S6K pathway. Representative western blot analysis of HER2 and HER3 downstream signaling pathways in spontaneous mammary tumors from Δ16HER2 mice, treated or not with resveratrol (left panels), and densitometry quantification from three independent experiments (right panels). (**a**) PI3K/AKT and (**b**) mTOR signaling pathways were analyzed. β-actin was used as loading control. The significance was determined by t-test (*$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). (**c**) Resveratrol promotes the direct coupling of Δ16HER2 to HER3 kinase in HER2+/ERα+ breast cancer. Δ16HER2 kinase co-immunoprecipitates with pHER3. Proteins were immunoprecipitated with anti-HER2 antibody and then probed by western blot for pHER3. Input represents 10% of the co-immunoprecipitation protein amount (30 μg).

receiving mice in comparison with controls (Figure 4e and 4f, $p<0.05$), giving further evidence of resveratrol-mediated proteasome inhibition. Noteworthy, such p53 augmentation inside tumors of resveratrol group did not trigger a concomitant up-regulation in the apoptotic cell death, as indicated by IHC analysis of cleaved caspase 3 (Figure S2). Moreover, the ability of resveratrol to inhibit in a concentration-dependent manner the activity of the proteasome was confirmed in both BT474 and CAM6 cell extracts (Figure S3).

Δ16HER2 accumulation induced by resveratrol results in a preferential activation of mTORC1/ p70S6K/4EBP1 pathway

To identify the signaling cascades beneath the resveratrol-induced Δ16HER2 accumulation, the main oncogenic pathways known to be involved in breast carcinogenesis were analyzed by western blot. As shown in Figure 5a, despite tumors from resveratrol-treated mice expressed significantly higher Δ16HER2 levels than vehicle counterparts, no relevant differences were detected in its phosphorylation status. Noteworthy, phosphorylated HER3 levels were significantly higher in tumor extracts from resveratrol-treated animals (Figure 5a). Consistent with the over-expression of Δ16HER2 and increased HER3 phosphorylation, tumors displayed high levels of Δ16HER2-HER3 heterodimer upon resveratrol treatment, as measured by co-immunoprecipitation assay (Figure 5c). This result suggests that resveratrol-induced accumulation of Δ16HER2 leads to Δ16HER2/HER3 heterodimer formation and consequently to HER3 transactivation. It is known that HER2/HER3 heterodimers are powerful oncogenic units, in part because phospho-HER3 boosts PI3K-AKT-mTOR signaling pathway [18].

Consistently, we found that resveratrol-induced Δ16HER2/HER3 heterodimerization caused a concomitant increase in the levels of PI3K p85 and PI3K p110α (PI3K-p85, $p<0.01$ and PI3K-p110α, $p<0.05$) and the up-regulation of the mammalian target of rapamycin complex 1 (mTORC1). The mTOR Ser/Thr kinase is the catalytic component of two structurally and functionally distinct signaling complexes. The rictor-containing mTORC2 regulates the actin cytoskeleton and activates Akt [19]. The raptor-containing mTORC1 regulates cell growth and nutrient signaling [20]. In particular, mTORC1 phosphorylates p70 S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), thereby regulating translation of proteins that are critical for progression from G1 into S phase [21]. Therefore, we examined levels of phospho-4EBP1 and phospho-S6K as readouts of mTORC1 activity. As shown in Figure 5b, resveratrol significantly increased both phospho-4EBP1 ($p<0.01$) and phospho-S6K levels ($p<0.05$) relative to vehicle. The phosphorylation of these two *par excellence* mTORC1 downstream effectors positively correlated with the level of mTOR Ser(P)-2481, while lower rictor and mTOR Ser(P)-2448 pointed toward inhibition of mTORC2. Interestingly, although resveratrol supplementation promoted the inhibiting phoshorylation of PTEN (pPTEN ser380/thr382/383) [22], no differences in terms of AKT activation (pAKT) were observed between resveratrol and control groups: this may be due to feedback loops that compensate PI3K-mediated AKT activation. Interestingly, other pathways known to be activated downstream Δ16HER2, such as the Δ16HER2/Src/STAT3 axis, did not vary significantly between the two experimental groups (Figure S4). It is worth to note that the levels of unphosphorylated proteins other than HER2, like mTOR (Figure 5b), Src and Erk1,2 (Figure S4) increased in tumor extracts from resveratrol group. This observation is consistent with the ubiquitin-proteasome system (UPS)-dependent degradation of these proteins [23-26] and supports our evidence about resveratrol acting as proteasome inhibitor.

DISCUSSION

The need for safe and low-toxic preventive and therapeutic strategies against cancer is still unmet. In this scenario phytoestrogens like resveratrol have received growing attention as valuable chemo-preventive tools. Contrarily to previously published results [12], describing an antitumor effect of resveratrol in a HER2+/ERα- breast cancer experimental model, here we have found that resveratrol, given at the same reported dose, regimen and administration way, did not prevent nor delay tumor onset; instead, significantly accelerated cancer development in Δ16HER2 females that spontaneously develop HER2+/ERα+ mammary carcinomas.

In Δ16HER2 mice, resveratrol intake not only anticipated the tumor onset, but significantly augmented tumor multiplicity, promoting the growth of a higher number of tumor masses per mouse. Moreover, consistently with its pro-proliferative effect, we provide evidence that resveratrol treatment induced a strong increase in Δ16HER2 expression, associated with a significant reduction of ERα protein level. In contrast to the observation by Provinciali et al., resveratrol did not affect Δ16HER2 mRNA levels, suggesting that Δ16HER2 protein accumulation was the consequence of a reduced degradation rather than an increased transcription. Indeed, it has been demonstrated that resveratrol can act as a potent proteasome inhibitor [27, 28]. In our experimental settings, resveratrol treatment

partially inhibited the chymotrypsin-like proteasome activity, which may elicit the observed accumulation of Δ16HER2 protein in mammary tumors, that in turn fuels cancer growth. Noteworthy, the same result was obtained *in vitro* using murine and human luminal B breast cancer cell lines (CAM6 and BT474 cells, respectively). Indeed, adding resveratrol to cell-free extracts of CAM6 and BT474 resulted in a concentration-dependent proteasome inhibition. On the whole, the *in vivo* and *in vitro* evidence we gathered suggests that resveratrol can act as a proteasome inhibitor as Qureshi et al. claimed [26]. Nonetheless, we cannot exclude that additional effects of resveratrol on signaling cascades might eventually mediate an indirect proteasome inhibition as well. Anyhow, the ability of resveratrol to inhibit the proteasome can be considered a double-edged sword. On one side, resveratrol has been reported to exert anti-inflammatory effects partially by counteracting the proteasome-mediated activation of NF-κB, thereby suppressing activation of pro-inflammatory cytokines and iNOS genes [26]. Since inflammation is a hallmark of neurodegenerative disorders and aging [29, 30], proteasome inhibition triggered by resveratrol might provide additional insights into its anti-aging properties as well. On the other side, as our evidence has demonstrated, proteasome impairment and the consequent HER2 accumulation can promote breast cancer development. Strikingly, this resveratrol-induced Δ16HER2 increase was associated with a concomitant ERα diminution. ERα is of critical importance in mammary cancer initiation and progression, having become an ideal target for anti-cancer therapies. However, expression of ERα in tumors is dynamic and can change during the course of tumor progression and following therapy [31, 32]. Loss of ERα represents a crucial mechanism for the acquisition of endocrine resistance [33-35]. Several clinical and preclinical studies have confirmed the existence of a remarkable crosstalk between HER2 and ERα that usually leads them to be inversely expressed inside breast cancer. Strikingly, such bidirectional fluctuations dictate the responsiveness to HER2- and/or ER-targeted therapies [36-38]. Accordingly, it has been shown that resistant breast cancer cells have higher HER2 expression than endocrine therapy-sensitive cells [39]. Our data perfectly fit in this scenario, suggesting that resveratrol treatment mirrors endocrine resistance acquirement, triggering HER2 up-regulation and ERα

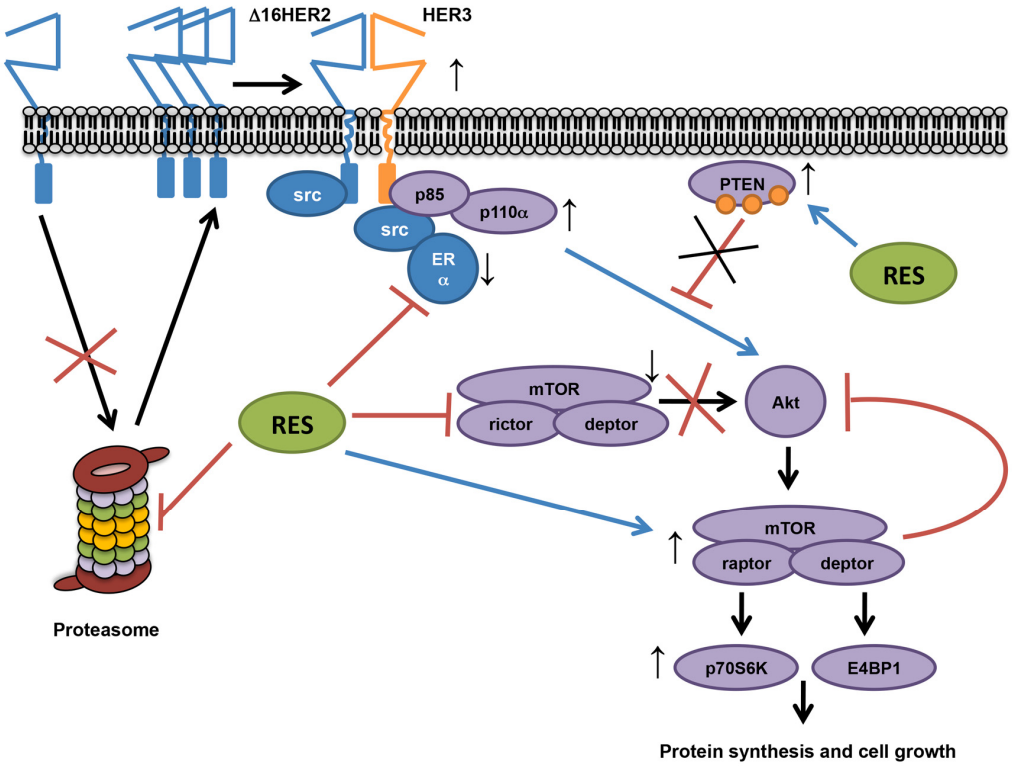

Figure 6. Proposed resveratrol's mechanism of action in a luminal B breast cancer model. Our data show that resveratrol down-regulates ERα and lowers the chymotrypsin-like activity of the 20S proteasome in HER2+/ERα+ breast cancer, leading to an increased accumulation of Δ16HER2, which efficiently couples to HER3 and activates the PI3K-AKT-mTOR pathway. In particular, Δ16HER2/HER3 heterodimers trigger the mTORC1/p70S6K/4EBP1 signaling axis inducing an up-regulation of protein synthesis and cell growth. On the other hand, resveratrol inhibits mTORC2 and promotes phosphorylation of PTEN, reducing its catalytic activity, thereby enhancing PI3K-mediated AKT activation, while feedback loops compensate it.

repression. Our results also support the hypothesis that resveratrol may exert its anti-ERα action by impairing proteasome-mediated ERα activation. Interestingly, UPS has been described as necessary for a correct activation of ERα [40] and some proteasome inhibitors have been shown to repress ERα gene expression [41, 42]. Moreover, a recently published work has proposed that mTORC1 inhibition activates the Ubiquitin/Proteasome System (UPS) and autophagy [43], thus sustaining an inverse correlation between mTOR signaling and proteasome activity. Consistently, we have reported the resveratrol-induced over-expression of Δ16HER2 eventually results in the activation of mTORC1/p70S6K/p4EBP1 axis. This observation recapitulates a relevant molecular pathway implicated in endocrine resistance development [44] and may explain why the combination of resveratrol and mTORC1 inhibitors have stood out as more effective anti-tumor therapy than single-agent approaches [45, 46].

Interestingly, despite the claimed anti-aging effect of resveratrol, longevity studies have demonstrated that combination of rapamycin and resveratrol was not able to prolong the overall mice survival as mTOR inhibition does [47]. This aspect further strengthens the connection between resveratrol and mTOR pathway and suggests that our data might be extended to a broader context such as aging-related dysfunctions exhibiting a preferential activation of mTORC1 pathway [48].

In conclusion, this study provides evidence that the effects of resveratrol treatment depend on intrinsic molecular cancer properties, exerting a tumor-promoting effect in luminal B breast cancer subtype by acting as proteasome inhibitor and inducing a multi-target molecular rearrangement (Figure 6).

MATERIALS AND METHODS

Animals

Δ16HER2 female mice were housed under controlled temperature (20 °C) and circadian cycle (12 hours light/12 hours dark) in the animal facility of University of Camerino. The animals were fed on chow diet and tap water *ad libitum.*

Cell culture

CAM6 cells, a Δ16HER2-expressing epithelial tumor cell line derived from a mammary carcinoma spontaneously arisen in a Δ16HER2 female [17] and BT474 cell line were maintained in DMEM (Lonza) supplemented with 10% FBS (Gibco, Life Technologies) and 1% penicillin-streptomycin (Gibco, Life Technologies). Cells were cultured at 37 °C under humidified atmosphere with 5% CO2.

Resveratrol treatment

8 week-old Δ16HER2 female mice were randomly divided into two experimental groups (n=9 each). One group underwent resveratrol treatment and the other was given control vehicle, as previously reported [12]. Briefly, resveratrol (Sigma) was dissolved in methanol to be then diluted in tap water to a final concentration of 1mg/l. Being the daily water intake of about 4 ml/mouse, 4 μg resveratrol/mouse was the daily dose assumed. The control mice were supplemented with 0.02% methanol in tap water (vehicle). Both vehicle- and resveratrol-containing water was renewed twice a week. During the treatment (from 8-weeks to 23-weeks of age), mice were weekly monitored for mammary tumor development by palpation and growing masses greater than 2 mm in mean diameter were regarded as tumors. Two perpendicular diameters (a and b) were measured on each tumor using caliper and volumes were calculated by the $V = \pi/6[(a+b)/2]^3$ formula. All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and were approved by the Ethic Committee on Animal Use of the University of Camerino (protocol number 14/2012). At the end of experiment mice were euthanized and tumors and lungs samples were collected for subsequent analyses. For the *in vitro* experiments resveratrol was dissolved in DMSO (Sigma) and then diluted in complete DMEM to usage concentrations (10-100 μM). 17β-estradiol (Calbiochem) was used as control estrogen treatment (stock solution 40 mM in 100% ethanol and diluted in complete DMEM to 10 nM), as previously described [41]. Cells were treated for 24 hours with resveratrol/17β-estradiol- or vehicle-containing medium (0.02% DMSO).

Cell viability assay

Resveratrol effect on cell viability was evaluated by seeding $4x10^4$ cells/well (CAM6 cells) or $2.5x10^4$ cells/well (BT474 cells) in 96-well plates in complete DMEM. The day after, fresh medium containing appropriate resveratrol concentrations was added. Cell viability was determined via MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma Aldrich (St. Louis, MO).

Immunohistochemistry (IHC) and confocal microscopy analyses

Tumors and lungs harvested from mice were fixed in 4% paraformaldehyde solution for 24-48 hours at room

temperature and then embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin or immunostained with anti-mouse PCNA monoclonal antibody (Dako) or with anti-cleaved caspase-3 monoclonal antibody (MAB835, R&D Systems, Milan, Italy). After washing, they were overlaid with appropriate secondary antibody. Immunostaining was developed with Vulcan Fast Red (Biocare) alkaline phosphatase method. The number of cleaved caspase-3 positive cells was evaluated on digital images of controls and resveratrol tumors (10 per group, 5 x 400 microscopic fields per tumor). As it regards confocal microscopy analysis, after incubation in blocking buffer for 20 minutes, tissue sections were incubated for 1 hour at 37 °C with anti-NEU polyclonal antibody (diluted 1:50, Santa Cruz). Nuclei staining was performed using DRAQ5 probe (Life technologies) according to the manufacturer instructions. After washing, the coverslips were incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (diluted 1:100, Life technologies) for 1 hour at 37 °C. Slides were viewed using a Zeiss LSM 510 Meta Confocal Microscope. Quantitative measures were obtained via ImageJ software.

Protein extraction and western blot analysis

Tumors and treated cells were homogenized in RIPA buffer (0.1% SDS, 1% NP40, 0.5% CHAPS) supplemented with protease inhibitors aprotinin, sodium orthovanadate and phenylmehylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). After 30 minutes' incubation on ice, whole tumor lysates were centrifuged at 14000 rpm, 4 °C, for 20 minutes. The supernatant was collected and proteins were quantified via Bradford assay (Bio-Rad). For western blot analysis equal amounts of protein lysates were separated onto Criterion™ TGX™ precast gels (Bio-Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) using Criterion™ Blotter (Bio-Rad). After blocking in 5% BSA-TBS-T for 1 hour, membranes were incubated with primary antibodies at 4 °C overnight. Secondary antibody-binding was performed at room temperature for 1 hour. After TBS-T washing, protein bands were incubated with LiteAblot PLUS reagent (Euroclone) and detected via ChemiDoc™ XRS+ System (Bio-Rad). Densitometry analysis was performed through ImageJ software. Detailed information about the used antibodies is available in Table S1, Supplementary Information.

Co-immunoprecipitation

300 µg of tumor cell lysates were incubated overnight with anti-NEU polyclonal antibody (diluted 1:200, Santa Cruz) at 4 °C. After washing in RIPA buffer at 2500 g, 4 °C for 5 minutes, 200 µl of protein G Sepharose 4 Fast Flow beads (GE Healthcare) were added to each tube. Incubation was performed for 4 hours at 4 °C under gentle rocking. Following washing procedure, the samples were boiled in Laemli loading buffer for 5 minutes: the collected supernatants underwent western blot procedure.

mRNA extraction, RT-PCR and qRT-PCR

Total RNA was extracted from liquid nitrogen cryo-preserved tumors via TRIzol® reagent (Life Technologies) following the manufacturer instructions. RNA was quantified by measuring 260 nm absorbance via NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA purity was considered good with A260/A280 ratio ≥ 2.0 and A260/A230 ratio ≥ 1.7. RNA (2 µg/tube) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Semi-quantitative RT-PCR was performed using Perfect Taq DNA polymerase (5-PRIME) and Eppendorf thermal Cycler. SYBR Premix Ex Taq (Tli RNaseH Plus) reagent (Takara) was used for qRT-PCR analysis. β-actin was taken as housekeeping gene and standard curves for target genes and β-actin were included to evaluate reaction efficiency. Experiments were performed in triplicates. A 2-steps amplification program was carried out on Bio-Rad iCycler Thermal Cycler with iQ5 Multicolor Real-Time PCR Detection System. Primers used: Δ16HER2 forward: 5'-CACCCACTCCCCTCTGAC-3'; Δ16HER2 reverse: 5'-GCTCCACCAGCTCCGTTTCCTG-3'; β-actin forward: 5'-CAAGGCCAACCGCGAGAAGAT-3'; β-actin reverse: 5'-GTCCCGGCCAGCCAGGTCC AG-3'.

Proteasome activity

The chymotrypsin-like activity of the 20S proteasome was assayed on tumor and cell extracts using the fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (sLLVY-NH-Mec, Sigma-Aldrich). Briefly, tissues/cells were homogenized on ice via a Potter-Elvehjem apparatus in a buffer consisting of 50 mM HEPES/KOH pH 7.8, 1 mM Dithiothreitol and 0.25 M Sucrose. The lysates were then cleared by centrifugation at 12.000 rpm for 10 minutes in a refrigerated Eppendorf centrifuge. Twenty and 40 µg of total proteins from each tissue sample were incubated at 37°C in 100 mM HEPES/KOH buffer, pH 7.8, 5 mM $MgCl_2$ and 10 mM KCl. Cell extracts (10 µg) were preincubated for 30 min at 37°C in the same buffer with the addition of different concentrations of resveratrol or with only the vehicle (DMSO). In both cases the reaction was initiated by addition of the fluorigenic substrate to a final concentration of 0.2 mM. The

breakdown of the peptide was monitored for 30/45 min using a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtech GmbH, Offenburg, Germany) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Proteasome activity in each sample, expressed as fluorimetric units min^{-1} mg^{-1}, was calculated by submitting data to linear regression analysis (R^2 >0.99).

Statistical analysis

Quantitative data are presented as means ± SEM from three independent experiments. The significance of differences was evaluated with two-tailed Students t-test, or one-way ANOVA followed by Bonferroni post-test. Statistical analysis was carried out with GraphPad Prism5.

AUTHOR CONTRIBUTIONS

AC, BC, WK performed the laboratory work. BM, CR performed UPS analysis. HA, IM performed IHC. AC, BC, AA, MC designed the experiments. AC, BC, CR, BM, MM, AA, MC analyzed the data. AC, BC, AA, MC conceived the study. AA, MC coordinated the study. AC, MC wrote the manuscript. All authors critically discussed the data, read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors have no conflict of interests to declare.

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REFERENCES

1. Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res. 2005; 49:462–71. doi: 10.1002/mnfr.200400094

2. Tang HY, Shih A, Cao HJ, Davis FB, Davis PJ, Lin HY. Resveratrol-induced cyclooxygenase-2 facilitates p53-dependent apoptosis in human breast cancer cells. Mol Cancer Ther. 2006; 5:2034–42. doi: 10.1158/1535-7163.MCT-06-0216

3. Shi Y, Yang S, Troup S, Lu X, Callaghan S, Park DS, Xing Y, Yang X. Resveratrol induces apoptosis in breast cancer cells by E2F1-mediated up-regulation of ASPP1. Oncol Rep. 2011; 25:1713–19. doi: 10.3892/or.2011.1248

4. Pozo-Guisado E, Alvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernandez-Salguero PM. The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. Biochem Pharmacol. 2002; 64:1375–86. doi: 10.1016/S0006-2952(02)01296-0

5. Garvin S, Ollinger K, Dabrosin C. Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo. Cancer Lett. 2006; 231:113–22. doi: 10.1016/j.canlet.2005.01.031

6. Fukui M, Yamabe N, Zhu BT. Resveratrol attenuates the anticancer efficacy of paclitaxel in human breast cancer cells in vitro and in vivo. Eur J Cancer. 2010; 46:1882–91. doi: 10.1016/j.ejca.2010.02.004

7. Castillo-Pichardo L, Cubano LA, Dharmawardhane S. Dietary grape polyphenol resveratrol increases mammary tumor growth and metastasis in immuno-compromised mice. BMC Complement Altern Med. 2013; 13:6. doi: 10.1186/1472-6882-13-6

8. Bhat KP, Lantvit D, Christov K, Mehta RG, Moon RC, Pezzuto JM. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. Cancer Res. 2001; 61:7456–63.

9. Levenson AS, Gehm BD, Pearce ST, Horiguchi J, Simons LA, Ward JE 3rd, Jameson JL, Jordan VC. Resveratrol acts as an estrogen receptor (ER) agonist in breast cancer cells stably transfected with ER alpha. Int J Cancer. 2003; 104:587–96. doi: 10.1002/ijc.10992

10. Singh B, Shoulson R, Chatterjee A, Ronghe A, Bhat NK, Dim DC, Bhat HK. Resveratrol inhibits estrogen-induced breast carcinogenesis through induction of NRF2-mediated protective pathways. Carcinogenesis. 2014; 35:1872–80. doi: 10.1093/carcin/bgu120

11. Bartolacci C, Andreani C, Amici A, Marchini C. Walking a Tightrope: A Perspective of Resveratrol Effects on Breast Cancer. Curr Protein Pept Sci. 2017. Epub ahead of print.

12. Provinciali M, Re F, Donnini A, Orlando F, Bartozzi B, Di Stasio G, Smorlesi A. Effect of resveratrol on the development of spontaneous mammary tumors in HER-2/neu transgenic mice. Int J Cancer. 2005; 115:36–45. doi: 10.1002/ijc.20874

13. Yee LD, Agarwal D, Rosol TJ, Lehman A, Tian M, Hatton J, Heestand J, Belury MA, Clinton SK. The

inhibition of early stages of HER-2/neu-mediated mammary carcinogenesis by dietary n-3 PUFAs. Mol Nutr Food Res. 2013; 57:320–27. doi: 10.1002/mnfr.201200445

14. Mitra D, Brumlik MJ, Okamgba SU, Zhu Y, Duplessis TT, Parvani JG, Lesko SM, Brogi E, Jones FE. An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance. Mol Cancer Ther. 2009; 8:2152–62. doi: 10.1158/1535-7163.MCT-09-0295

15. Alajati A, Sausgruber N, Aceto N, Duss S, Sarret S, Voshol H, Bonenfant D, Bentires-Alj M. Mammary tumor formation and metastasis evoked by a HER2 splice variant. Cancer Res. 2013; 73:5320–27. doi: 10.1158/0008-5472.CAN-12-3186

16. Marchini C, Gabrielli F, Iezzi M, Zenobi S, Montani M, Pietrella L, Kalogris C, Rossini A, Ciravolo V, Castagnoli L, Tagliabue E, Pupa SM, Musiani P, et al. The human splice variant Δ16HER2 induces rapid tumor onset in a reporter transgenic mouse. PLoS One. 2011; 6:e18727. doi: 10.1371/journal.pone.0018727

17. Tilio M, Gambini V, Wang J, Garulli C, Kalogris C, Andreani C, Bartolacci C, Elexpuru Zabaleta M, Pietrella L, Hysi A, Iezzi M, Belletti B, Orlando F, et al. Irreversible inhibition of Δ16HER2 is necessary to suppress Δ16HER2-positive breast carcinomas resistant to Lapatinib. Cancer Lett. 2016; 381:76–84. doi: 10.1016/j.canlet.2016.07.028

18. Vaught DB, Stanford JC, Young C, Hicks DJ, Wheeler F, Rinehart C, Sánchez V, Koland J, Muller WJ, Arteaga CL, Cook RS. HER3 is required for HER2-induced preneoplastic changes to the breast epithelium and tumor formation. Cancer Res. 2012; 72:2672–82. doi: 10.1158/0008-5472.CAN-11-3594

19. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004; 14:1296–302. doi: 10.1016/j.cub.2004.06.054

20. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002; 110:163–75. doi: 10.1016/S0092-8674(02)00808-5

21. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004; 18:1926–45. doi: 10.1101/gad.1212704

22. Vazquez F, Ramaswamy S, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol. 2000; 20:5010–18. doi: 10.1128/MCB.20.14.5010-5018.2000

23. Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, Balmain A. FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science. 2008; 321:1499–502. doi: 10.1126/science.1162981

24. Harris KF, Shoji I, Cooper EM, Kumar S, Oda H, Howley PM. Ubiquitin-mediated degradation of active Src tyrosine kinase. Proc Natl Acad Sci USA. 1999; 96:13738–43. doi: 10.1073/pnas.96.24.13738

25. Hakak Y, Martin GS. Ubiquitin-dependent degradation of active Src. Curr Biol. 1999; 9:1039–42. doi: 10.1016/S0960-9822(99)80453-9

26. Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol Cell. 2002; 9:945–56. doi: 10.1016/S1097-2765(02)00519-1

27. Qureshi AA, Guan XQ, Reis JC, Papasian CJ, Jabre S, Morrison DC, Qureshi N. Inhibition of nitric oxide and inflammatory cytokines in LPS-stimulated murine macrophages by resveratrol, a potent proteasome inhibitor. Lipids Health Dis. 2012; 11:76. doi: 10.1186/1476-511X-11-76

28. Kwon KJ, Kim JN, Kim MK, Lee J, Ignarro LJ, Kim HJ, Shin CY, Han SH. Melatonin synergistically increases resveratrol-induced heme oxygenase-1 expression through the inhibition of ubiquitin-dependent proteasome pathway: a possible role in neuroprotection. J Pineal Res. 2011; 50:110–23.

29. Tung BT, Rodríguez-Bies E, Talero E, Gamero-Estévez E, Motilva V, Navas P, López-Lluch G. Anti-inflammatory effect of resveratrol in old mice liver. Exp Gerontol. 2015; 64:1–7. doi: 10.1016/j.exger.2015.02.004

30. Pugazhenthi S, Qin L, Reddy PH. Common neurodegenerative pathways in obesity, diabetes, and Alzheimer's disease. Biochim Biophys Acta. 2016S0925-4439(16)30097-7.

31. Liedtke C, Broglio K, Moulder S, Hsu L, Kau SW, Symmans WF, Albarracin C, Meric-Bernstam F, Woodward W, Theriault RL, Kiesel L, Hortobagyi GN, Pusztai L, Gonzalez-Angulo AM. Prognostic impact of discordance between triple-receptor measurements in primary and recurrent breast cancer. Ann Oncol. 2009; 20:1953–58. doi: 10.1093/annonc/mdp263

32. Nomura Y, Tashiro H, Shinozuka K. Changes of steroid hormone receptor content by chemotherapy and/or endocrine therapy in advanced breast cancer. Cancer. 1985; 55:546–51.

doi: 10.1002/1097-0142(19850201)55:3<546::AID-CNCR2820550313>3.0.CO;2-V

33. Kuukasjärvi T, Kononen J, Helin H, Holli K, Isola J. Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. J Clin Oncol. 1996; 14:2584–89.

34. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med. 2011; 62:233–47. doi: 10.1146/annurev-med-070909-182917

35. Creighton CJ, Massarweh S, Huang S, Tsimelzon A, Hilsenbeck SG, Osborne CK, Shou J, Malorni L, Schiff R. Development of resistance to targeted therapies transforms the clinically associated molecular profile subtype of breast tumor xenografts. Cancer Res. 2008; 68:7493–501. doi: 10.1158/0008-5472.CAN-08-1404

36. Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, Paulazzo G, Lyass L, Trusk P, Hill J, Harris J, Spector NL. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. Proc Natl Acad Sci USA. 2006; 103:7795–800. doi: 10.1073/pnas.0602468103

37. Munzone E, Curigliano G, Rocca A, Bonizzi G, Renne G, Goldhirsch A, Nolè F. Reverting estrogen-receptor-negative phenotype in HER-2-overexpressing advanced breast cancer patients exposed to trastuzumab plus chemotherapy. Breast Cancer Res. 2006; 8:R4. doi: 10.1186/bcr1366

38. Leary AF, Drury S, Detre S, Pancholi S, Lykkesfeldt AE, Martin LA, Dowsett M, Johnston SR. Lapatinib restores hormone sensitivity with differential effects on estrogen receptor signaling in cell models of human epidermal growth factor receptor 2-negative breast cancer with acquired endocrine resistance. Clin Cancer Res. 2010; 16:1486–97. doi: 10.1158/1078-0432.CCR-09-1764

39. Engel RH, Kaklamani VG. HER2-positive breast cancer: current and future treatment strategies. Drugs. 2007; 67:1329–41. doi: 10.2165/00003495-200767090-00006

40. Lonard DM, Nawaz Z, Smith CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell. 2000; 5:939–48. doi: 10.1016/S1097-2765(00)80259-2

41. Thaler S, Thiede G, Hengstler JG, Schad A, Schmidt M, Sleeman JP. The proteasome inhibitor Bortezomib (Velcade) as potential inhibitor of estrogen receptor-positive breast cancer. Int J Cancer. 2015; 137:686–97. doi: 10.1002/ijc.29404

42. Powers GL, Ellison-Zelski SJ, Casa AJ, Lee AV, Alarid ET. Proteasome inhibition represses ERalpha gene expression in ER+ cells: a new link between proteasome activity and estrogen signaling in breast cancer. Oncogene. 2010; 29:1509–18. doi: 10.1038/onc.2009.434

43. Zhao J, Zhai B, Gygi SP, Goldberg AL. mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. Proc Natl Acad Sci USA. 2015; 112:15790–97. doi: 10.1073/pnas.1521919112

44. Yamnik RL, Digilova A, Davis DC, Brodt ZN, Murphy CJ, Holz MK. S6 kinase 1 regulates estrogen receptor alpha in control of breast cancer cell proliferation. J Biol Chem. 2009; 284:6361–69. doi: 10.1074/jbc.M807532200

45. Alayev A, Berger SM, Holz MK. Resveratrol as a novel treatment for diseases with mTOR pathway hyperactivation. Ann N Y Acad Sci. 2015; 1348:116–23. doi: 10.1111/nyas.12829

46. He X, Wang Y, Zhu J, Orloff M, Eng C. Resveratrol enhances the anti-tumor activity of the mTOR inhibitor rapamycin in multiple breast cancer cell lines mainly by suppressing rapamycin-induced AKT signaling. Cancer Lett. 2011; 301:168–76. doi: 10.1016/j.canlet.2010.11.012

47. Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, et al. Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. J Gerontol A Biol Sci Med Sci. 2011; 66:191–201. doi: 10.1093/gerona/glq178

48. Sharp Z, Hasty P. (2015). mTOR, aging, and cancer: a dangerous link. mTOR inhibition for cancer therapy: past, present and future. Springer-Verlag France, pp. 277-292.

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Review

Regulation of Cell Signaling Pathways and miRNAs by Resveratrol in Different Cancers

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Abstract: Genomic and proteomic studies have helped improve our understanding of the underlying mechanism(s) of cancer development and progression. Mutations, overexpressed oncogenes, inactivated/downregulated tumor suppressors, loss of apoptosis, and dysregulated signal transduction cascades are some of the well-studied areas of research. Resveratrol has gained considerable attention in the last two decades because of its pleiotropic anticancer activities. In this review, we have summarized the regulation of WNT, SHH (sonic hedgehog)/GLI (glioma-associated oncogene homolog), TGFβ1 (transforming growth factor beta 1)/SMAD, NOTCH, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), STAT (signal transducer and activator of transcription), and microRNAs by resveratrol in different cancers. The importance of these signaling pathways in cancer progression, along with their modulation by resveratrol, is discussed. Further, we also evaluate the mechanisms and implications of the downregulation of oncogenic miRNAs and the upregulation of tumor suppressor miRNAs by resveratrol, both of which also define its ability to inhibit tumor growth and metastasis. It is envisioned that designing effective clinical trials will be helpful for the identification of resveratrol responders and non-responders and the elucidation of how this phytochemical can be combined with current therapeutic options to improve their clinical efficacy and reduce off-target effects.

Keywords: resveratrol; cell signaling; miRNAs

1. Introduction

Using high-throughput technologies, it has been shown that the bulk tumor includes a diversified collection of cells that harbor characteristically unique molecular features with variable levels of sensitivity to therapeutic options [1,2]. This heterogeneity results in an uneven distribution of genetically distinct subpopulations of tumor cells across and within disease sites (spatial heterogeneity) [3,4]. In addition, tumors are marked by temporal heterogeneity, wherein cells temporally change their molecular makeup [3,4]. Combining high-throughput genotyping platforms and next-generation sequencing technologies has brought us one step closer in the pursuit for genetic variants, from SNPs (single nucleotide polymorphisms) to large-scale copy number variants. Translational and functional studies have demystified the underlying mechanisms and bio-molecular signatures of difficult-to-treat cancers. Natural product chemistry and the discovery of drugs from plants (phytochemicals) has enjoyed a renaissance in the past decade [5,6]. Based on the insights gleaned from decades of research, it seems clear that off-target effects of modern medicines and rapidly developing resistance against different therapeutics are some of the challenges that must be overcome.

Much attention is currently being given to the identification of natural products that can modulate multiple targets, are significantly effective, and minimize off-target effects [6,7]. In accordance with this approach, there is a rapidly growing list of natural products with considerable medicinal importance and substantial pharmacological properties [8].

Resveratrol, a polyphenolic phytoalexin, is an extensively studied phytochemical with remarkable pharmacological properties and a unique ability to modulate multiple targets in different cancers [9]. It regulates oxidative stress, which is an interesting aspect of its anticancer activity [10,11]. Resveratrol has a molecular weight of 228.247 g/mol and a topological polar surface area of 60.7 A^2 [12]. Because of the rapidly accumulating wealth of information about resveratrol, many review papers have summarized important biological mechanisms targeted by resveratrol for the treatment of different cancers [13–16]. Most recently published reviews have provided a summary of the role of resveratrol in different cancers; however, in recent years, none of the reviews have provided an in-depth and comprehensive overview of the cell signaling pathways targeted by resveratrol in different cancers [17–19]. We have exclusively focused on various signal transduction cascades and the miRNAs that are modulated by resveratrol in different cancers. In particular, in this review, we have summarized the regulation of TGFβ1/SMAD, WNT, SHH/GLI, NOTCH, TRAIL, STAT pathways, and the oncogenic as well as tumor suppressor miRNAs, by resveratrol.

2. Regulation of the TGFβ1/SMAD Pathway

TGFβ1/SMAD pathway is well known to contribute to cancer development and progression (Figure 1). Signalosome formed by phosphorylated receptor-mediated SMADs triggers the accumulation of active SMAD complexes in the nucleus, which transcriptionally upregulate target genes in conjunction with transcriptional factors, histone modifiers, and chromatin-remodeling machinery [20]. In this section, we will provide an overview of the mode of action of resveratrol to inhibit SMAD-mediated intracellular signaling. Resveratrol effectively inhibits SMAD proteins by either affecting post-translational modifications by different enzymes to target SMAD proteins for degradation or by inhibiting the phosphorylation and activation of SMADs.

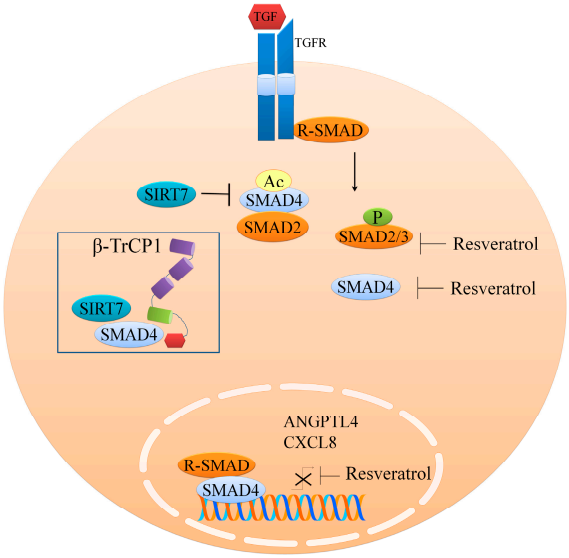

Figure 1. TGF/TGFR signaling axis. SMAD proteins play a central role in transducing the signals intracellularly. SIRT7 modulates SMAD4 degradation via β-transducin repeat-containing protein (β-TrCP1). SMAD4, β-TrCP1, and SIRT7 complex formation results in shutdown of the TGF pathway. SMAD: Sma Mothers Against Decapentaplegic; β-TrCP: β-transducin repeat-containing protein; CXCL8: chemokine CXC motif, ligand 8; TGF: transforming growth factor; ANGPTL4: angiopoietin-like 4.

In a study demonstrating regulation of the TGFβ1/SMAD pathway by resveratrol, lung metastases were developed in Balb/c mice by intravenous injection of highly metastatic murine 4T1 cells [21]. Further, intravenous injection of the SIRT7-inhibited development of these metastatic nodules and SIRT7-knockdown induced activation of the TGF-β pathway in 4T1 and MDA-MB-231 cells, while SIRT7 overexpression exerted inhibitory effects on the TGF-β pathway. SIRT7 physically interacted with and de-acetylated SMAD4 [21]. Significantly increased levels of acetylated lysine residues of SMAD4 were noted in SIRT7-silenced cancer cells. The binding affinity of SMAD4 for SMAD2 markedly decreased in cells that ectopically expressed SIRT7. When protein synthesis was inhibited by cycloheximide, ectopically expressed SIRT7 promoted SMAD4 degradation, and SIRT7 inhibition protected SMAD4 from degradation machinery [21]. Furthermore, K428R, which mimicked a hypo-acetylated form of SMAD4, accelerated its degradation, whereas K428Q, which resembled a hyper-acetylated form of SMAD4, inhibited its degradation. SIRT7 deletion by CRISPR/Cas9 inhibited HA-SMAD4 ubiquitination, whereas SIRT7 overexpression increased ubiquitination. SIRT7 modulated SMAD4 degradation by β-transducin repeat-containing protein (β-TrCP1). Protein studies have provided evidence of the interaction of SMAD4, β-TrCP1, and SIRT7 [21] (Figure 1). β-TrCP1 inhibition abolished the increased ubiquitination and accelerated SMAD4 degradation caused by ectopically expressed SIRT7. Resveratrol dose-dependently enhanced deacetylation of HA-SMAD4. It also exerted repressive effects on TGF-β1-induced mesenchymal morphology, downregulated SMAD4, and suppressed cellular migration. TGF-β responsive genes *ANGPTL4* and *CXCL8*, required for breast cancer lung metastases and the development of resistance against drugs, were considerably repressed by resveratrol [21].

Resveratrol has been reported to reduce phosphorylated-SMAD2/3 in colorectal cancer LoVo cells [22]. Resveratrol (150 mg/Kg) reduced metastatic lesions in the lungs and liver of mice orthotopically transplanted with LoVo cells. In alveolar rhabdomyosarcoma PLA-802 cells, resveratrol (40 μmol/L) downregulated SMAD4 [23]. Upon treatment with resveratrol (20 μmol/L), no significant changes in expression of TGF-β1 and SMAD4 were noticed; however, at a dose of 40 μmol/L, resveratrol significantly decreased the expression of SMAD4 and TGF-β1 [23]. Resveratrol decreased expression of TGFβ-2 in SCCs and skin of $p53^{+/-}$/SKH-1 mice chronically irradiated with UVB [24]. There was a 2–3-fold increase in the invasive capacity of cells that expressed constitutively active Akt. Overexpression of active Akt induced TGF-β2 in the absence of resveratrol, which suggested a clear association between TGFβ2 and Akt. Active Akt significantly impaired resveratrol-induced inhibitory effects on TGF-β2. Resveratrol dose-dependently reduced phospho-SMAD2/3 levels and completely abolished the phosphorylation of SMAD2/3 at a dose of 100 μM [24].

These findings indicate that resveratrol affects the TGFβ1/SMAD pathway via post-translational modifications, either by degrading SMADs or inhibiting their activity by blocking the phosphorylation of SMADs.

3. Regulation of the WNT Pathway

A WNT ligand is a secreted glycoprotein that binds to Frizzled receptors and results in the formation of a larger cell surface complex with low-density lipoprotein-receptor-related protein (LRP5/6) [25] (Figure 2). In the absence of any signal, β-catenin is post-translationally modified by the APC/AXIN/GSK-3β-complex leading to its ubiquitination and degradation by proteasomal machinery. When the WNT pathway is active, β-catenin gets sequestered away from degradation machinery and moves into the nucleus to trigger the expression of target genes. There has been a focus on strategies to inhibit the transportation of β-catenin into the nucleus [25,26].

Resveratrol can downregulate WNT2 and upregulate AXIN2 in Colo16 cells [27]. Furthermore, there was an earlier induction of apoptosis in resveratrol-treated β-catenin-silenced Colo16 cells [27]. Resveratrol dose-dependently decreased exogenously overexpressed Myc-tagged TCF4 protein in colorectal cancer cells [28] and increased TCF4 phosphorylation through ERK- and p38-MAPK-modulated pathways. Knockdown of TCF4 reduced TCF/β-catenin-regulated transcriptional activities and sensitized cancer cells to resveratrol-mediated apoptosis [28].

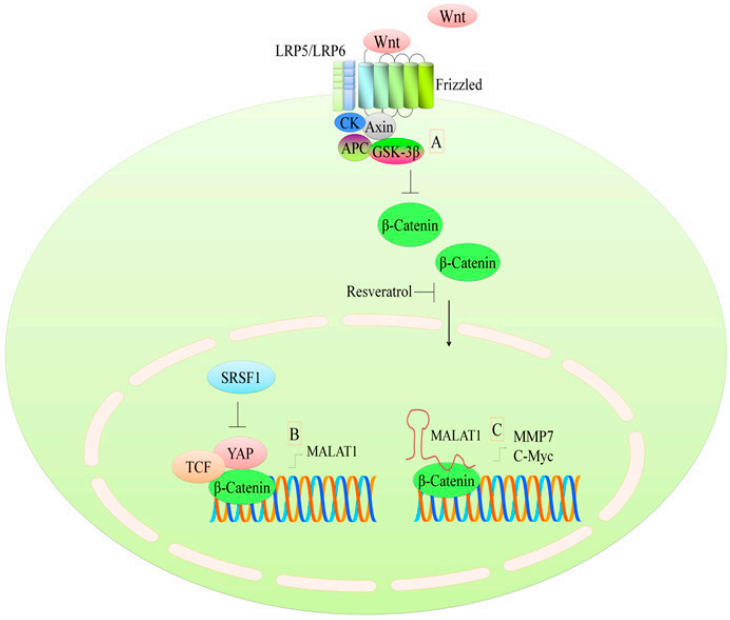

Figure 2. (**A**) WNT/β-catenin signaling axis. Resveratrol effectively inhibits the nuclear accumulation of β-catenin. β-catenin works synchronously with various proteins to modulate the transcription of different genes; (**B**) SRSF1 inhibits YAP and represses the β-catenin-mediated upregulation of MALAT1; (**C**) β-catenin works with MALAT1 and increases the expression of MMP7 and C-Myc. APC: adenomatous polyposis coli; CK: casein kinase; GSK-3β: glycogen synthase kinase 3 beta; LRP: low-density lipoprotein-receptor-related protein; MALAT1: metastasis-associated lung adenocarcinoma transcript 1; MMP&: matrix metalloproteinase-7; SRSF1: serine and arginine rich splicing factor 1; TCF: T-cell factor; YAP: YES-associated protein; 65-KD.

Response to resveratrol was noted to be highly heterogeneous among glioma stem cells. Resveratrol inhibited cell proliferation, increased cell mortality, and strongly reduced the motility of the cells [29]. In GBM2, G166, GBM7, and G179 cell lines, both WNT1 and MYC were upregulated, while in G144 cells these two genes were downregulated by resveratrol. FZD4 and TCF7 changed only in 3 out of 7 GSC lines after resveratrol treatment [29]. Resveratrol significantly reduced cyclin D1 and β-catenin in xenograft breast tumors [30]. β-catenin overexpression markedly reduced resveratrol-mediated cytotoxic effects, which indicated that resveratrol inhibited breast cancer stem-like cells and induced autophagy, at least partially, by suppression of the Wnt/β-catenin cascade [30]. In HCT116 cells, resveratrol reduced β-catenin [31]. Resveratrol did not appear to influence the total levels of GSK-3β but did decrease GSK-3β phosphorylation. A β-catenin/Tcf4 reporter assay provided evidence that resveratrol, concentration-dependently, inhibited reporter activity [31].

β-catenin has been shown to interact with different non-coding RNAs and transcriptionally upregulate their expression, thereby stimulating the expression of a target gene network. Resveratrol increases the cytosolic levels of β-catenin with a concomitant inhibition of its nuclear accumulation (Figure 2), with little effects on the total cellular β-catenin [32]. Resveratrol exerted inhibitory effects on the Wnt/β-catenin pathway through the regulation of MALAT1. The use of lithium chloride inhibited GSK3β from binding to β-catenin and triggered Wnt/β-catenin pathway activation. Resveratrol dose-dependently downregulated long non-coding RNA-MALAT1 [32]. Knockdown of MALAT1 impaired the nuclear accumulation of β-catenin, which resulted in the transcriptional repression of MMP7 and c-Myc, while MALAT1 overexpression promoted the transcriptional upregulation of MMP7 and c-Myc (Figure 2). The data clearly suggest that MALAT1 and β-catenin co-existed in the nucleus, and the targeted inhibition of MALAT1 is necessary to inhibit synchronous activity of MALAT1 and β-catenin for the transcriptional repression of target genes [32]. YES-associated protein (YAP)

upregulated MALAT1 transcriptional and post-transcriptional levels, whereas serine/arginine-rich splicing factor 1 (SRSF1) resisted YAP-mediated effects [33]. SRSF1 exerted inhibitory effects on YAP activity and prevented its co-existence with TCF/β-catenin on the promoter region of MALAT1 [33] (Figure 2). These two reports together are helpful in putting different pieces of an incomplete jigsaw puzzle together. It seems clear that β-catenin co-operates with different proteins to trigger the expression of MALAT1 and works synchronously with MALAT1 to drive the expression of WNT signaling target genes.

Despite these studies on the regulation of the WNT pathway by resveratrol, there remain a few outstanding questions that need to be adequately addressed to understand mechanistic details. For example, the modulation of LRP5/6 and SFRPs by resveratrol has not been sufficiently studied. Future studies need to comprehensively focus on the detailed mechanism(s) of regulation of WNT signaling by resveratrol so that the information can be utilized for the targeting of WNT signaling by resveratrol in clinical settings.

4. Regulation of the SHH/GLI Pathway

Inappropriate activation of the Sonic hedgehog–GLI signaling cascade has been extensively studied in different cancers [34–36]. BCR-ABL is an oncoprotein that re-wires intracellular signaling cascades in chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia [37]. In vitro and preclinical studies have shown that imatinib exerted preferential inhibitory effects on the viability and expression of BCR-ABL in imatinib-sensitive K562 cells, but imatinib was not effective against K562R cells that overexpressed BCR-ABL. Both K562R (imatinib-resistant) and parental K562 cells expressed mRNAs of molecules involved in the SHH pathway, including SHH, Smoothened (SMO), Patched (PTCH), and GLI-1. There was a significant reduction in BCR-ABL in GLI-1-silenced K562R and K562 cells. The use of SHH peptide not only enhanced GLI-1 and SHH but also upregulated BCR-ABL in tested cell lines. Resveratrol effectively inhibited SMO and BCR-ABL and exerted inhibitory effects on the nuclear accumulation of GLI proteins to transcriptionally upregulate the expression of cancer promoting genes [37]. PTCH, SMO, and GLI-1 were also inhibited in resveratrol-treated colorectal cancer HCT116 cells [38]. Resveratrol inhibited the nuclear accumulation of GLI-1 in interleukin-6 (IL-6)-stimulated HL-60 cells [39].

Resveratrol and cyclopamine have been reported to synergistically inhibit metastases and reduce the invasive potential of gastric SGC-7901 cancer cells [40]. Resveratrol was noted to inhibit GLI-1 activity and downregulate Snail and *N*-cadherin in SGC-7901 cells. Previously, it has been reported that Snail repressed the expression of E-cadherin, but resveratrol upregulated E-cadherin and markedly reduced the metastasizing potential of SGC-7901 cells [40]. Based on the reports discussed above, resveratrol seems to significantly affects the SHH-Gli pathway.

It is now well established that CpG islands are heavily hypomethylated in cancer cells [41]. Resveratrol enhances the methylation levels of CpG sites within enhancers of MAML2 and GLI2. Interestingly, active enhancers are enriched with H3K27ac. Data obtained through the chromatin-immunoprecipitation technique provided information about the presence of histone marks at MAML2 in cancer cells but its occupancy reduced considerably in resveratrol-treated cancer cells [41]. Occupancy of activating histone marks (H3K9acetylation) and repressive histone marks (H3K27tri-methylation) was biochemically analyzed. Data suggested a 30% decrease in H3K9ac and a 2-fold increase in H3K27me3 occupancy within the MAML2 enhancer, whereas resveratrol induced an increase in DNA methylation levels. OCT1, a transcription factor, transcriptionally upregulated MAML2; however, resveratrol inhibited the binding of OCT1 to the MAML2 enhancer. Resveratrol with a concentration of 15 μM promoted the accumulation of DNMT3B in the MAML2 enhancer in MCF10CA1a cells, resulting in epigenetic inactivation [41].

5. Regulation of the NOTCH Pathway: A Double-Edged Sword

The NOTCH pathway is involved in the regulation of different stages of cancer [42,43]. Although some reports document efficient inhibition of different proteins of the NOTCH pathway by resveratrol to inhibit cancer, there are conflicting reports that resveratrol can activate the NOTCH pathway, leading to its anticancer activity. Resveratrol has been noted to induce differentiation-associated genes in anaplastic thyroid carcinoma mainly through Notch1 activation [44]. Resveratrol was a strong inducer of TTF1, which increased 4-fold at 25 μmol/L and 6-fold at 50 μmol/L in tested cell lines. Significantly elevated TTF2 was observed at 50 μmol/L in thyroid cancer cells. Moreover, there was an increase (5-fold) in Pax8 in HTh7 cells at a 50 μmol/L dose of resveratrol. The upregulation of Pax8, TTF1, and TTF2 was observed in resveratrol-treated NOTCH1-competent 8505C cells. However, no detectable rise has been found in the levels of Pax8 or TTF1 in resveratrol-treated, NOTCH1-silenced ATC cells. Tumor volume decreased considerably (~69%) after five injections of resveratrol in mice xenografted with either HTh7 or 8505C cells [44].

Resveratrol (50 μM) increased both active p53 and NOTCH-1 in glioblastoma cells [45]. Western blot data suggest that expression of the Notch-1 intracellular domain (NICD) was 3.2-fold and 2.9-fold higher in the A172 and T98G cells, respectively, after resveratrol treatment. Similar patterns were observed and p53 was also found to be upregulated as evidenced by 1.2-fold and 1.1-fold higher expressions in A172 and T98G cells, respectively, after treatment with resveratrol [45]. Taken together, the effect of resveratrol on NOTCH signaling seems to be context-dependent. When NOTCH signaling is oncogenic, it is effectively inhibited by resveratrol; however, when it leans towards a tumor-suppressive action, it is potentiated by resveratrol.

6. Regulation of TRAIL Signaling

The discovery of TRAIL revolutionized the field of molecular oncology because of its ability to differentially target cancer cells while leaving normal cells intact [46]. However, circumstantial evidence has also indicated the development of resistance against TRAIL-based therapeutics. TRAIL resistance emerged as a major setback in the standardization of therapy, and attempts have been made to sensitize resistant cancer cells to TRAIL. Analyses of different cancers have shown an imbalance in stoichiometric ratios of pro- and anti-apoptotic proteins. However, over the past 10 years, there has been a worldwide resurgence of studies emphasizing different strategies for restoring TRAIL-induced apoptosis in resistant cancer cells. Resveratrol has shown potential as a TRAIL sensitizer.

Resveratrol has been found to increase the cell-surface expression of NKG2D ligands and DR4 along with the downregulation of decoy receptor 1 (DcR1) in KG-1a cells [47]. A suppressor of cytokine signaling (SOCS3) was found to considerably impair TRAIL-driven apoptosis in cancer cells [48]. SOCS3 overexpression reduced apoptosis in DU145 cells co-treated with resveratrol and TRAIL. Moreover, SOCS3 inhibition increased apoptosis in TRAIL-treated LNCaP and PC-3 cells [48]. Survivin is a frequently overexpressed protein in TRAIL-resistant cancer cells. Resveratrol dose-dependently downregulated survivin in HepG2 cells. Moreover, 10 ng/mL TRAIL and 50 μmol/L resveratrol worked with effective synergy and increased the apoptotic rate up to 49.6% in HepG2 cells [49]. In xenografted tumors, resveratrol upregulated DR4, DR5, Bax, and p27(/KIP1) and inhibited the expression of cyclin D1 and Bcl-2 [50].

7. Regulation of STAT Signaling

Prostate cancer associated transcript 29 (PCAT29), a tumor suppressor, is frequently overexpressed in prostate cancer and downregulated by interleukin (IL-6) in DU145 and LNCaP cells [51]. Significantly enhanced phopsphorylated-STAT3 has been observed following treatment with IL-6 (10 ng/mL) in DU145 and LNCaP cells [51]. STAT3 knockdown induced an increase in PCAT29 expression in both DU145 and LNCaP cells. Mechanistically, it was shown that STAT3 triggered the upregulation of miR-21, which consequently negatively regulated PCAT29 in PCa cells. Resveratrol repressed STAT

signaling and stimulated expression of PCAT29 via repression of miR-21 [51] (Figure 3). Further, protein inhibitor of activated STAT3 (PIAS3) has been found to be downregulated in medulloblastomas, and resveratrol shown to significantly upregulate PIAS3 in UW228-2, UW228-3, and DAOY cells with the repression of p-STAT3 levels [52].

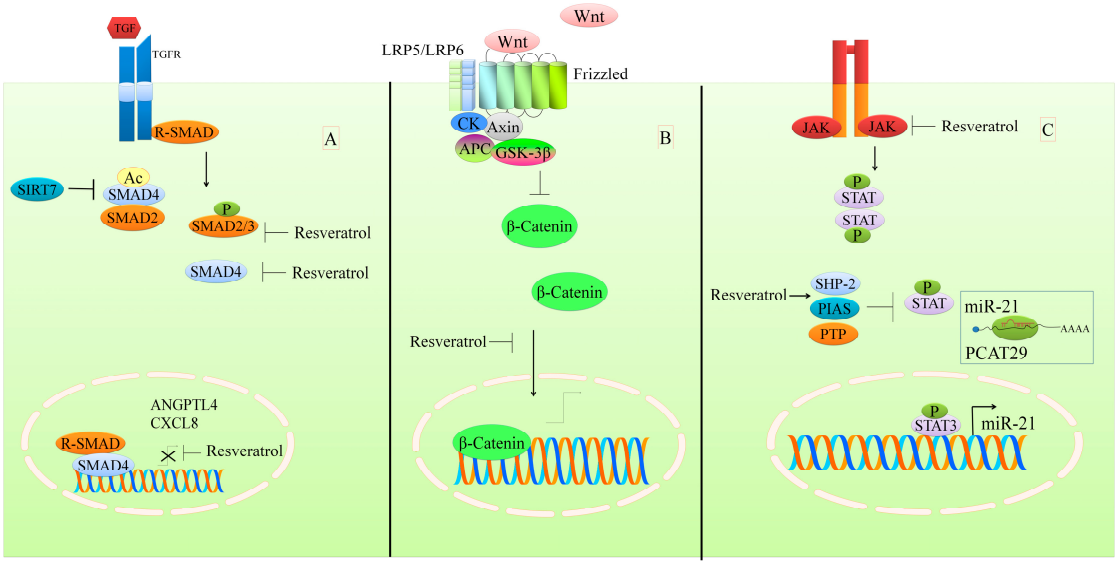

Figure 3. An overview of resveratrol-mediated targeting of multiple signaling pathways. (**A**) TGF/SMAD signaling pathway regulation by resveratrol; (**B**) WNT/Catenin pathway regulation by resveratrol; (**C**) Resveratrol increases negative regulators of STAT signaling (SHP-2, PIAS, and PTP) and inhibits JAK-mediated STAT phosphorylation. STAT3 transcriptionally upregulates miR-21, and targets the tumor suppressor gene PCAT29.

Resveratrol exerts inhibitory effects on the constitutive activation of STAT3 and STAT5. It inhibits the shuttling of STAT3 and STAT5 into the nucleus and significantly reduces the DNA binding affinity of STAT proteins in 786-O and Caki-1 cells [53]. Resveratrol was also found to markedly reduce the constitutive activation of Janus kinases (JAK1 and JAK2) in 786-O and Caki-1 cells. Protein tyrosine phosphatases (PTP and SHP-2) were considerably upregulated in resveratrol-treated 786-O and Caki-1 cells (Figure 3). Targeted inhibition of either PTP or SHP-2 substantially impaired the resveratrol-mediated decrease in the level of phosphorylated-STAT [53]. Resveratrol has also been shown to prevent the activation of JAK, resulting in the inhibition of the JAK-mediated phosphorylation of STAT1 [54].

8. Regulation of Other Signaling Pathways

8.1. AKT and MAPK

Resistance against different chemotherapeutic drugs is a frequently noted mechanism [55]. Docetaxel increased HER-2 in SK-BR-3 cells and induced Akt (Ser 473) phosphorylation within a time period as short as 30 min. Docetaxel induced extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and P38 phosphorylation, which was significantly inhibited by resveratrol [55]. It has also been noted that a high glucose-driven increase in the levels of phosphorylated-ERK and phosphorylated-p38 were inhibited by resveratrol in Panc-1 cells [56].

8.2. The ATM/p53 Pathway

ATM (ataxia telangiectasia mutated) is an important sensor of DNA damage and transduces the signals to downstream effectors, particularly p53 [57]. In glioblastoma-initiating cells treated with

a combination of temozolomide and resveratrol, phosphorylated-ATM was found to be moderately upregulated, while p53 and phosphorylated-p53 were found to significantly increase. Resveratrol has also been found to be more effective against HCT-116 p53 wild-type colon carcinoma cells, as compared to HCT-116 $p53^{-/-}$ cells [58]. Resveratrol induced DNA damage, as evidenced by the presence of multiple γ-H2AX foci after treatment with 25 μM resveratrol. DNA-damage-associated signaling was initiated through the activation of ATM kinase. p53 was found to be phosphorylated on serine 15 by ATM in response to resveratrol [58].

8.3. Multiple Signaling Pathways

Being a pleiotropic agent, resveratrol has been reported to target multiple proteins in ovarian cancer, markedly reducing NOTCH2 and HES1 in OVCAR-3 and CAOV-3 cells [59]. In CAOV-3 cells, resveratrol downregulated WNT2 and reduced the nuclear accumulation of β-catenin [59]. Moreover, the accumulation of STAT3 was evident in the nuclei of naïve OVCAR-3 cells, and resveratrol significantly reduced this nuclear accumulation. Although there is general agreement about the multi-protein targeting of resveratrol, there are still important mechanistic details related to the interwoven network of signal transduction pathways that remain to be elucidated.

9. Regulation of MicroRNAs by Resveratrol

In just over two decades, since the discovery of the first microRNA (miRNA), there has been an exponential rise in the number of reports that provide in-depth analysis of regulation of signaling pathways by miRNAs in different cancers [60]. Categorically, miRNAs are classified into tumor suppressor and oncogenic miRNAs. Different strategies are currently being used to increase the expression of tumor suppressor miRNAs and downregulate oncogenic miRNAs. In this section, we will focus on the regulation of oncogenic and tumor suppressor miRNAs by resveratrol (Figure 4).

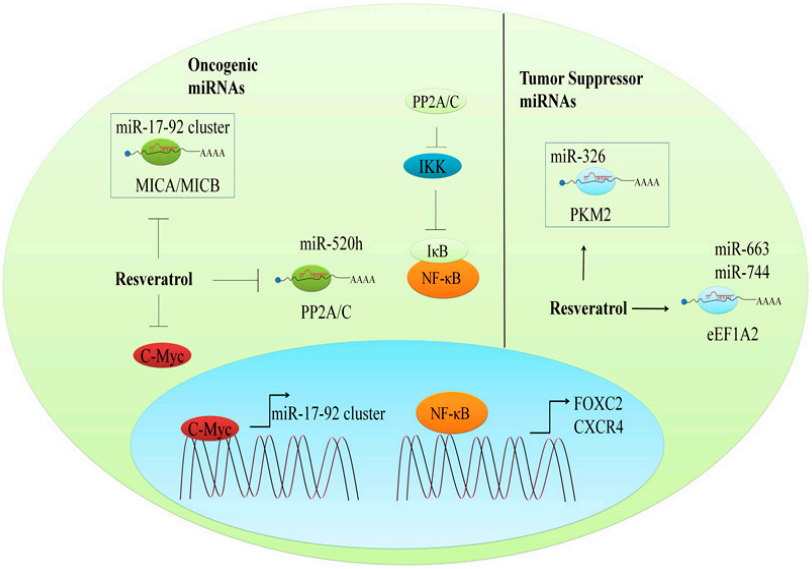

Figure 4. Resveratrol profoundly affects the miRNA machinery. It inhibits oncogenic as well as tumor suppressor miRNAs to affect the cellular oncogenic machinery, resulting in reduced expression of oncogenes and increased expression of tumor suppressor genes.

9.1. Regulation of Oncogenic miRNAs

Insulin-like growth factor binding protein-3 (IGFBP3) has been noted to be frequently downregulated in acute lymphocytic leukemia (ALL) cells [61]. Detailed mechanistic insights revealed that miR-1290 and miR-196b quantitatively controlled IGFBP3 in the ALL cell line. More importantly,

resveratrol considerably reduced the expression of miRNA-196b/miRNA-1290 in TALL-104/SUP-B15 cell lines. The research findings were suggestive of a resveratrol-induced upregulation of IGFBP3 (mRNA, protein) in SUP-B15 as well as TALL-104 cells. Resveratrol arrested TALL-104 cells at the G1 phase and arrested SUP-B15 cells at the S phase. Resveratrol strongly induced apoptosis in the SUP-B15 and TALL-104 cells [61].

Activation of natural killer cells is a well-coordinated mechanism, triggered by signals derived from activating receptor ligation [62]. Natural killer group-2 member-D (NKG2D) or killer cell lectin-like receptor, subfamily-K, member-1 (KLRK1) is an extensively studied activating receptor of natural killer (NK) cells. In humans, the NKG2D ligand (NKG2DLs) family has eight glycoproteins present on the cell surface, including the major histocompatibility complex class I chain-related proteins A and B (MICA/B) [62]. Resveratrol was found to dose-dependently enhance MICA and MICB on the surface of breast cancer cells. Resveratrol-mediated increases in MICA and MICB were more pronounced in the MDA-MB-231 and BCap37 cell lines. Transfection of BCap37 cells with miRNA-17 inhibitors resulted in the upregulation of MICA and MICB, while transfection of BCap37 cells with miRNA-17 mimics resulted in the downregulation of MICA and MICB. c-Myc binding sites had previously been identified in transcriptional initiation sites in miR-17-92 clusters, and c-Myc triggered an increase in the expression of this gene cluster [62]. Resveratrol dose-dependently repressed c-Myc (mRNA, protein) in treated breast cancer cells, which consequently resulted in a reduction of miR-17. c-Myc knockdown resulted in a reduction of pri-miR-17-92 and miR-17, whereas c-Myc-overexpression markedly enhanced pri-miR-17-92 and miR-17 [62].

A chromatin immunoprecipitation assay revealed that resveratrol decreases the association of NF-κB with FOXC2 promoter [63]. Specific Akt inhibition resulted in the suppression of phosphorylated-Akt, FOXC2, and mobility of A549 cells. Resveratrol-mediated inhibitory effects on NF-κB activity were impaired in cancer cells with constitutively active Akt [63]. PP2A/C, a catalytic subunit of serine/threonine phosphatase PP2A, inhibited Akt activity by Akt dephosphorylation. Resveratrol, dose- and time-dependently, increased the expression and activity of PP2A/C protein in cancer cells. Resveratrol enhanced the levels of PP2A/C via the inhibition of miR-520h. Intriguingly, the resveratrol-mediated increase in PP2A/C was dramatically abolished after restoration of miR-520h expression [63]. For a deeper understanding of the crosstalk among these proteins, molecular analysis was conducted in cervical cancer cells, and the results suggested that the PP2A/C-driven dephosphorylation of the inhibitor of kappa-B kinase (IKK) resulted in its inactivation [64]. Inactivation of IKK protected IκB from phosphorylation by IKK; consequently, activation of NF-κB was inhibited. Inactivation of NF-κB resulted in transcriptional repression of its target gene, CXCR4 [64]. These findings provide insight into the mechanisms by which miR-520h quantitatively reduces PP2A/C to facilitate the tumor-promoting activities of NF-κB via the transcriptional upregulation of its target genes.

9.2. The Regulation of Tumor Suppressor miRNAs

Pyruvate kinase M2 (PKM2) has been found to be overexpressed in different cancers [65]. Resveratrol, dose-dependently, increased the mRNA and protein expression levels of GRP78, CHOP, and caspase-12 in both HeLa and DLD1 cells. A reversal of these changes was observed in PKM2-overexpressing cancer cells. Mechanistically, it was shown that resveratrol repressed PKM2 by increasing the expression of miR-326. Interestingly, resveratrol-mediated repressive effects on PKM2 were impaired in miR-326 inhibitor-transfected cancer cells [65].

Stem cell factor (SCF, KITLG) is frequently overexpressed in colorectal cancer cells [66]. KITLG is quantitatively controlled by miR-34c. Studies have shown that p53 transcriptionally upregulated miR-34c in colorectal cancer tissues and cell lines. Resveratrol was noted to increase p53 in p53-positive HT-29 cells, and miR-34c upregulation was more pronounced in p53-positive HT-29 cells, as compared to p53-negative HCT-116 cells [66]. Overexpression of Isoform A2 of eukaryotic translation elongation factor (eEF1A2) played a contributory role in breast cancer progression [67]. miR-663 and miR-744

have been found to negatively regulate eEF1A2 in breast cancer. Resveratrol induced a 4.5-fold upregulation of miR-663 and a two-fold increase in miR-744. It also reduced EEF1A2 in breast cancer cells. A resveratrol-induced decrease in EEF1A2 levels was not found in MCF7 cells transfected with inhibitors of miR-663 and miR-744 [67]. Resveratrol was noted to reduce MMP2 via upregulation of miR-328 in osteosarcoma cells [68]. The resveratrol-mediated suppressive effects on MMP2 were impaired in HOS cells transfected with miR-328 inhibitors, whereas transient or stable overexpression of miR-328 markedly reduced the invasive potential of HOS cells [68].

10. Resveratrol in Clinical Trials

MPX, powdered muscadine grape skin that contains ellagic acid, resveratrol, and quercetin, was tested for efficacy in biochemically recurrent prostate cancer patients [69]. 6 out of 14 patients dropped out of the ongoing trial because the disease progressed post-treatment for a median of 15 months. Seven patients remained in study. More importantly, the lack of dose-limiting toxicities was encouraging even at a high dose of 4000 mg/day [69].

Randomized double blind placebo control trials have gained appreciation as the "gold standard" of epidemiological studies. Recently, a trial was conducted in which 22 biochemically recurrent prostate cancer patients were enrolled [70]. These patients had a moderate rise rate. Doubling time of prostate specific antigen (PSA) was 4–15 months with no evidence of metastasis. A non-significant rise in the log-slope of PSA was experienced by the treatment group. Pre-treatment doubling time was 10.2 months, whereas post-treatment doubling time was 5.5 months. However, no change in the log-slope of PSA was experienced by the placebo group. Doubling time before treatment was 10.8 months, while the doubling time after treatment was 10.9 months [70].

A Phase I clinical trial was conducted where colon cancer patients were enrolled in a study designed to evaluate the effects of low doses of resveratrol-containing freeze-dried grape powder (GP) and a plant-derived resveratrol formulation on the Wnt signal transduction cascade in the colon [71]. Patients treated with resveratrol/GP did not show any change in the Wnt target gene expression [71]. In another study, SRT501, micronized resveratrol, was given 5.0 g daily for 2 weeks to colorectal cancer patients. SRT501 was also given to patients with liver metastases scheduled to undergo hepatectomy [72]. The mean plasma level of resveratrol after single administrations of SRT501 was 1942 $\pm$ 1422 ng/mL. Resveratrol was detected in hepatic tissues after the administration of SRT501 (up to 2287 ng/g) [72]. The promising pre-clinical data has led to the interest in clinical evaluation of resveratrol. More elaborate trials need to be conducted to further establish the clinical relevance of resveratrol's anticancer properties.

11. Concluding Remarks

Deregulation of spatio-temporally controlled cell signaling pathways contributes to cancer initiation, progression, and its metastatic spread. Therefore, the identification of novel anticancer therapeutics, such as natural products with significant anticancer activity and an ability to target multiple proteins of oncogenic pathways, is required. Resveratrol has been shown to modulate key regulators of oncogenic signaling pathways. It strongly inhibits the TGF/SMAD pathway and reduces phosphorylated SMADs, resulting in the inhibition of cancer cell proliferation and metastasis. Resveratrol has shown experimentally verified activity against WNT signaling and can inhibit the translocation of β-catenin into the nucleus. Resveratrol, context-dependently, regulates the NOTCH pathway in cancer cells, all of which underlines its flexible and potent anticancer activity. Reduced apoptosis is a hallmark of aggressive cancer cells, and resveratrol re-balances pro- and anti-apoptotic proteins to improve the efficacy of TRAIL-based therapeutics. It seems exciting to note that resveratrol may prove to be a clinically effective agent; however, lower bioavailability is a major shortcoming that needs to be overcome. In accordance with this idea, nanotechnological strategies are currently being tested to enhance the bioavailability of resveratrol. Resveratrol has been shown to efficiently upregulate various tumor suppressor miRNAs in different cancers, and its suppressive effects on

oncogenic miRNAs have also been well documented. Keeping in view the multi-targeted approach and its robust anticancer effects involving multiple signaling pathways and molecular targets, resveratrol has enormous potential to be considered as an important and pharmacologically effective agent in the fight against cancer.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Califano, A.; Alvarez, M.J. The recurrent architecture of tumour initiation, progression and drug sensitivity. *Nat. Rev. Cancer* **2017**, *17*, 116–130. [CrossRef] [PubMed]
2. Holohan, C.; Van Schaeybroeck, S.; Longley, D.B.; Johnston, P.G. Cancer drug resistance: An evolving paradigm. *Nat. Rev. Cancer* **2013**, *13*, 714–726. [CrossRef] [PubMed]
3. De Palma, M.; Biziato, D.; Petrova, T.V. Microenvironmental regulation of tumour angiogenesis. *Nat. Rev. Cancer* **2017**, *17*, 457–474. [CrossRef] [PubMed]
4. Joyce, J.A.; Pollard, J.W. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* **2009**, *9*, 239–252. [CrossRef] [PubMed]
5. Zubair, H.; Azim, S.; Ahmad, A.; Khan, M.A.; Patel, G.K.; Singh, S.; Singh, A.P. Cancer chemoprevention by phytochemicals: Nature's healing touch. *Molecules* **2017**, *22*, 395. [CrossRef] [PubMed]
6. Ahmad, A.; Ginnebaugh, K.R.; Li, Y.; Padhye, S.B.; Sarkar, F.H. Molecular targets of naturopathy in cancer research: Bridge to modern medicine. *Nutrients* **2015**, *7*, 321–334. [CrossRef] [PubMed]
7. Ahmad, A.; Sakr, W.A.; Rahman, K.M. Novel targets for detection of cancer and their modulation by chemopreventive natural compounds. *Front. Biosci. (Elite Ed.)* **2012**, *4*, 410–425. [CrossRef] [PubMed]
8. Ahmad, A.; Li, Y.; Sarkar, F.H. The bounty of nature for changing the cancer landscape. *Mol. Nutr. Food Res.* **2016**, *60*, 1251–1263. [CrossRef] [PubMed]
9. Kim, C.W.; Hwang, K.A.; Choi, K.C. Anti-metastatic potential of resveratrol and its metabolites by the inhibition of epithelial-mesenchymal transition, migration, and invasion of malignant cancer cells. *Phytomedicine* **2016**, *23*, 1787–1796. [CrossRef] [PubMed]
10. Ahmad, A.; Farhan Asad, S.; Singh, S.; Hadi, S.M. DNA breakage by resveratrol and Cu(II): Reaction mechanism and bacteriophage inactivation. *Cancer Lett.* **2000**, *154*, 29–37. [CrossRef]
11. Ahmad, A.; Syed, F.A.; Singh, S.; Hadi, S.M. Prooxidant activity of resveratrol in the presence of copper ions: Mutagenicity in plasmid DNA. *Toxicol. Lett.* **2005**, *159*, 1–12. [CrossRef] [PubMed]
12. Frazzi, R.; Guardi, M. Cellular and molecular targets of resveratrol on lymphoma and leukemia cells. *Molecules* **2017**, *22*, 885. [CrossRef]
13. Aziz, S.W.; Aziz, M.H. Protective molecular mechanisms of resveratrol in UVR-induced skin carcinogenesis. *Photodermatol. Photoimmunol. Photomed.* **2018**, *34*, 35–41. [CrossRef] [PubMed]
14. Ko, J.H.; Sethi, G.; Um, J.Y.; Shanmugam, M.K.; Arfuso, F.; Kumar, A.P.; Bishayee, A.; Ahn, K.S. The role of resveratrol in cancer therapy. *Int. J. Mol. Sci.* **2017**, *18*, 2589. [CrossRef] [PubMed]
15. Sinha, D.; Sarkar, N.; Biswas, J.; Bishayee, A. Resveratrol for breast cancer prevention and therapy: Preclinical evidence and molecular mechanisms. *Semin. Cancer Biol.* **2016**, *40–41*, 209–232. [CrossRef] [PubMed]
16. Hadi, S.M.; Ullah, M.F.; Azmi, A.S.; Ahmad, A.; Shamim, U.; Zubair, H.; Khan, H.Y. Resveratrol mobilizes endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: A putative mechanism for chemoprevention of cancer. *Pharm. Res.* **2010**, *27*, 979–988. [CrossRef] [PubMed]
17. Zhang, L.; Wen, X.; Li, M.; Li, S.; Zhao, H. Targeting cancer stem cells and signaling pathways by resveratrol and pterostilbene. *Biofactors* **2018**, *44*, 61–68. [CrossRef] [PubMed]
18. Jiang, Z.; Chen, K.; Cheng, L.; Yan, B.; Qian, W.; Cao, J.; Li, J.; Wu, E.; Ma, Q.; Yang, W. Resveratrol and cancer treatment: Updates. *Ann. N. Y. Acad. Sci.* **2017**, *1403*, 59–69. [CrossRef] [PubMed]
19. Ferraz da Costa, D.C.; Fialho, E.; Silva, J.L. Cancer chemoprevention by resveratrol: The p53 tumor suppressor protein as a promising molecular target. *Molecules* **2017**, *22*, 1014. [CrossRef] [PubMed]
20. Ikushima, H.; Miyazono, K. Tgfbeta signalling: A complex web in cancer progression. *Nat. Rev. Cancer* **2010**, *10*, 415–424. [CrossRef] [PubMed]

21. Tang, X.; Shi, L.; Xie, N.; Liu, Z.; Qian, M.; Meng, F.; Xu, Q.; Zhou, M.; Cao, X.; Zhu, W.G.; et al. SIRT7 antagonizes tgf-beta signaling and inhibits breast cancer metastasis. *Nat. Commun.* **2017**, *8*, 318. [CrossRef] [PubMed]
22. Ji, Q.; Liu, X.; Han, Z.; Zhou, L.; Sui, H.; Yan, L.; Jiang, H.; Ren, J.; Cai, J.; Li, Q. Resveratrol suppresses epithelial-to-mesenchymal transition in colorectal cancer through TGF-beta1/smads signaling pathway mediated snail/e-cadherin expression. *BMC Cancer* **2015**, *15*, 97. [CrossRef] [PubMed]
23. Yang, H.; Yuan, Y.; Luo, C.; He, H.; Zhou, Y. Inhibitory effects of resveratrol on the human alveolar rhabdomyosarcoma cell line PLA-802 through inhibition of the TGF-beta1/smad signaling pathway. *Pharmacology* **2016**, *98*, 35–41. [CrossRef] [PubMed]
24. Kim, K.H.; Back, J.H.; Zhu, Y.; Arbesman, J.; Athar, M.; Kopelovich, L.; Kim, A.L.; Bickers, D.R. Resveratrol targets transforming growth factor-beta2 signaling to block UV-induced tumor progression. *J. Investig. Dermatol.* **2011**, *131*, 195–202. [CrossRef] [PubMed]
25. Anastas, J.N.; Moon, R.T. Wnt signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer* **2013**, *13*, 11–26. [CrossRef] [PubMed]
26. Ahmad, A.; Sarkar, S.H.; Bitar, B.; Ali, S.; Aboukameel, A.; Sethi, S.; Li, Y.; Bao, B.; Kong, D.; Banerjee, S.; et al. Garcinol regulates emt and wnt signaling pathways in vitro and in vivo, leading to anticancer activity against breast cancer cells. *Mol. Cancer Ther.* **2012**, *11*, 2193–2201. [CrossRef] [PubMed]
27. Liu, Z.L.; Li, H.; Liu, J.; Wu, M.L.; Chen, X.Y.; Liu, L.H.; Wang, Q. Inactivated wnt signaling in resveratrol-treated epidermal squamous cancer cells and its biological implication. *Oncol. Lett.* **2017**, *14*, 2239–2243. [CrossRef] [PubMed]
28. Jeong, J.B.; Lee, J.; Lee, S.H. TCF4 is a molecular target of resveratrol in the prevention of colorectal cancer. *Int. J. Mol. Sci.* **2015**, *16*, 10411–10425. [CrossRef] [PubMed]
29. Cilibrasi, C.; Riva, G.; Romano, G.; Cadamuro, M.; Bazzoni, R.; Butta, V.; Paoletta, L.; Dalpra, L.; Strazzabosco, M.; Lavitrano, M.; et al. Resveratrol impairs glioma stem cells proliferation and motility by modulating the wnt signaling pathway. *PLoS ONE* **2017**, *12*, e0169854. [CrossRef] [PubMed]
30. Fu, Y.; Chang, H.; Peng, X.; Bai, Q.; Yi, L.; Zhou, Y.; Zhu, J.; Mi, M. Resveratrol inhibits breast cancer stem-like cells and induces autophagy via suppressing wnt/beta-catenin signaling pathway. *PLoS ONE* **2014**, *9*, e102535.
31. Liu, Y.Z.; Wu, K.; Huang, J.; Liu, Y.; Wang, X.; Meng, Z.J.; Yuan, S.X.; Wang, D.X.; Luo, J.Y.; Zuo, G.W.; et al. The PTEN/PI3K/Akt and wnt/beta-catenin signaling pathways are involved in the inhibitory effect of resveratrol on human colon cancer cell proliferation. *Int. J. Oncol.* **2014**, *45*, 104–112. [CrossRef] [PubMed]
32. Ji, Q.; Liu, X.; Fu, X.; Zhang, L.; Sui, H.; Zhou, L.; Sun, J.; Cai, J.; Qin, J.; Ren, J.; et al. Resveratrol inhibits invasion and metastasis of colorectal cancer cells via malat1 mediated wnt/beta-catenin signal pathway. *PLoS ONE* **2013**, *8*, e78700. [CrossRef] [PubMed]
33. Wang, J.; Wang, H.; Zhang, Y.; Zhen, N.; Zhang, L.; Qiao, Y.; Weng, W.; Liu, X.; Ma, L.; Xiao, W.; et al. Mutual inhibition between yap and SRSF1 maintains long non-coding rna, malat1-induced tumourigenesis in liver cancer. *Cell. Signal.* **2014**, *26*, 1048–1059. [CrossRef] [PubMed]
34. Pasca di Magliano, M.; Hebrok, M. Hedgehog signalling in cancer formation and maintenance. *Nat. Rev. Cancer* **2003**, *3*, 903–911. [CrossRef] [PubMed]
35. Ahmad, A.; Maitah, M.Y.; Ginnebaugh, K.R.; Li, Y.; Bao, B.; Gadgeel, S.M.; Sarkar, F.H. Inhibition of hedgehog signaling sensitizes nsclc cells to standard therapies through modulation of emt-regulating mirnas. *J. Hematol. Oncol.* **2013**, *6*, 77. [CrossRef] [PubMed]
36. Li, Y.; Maitah, M.Y.; Ahmad, A.; Kong, D.; Bao, B.; Sarkar, F.H. Targeting the hedgehog signaling pathway for cancer therapy. *Expert Opin. Ther. Targets* **2012**, *16*, 49–66. [CrossRef] [PubMed]
37. Liao, H.F.; Su, Y.C.; Zheng, Z.Y.; Jhih Cai, C.; Hou, M.H.; Chao, K.S.; Chen, Y.J. Sonic hedgehog signaling regulates Bcr-Abl expression in human chronic myeloid leukemia cells. *Biomed. Pharmacother.* **2012**, *66*, 378–383. [CrossRef] [PubMed]
38. Du, Z.; Zhou, F.; Jia, Z.; Zheng, B.; Han, S.; Cheng, J.; Zhu, G.; Huang, P. The hedgehog/gli-1 signaling pathways is involved in the inhibitory effect of resveratrol on human colorectal cancer hct116 cells. *Iran. J. Basic Med. Sci.* **2016**, *19*, 1171–1176. [PubMed]
39. Su, Y.C.; Li, S.C.; Wu, Y.C.; Wang, L.M.; Chao, K.S.; Liao, H.F. Resveratrol downregulates interleukin-6-stimulated sonic hedgehog signaling in human acute myeloid leukemia. *Evid.-Based Complement. Altern. Med.* **2013**, *2013*, 547430. [CrossRef] [PubMed]

40. Gao, Q.; Yuan, Y.; Gan, H.Z.; Peng, Q. Resveratrol inhibits the hedgehog signaling pathway and epithelial-mesenchymal transition and suppresses gastric cancer invasion and metastasis. *Oncol. Lett.* **2015**, *9*, 2381–2387. [CrossRef] [PubMed]
41. Lubecka, K.; Kurzava, L.; Flower, K.; Buvala, H.; Zhang, H.; Teegarden, D.; Camarillo, I.; Suderman, M.; Kuang, S.; Andrisani, O.; et al. Stilbenoids remodel the DNA methylation patterns in breast cancer cells and inhibit oncogenic notch signaling through epigenetic regulation of MAML2 transcriptional activity. *Carcinogenesis* **2016**, *37*, 656–668. [CrossRef] [PubMed]
42. Ruiz i Altaba, A.; Sanchez, P.; Dahmane, N. Gli and hedgehog in cancer: Tumours, embryos and stem cells. *Nat. Rev. Cancer* **2002**, *2*, 361–372. [CrossRef] [PubMed]
43. Wang, Z.; Li, Y.; Ahmad, A.; Azmi, A.S.; Banerjee, S.; Kong, D.; Sarkar, F.H. Targeting notch signaling pathway to overcome drug resistance for cancer therapy. *Biochim. Biophys. Acta* **2010**, *1806*, 258–267. [CrossRef] [PubMed]
44. Yu, X.M.; Jaskula-Sztul, R.; Ahmed, K.; Harrison, A.D.; Kunnimalaiyaan, M.; Chen, H. Resveratrol induces differentiation markers expression in anaplastic thyroid carcinoma via activation of notch1 signaling and suppresses cell growth. *Mol. Cancer Ther.* **2013**, *12*, 1276–1287. [CrossRef] [PubMed]
45. Lin, H.; Xiong, W.; Zhang, X.; Liu, B.; Zhang, W.; Zhang, Y.; Cheng, J.; Huang, H. Notch-1 activation-dependent p53 restoration contributes to resveratrol-induced apoptosis in glioblastoma cells. *Oncol. Rep.* **2011**, *26*, 925–930. [PubMed]
46. Von Karstedt, S.; Montinaro, A.; Walczak, H. Exploring the trails less travelled: Trail in cancer biology and therapy. *Nat. Rev. Cancer* **2017**, *17*, 352–366. [CrossRef] [PubMed]
47. Hu, L.; Cao, D.; Li, Y.; He, Y.; Guo, K. Resveratrol sensitized leukemia stem cell-like kg-1a cells to cytokine-induced killer cells-mediated cytolysis through NKG2D ligands and trail receptors. *Cancer Biol. Ther.* **2012**, *13*, 516–526. [CrossRef] [PubMed]
48. Horndasch, M.; Culig, Z. SOCS-3 antagonizes pro-apoptotic effects of trail and resveratrol in prostate cancer cells. *Prostate* **2011**, *71*, 1357–1366. [CrossRef] [PubMed]
49. Tameda, M.; Sugimoto, K.; Shiraki, K.; Inagaki, Y.; Ogura, S.; Kasai, C.; Yoneda, M.; Okamoto, R.; Yamamoto, N.; Takei, Y.; et al. Resveratrol sensitizes HEPG2 cells to trail-induced apoptosis. *Anticancer Drugs* **2014**, *25*, 1028–1034. [CrossRef] [PubMed]
50. Ganapathy, S.; Chen, Q.; Singh, K.P.; Shankar, S.; Srivastava, R.K. Resveratrol enhances antitumor activity of trail in prostate cancer xenografts through activation of foxo transcription factor. *PLoS ONE* **2010**, *5*, e15627. [CrossRef] [PubMed]
51. Al Aameri, R.F.H.; Sheth, S.; Alanisi, E.M.A.; Borse, V.; Mukherjea, D.; Rybak, L.P.; Ramkumar, V. Tonic suppression of PCAT29 by the IL-6 signaling pathway in prostate cancer: Reversal by resveratrol. *PLoS ONE* **2017**, *12*, e0177198. [CrossRef] [PubMed]
52. Li, C.; Li, H.; Zhang, P.; Yu, L.J.; Huang, T.M.; Song, X.; Kong, Q.Y.; Dong, J.L.; Li, P.N.; Liu, J. SHP2, SOCS3 and PIAS3 expression patterns in medulloblastomas: Relevance to STAT3 activation and resveratrol-suppressed STAT3 signaling. *Nutrients* **2017**, *9*, 3. [CrossRef] [PubMed]
53. Kim, C.; Baek, S.H.; Um, J.Y.; Shim, B.S.; Ahn, K.S. Resveratrol attenuates constitutive STAT3 and STAT5 activation through induction of ptpepsilon and SHP-2 tyrosine phosphatases and potentiates sorafenib-induced apoptosis in renal cell carcinoma. *BMC Nephrol.* **2016**, *17*, 19. [CrossRef] [PubMed]
54. Madan, E.; Prasad, S.; Roy, P.; George, J.; Shukla, Y. Regulation of apoptosis by resveratrol through JAK/STAT and mitochondria mediated pathway in human epidermoid carcinoma A431 cells. *Biochem. Biophys. Res. Commun.* **2008**, *377*, 1232–1237. [CrossRef] [PubMed]
55. Vinod, B.S.; Nair, H.H.; Vijayakurup, V.; Shabna, A.; Shah, S.; Krishna, A.; Pillai, K.S.; Thankachan, S.; Anto, R.J. Resveratrol chemosensitizes HER-2-overexpressing breast cancer cells to docetaxel chemoresistance by inhibiting docetaxel-mediated activation of HER-2-Akt axis. *Cell Death Discov.* **2015**, *1*, 15061. [CrossRef] [PubMed]
56. Cao, L.; Chen, X.; Xiao, X.; Ma, Q.; Li, W. Resveratrol inhibits hyperglycemia-driven ros-induced invasion and migration of pancreatic cancer cells via suppression of the erk and p38 mapk signaling pathways. *Int. J. Oncol.* **2016**, *49*, 735–743. [CrossRef] [PubMed]
57. Li, H.; Liu, Y.; Jiao, Y.; Guo, A.; Xu, X.; Qu, X.; Wang, S.; Zhao, J.; Li, Y.; Cao, Y. Resveratrol sensitizes glioblastoma-initiating cells to temozolomide by inducing cell apoptosis and promoting differentiation. *Oncol. Rep.* **2016**, *35*, 343–351. [CrossRef] [PubMed]

58. Demoulin, B.; Hermant, M.; Castrogiovanni, C.; Staudt, C.; Dumont, P. Resveratrol induces DNA damage in colon cancer cells by poisoning topoisomerase ii and activates the atm kinase to trigger p53-dependent apoptosis. *Toxicol. In Vitro* **2015**, *29*, 1156–1165. [CrossRef] [PubMed]
59. Zhong, L.X.; Li, H.; Wu, M.L.; Liu, X.Y.; Zhong, M.J.; Chen, X.Y.; Liu, J.; Zhang, Y. Inhibition of STAT3 signaling as critical molecular event in resveratrol-suppressed ovarian cancer cells. *J. Ovarian Res.* **2015**, *8*, 25. [CrossRef] [PubMed]
60. Tang, J.; Ahmad, A.; Sarkar, F.H. Micrornas in breast cancer therapy. *Curr. Pharm. Des.* **2014**, *20*, 5268–5274. [CrossRef] [PubMed]
61. Zhou, W.; Wang, S.; Ying, Y.; Zhou, R.; Mao, P. miR-196b/miR-1290 participate in the antitumor effect of resveratrol via regulation of IGFBP3 expression in acute lymphoblastic leukemia. *Oncol. Rep.* **2017**, *37*, 1075–1083. [CrossRef] [PubMed]
62. Pan, J.; Shen, J.; Si, W.; Du, C.; Chen, D.; Xu, L.; Yao, M.; Fu, P.; Fan, W. Resveratrol promotes MICA/B expression and natural killer cell lysis of breast cancer cells by suppressing c-Myc/miR-17 pathway. *Oncotarget* **2017**, *8*, 65743–65758. [CrossRef] [PubMed]
63. Yu, Y.H.; Chen, H.A.; Chen, P.S.; Cheng, Y.J.; Hsu, W.H.; Chang, Y.W.; Chen, Y.H.; Jan, Y.; Hsiao, M.; Chang, T.Y.; et al. miR-520h-mediated FOXC2 regulation is critical for inhibition of lung cancer progression by resveratrol. *Oncogene* **2013**, *32*, 431–443. [CrossRef] [PubMed]
64. Chang, Y.W.; Chen, M.W.; Chiu, C.F.; Hong, C.C.; Cheng, C.C.; Hsiao, M.; Chen, C.A.; Wei, L.H.; Su, J.L. Arsenic trioxide inhibits CXCR4-mediated metastasis by interfering miR-520h/PP2A/NF-kappaB signaling in cervical cancer. *Ann. Surg. Oncol.* **2014**, *21* (Suppl. 4), S687–S695. [CrossRef] [PubMed]
65. Wu, H.; Wang, Y.; Wu, C.; Yang, P.; Li, H.; Li, Z. Resveratrol induces cancer cell apoptosis through miR-326/PKM2-mediated er stress and mitochondrial fission. *J. Agric. Food Chem.* **2016**, *64*, 9356–9367. [CrossRef] [PubMed]
66. Yang, S.; Li, W.; Sun, H.; Wu, B.; Ji, F.; Sun, T.; Chang, H.; Shen, P.; Wang, Y.; Zhou, D. Resveratrol elicits anti-colorectal cancer effect by activating miR-34c-KITLG in vitro and in vivo. *BMC Cancer* **2015**, *15*, 969. [CrossRef] [PubMed]
67. Vislovukh, A.; Kratassiouk, G.; Porto, E.; Gralievska, N.; Beldiman, C.; Pinna, G.; El'skaya, A.; Harel-Bellan, A.; Negrutskii, B.; Groisman, I. Proto-oncogenic isoform A2 of eukaryotic translation elongation factor eEF1 is a target of miR-663 and miR-744. *Br. J. Cancer* **2013**, *108*, 2304–2311. [CrossRef] [PubMed]
68. Yang, S.F.; Lee, W.J.; Tan, P.; Tang, C.H.; Hsiao, M.; Hsieh, F.K.; Chien, M.H. Upregulation of miR-328 and inhibition of CREB-DNA-binding activity are critical for resveratrol-mediated suppression of matrix metalloproteinase-2 and subsequent metastatic ability in human osteosarcomas. *Oncotarget* **2015**, *6*, 2736–2753. [CrossRef] [PubMed]
69. Paller, C.J.; Rudek, M.A.; Zhou, X.C.; Wagner, W.D.; Hudson, T.S.; Anders, N.; Hammers, H.J.; Dowling, D.; King, S.; Antonarakis, E.S.; et al. A phase i study of muscadine grape skin extract in men with biochemically recurrent prostate cancer: Safety, tolerability, and dose determination. *Prostate* **2015**, *75*, 1518–1525. [CrossRef] [PubMed]
70. Van Die, M.D.; Williams, S.G.; Emery, J.; Bone, K.M.; Taylor, J.M.; Lusk, E.; Pirotta, M.V. A placebo-controlled double-blinded randomized pilot study of combination phytotherapy in biochemically recurrent prostate cancer. *Prostate* **2017**, *77*, 765–775. [CrossRef] [PubMed]
71. Nguyen, A.V.; Martinez, M.; Stamos, M.J.; Moyer, M.P.; Planutis, K.; Hope, C.; Holcombe, R.F. Results of a phase i pilot clinical trial examining the effect of plant-derived resveratrol and grape powder on wnt pathway target gene expression in colonic mucosa and colon cancer. *Cancer Manag. Res.* **2009**, *1*, 25–37. [PubMed]
72. Howells, L.M.; Berry, D.P.; Elliott, P.J.; Jacobson, E.W.; Hoffmann, E.; Hegarty, B.; Brown, K.; Steward, W.P.; Gescher, A.J. Phase i randomized, double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases—Safety, pharmacokinetics, and pharmacodynamics. *Cancer Prev. Res.* **2011**, *4*, 1419–1425. [CrossRef] [PubMed]

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Review

Cancer Chemoprevention by Resveratrol: The p53 Tumor Suppressor Protein as a Promising Molecular Target

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Abstract: Increasing epidemiological and experimental evidence has demonstrated an inverse relationship between the consumption of plant foods and the incidence of chronic diseases, including cancer. Microcomponents that are naturally present in such foods, especially polyphenols, are responsible for the benefits to human health. Resveratrol is a diet-derived cancer chemopreventive agent with high therapeutic potential, as demonstrated by different authors. The aim of this review is to collect and present recent evidence from the literature regarding resveratrol and its effects on cancer prevention, molecular signaling (especially regarding the involvement of p53 protein), and therapeutic perspectives with an emphasis on clinical trial results to date.

Keywords: resveratrol; cancer; signal transduction; p53; clinical trials

1. Introduction

Cancer is a source of significant and growing mortality worldwide, with an increase to 19.3 million new cancer cases per year projected for 2025. More than half of cancer cases and mortality occur in low- and middle-income countries [1,2]. Current treatments for cancer include surgery, radiotherapy and systemic treatments comprising cytotoxic chemotherapy, hormonal therapy, immunotherapy, and targeted therapies [3].

Epidemiologic and experimental studies have suggested favorable effects of dietary polyphenols through their anti-carcinogenic properties. Resveratrol represents a group of diet-derived cancer chemopreventive agents encompassing, among others, curcumin, tea polyphenols, probiotics, and lycopene, which have attracted great interest in the cancer chemoprevention community because of their ability to minimally engage this process. Thus, these agents have been explored as potential therapeutic agents in humans [4].

The aim of this review is to collect and present recent evidence in the literature regarding resveratrol and its effects on cancer prevention, molecular signaling (especially regarding the involvement of the p53 protein), and therapeutic perspectives, with an emphasis on clinical trial results.

2. Cell Signaling Pathways Regulated by Resveratrol in Cancer

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a naturally occurring polyphenol that is found at low concentrations in more than 70 plant species, including grapes, cranberries and peanuts, as

well as in a number of herbal remedies [5]. Resveratrol from grapes is efficiently extracted during the wine-making process, and it has been speculated that red wine, in particular, may be the most important dietary source of this microcomponent [6].

In PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), a search for "Resveratrol and Cancer" generated 2325 hits (April 2017). Among its wide range of biological activities, resveratrol has attracted considerable attention due to its role in regulating multiple signal transduction pathways involved in carcinogenesis. Since 1997, when Jang et al. [7] first reported the in vivo antitumor properties of resveratrol, accumulating data have demonstrated its ability to modulate a number of intracellular mediators in cancer initiation, promotion and progression. Therefore, many chemopreventive and chemotherapeutic mechanisms to prevent, arrest, or delay tumor development by this microcomponent have been proposed [5,8–10].

Resveratrol regulates a variety of processes and signaling pathways that involves procarcinogen bioactivation and carcinogen detoxification [11,12]; reduction of oxidative stress [13] and inflammation [14]; apoptosis induction through the activation of both extrinsic and intrinsic pathways [15–20]; and other anticancer effects [21,22]. Resveratrol affects the three phases of carcinogenesis, tumor initiation, promotion and progression, and it also suppresses the final steps of carcinogenesis, e.g., angiogenesis and metastasis [11,23]. Resveratrol also impacts mitochondrial functions (the respiratory chain, oncoproteins, gene expression, among others), including those directly involving the p53 protein tumor suppressor protein [24]. Finally, in various cancer types, resveratrol behaves as a chemosensitizer that lowers the threshold of cell death induction by classical anticancer agents and counteracts tumor cell chemoresistance [25,26].

In vitro and in vivo studies have shown that resveratrol can act as a tumor-initiation suppressor by modulating phase I and phase II cytochrome P450 enzymes (CYPs) [5,12,27–30]. Resveratrol blocks the transcriptional activation of CYPs and inhibits the activity of CYP1A1, CYP1B1 and CYP1A2, which are phase I enzymes responsible for activating xenobiotics, thus halting the transformation of procarcinogen agents into potential carcinogens [12,27,28]. Additionally, resveratrol enhances the expression and/or activity of phase II enzymes, including glutathione peroxidase, glutathione S-transferase, UDP-glucuronyltransferase, NAD(P)H:quinone oxidoreductase, and heme oxygenase, among others [5,29,31], subsequently stimulating carcinogen detoxification processes.

The role of resveratrol in the intracellular redox status has been described in many studies. Like other polyphenols, this microcomponent acts as an important cellular antioxidant, and its effects are dependent on the concentration tested and the cell type used. However, it has been proposed that resveratrol may also act as a pro-oxidant agent, representing one of the antineoplastic mechanisms of action involved in tumor cell death. Likewise, resveratrol has been shown to decrease the mitochondrial membrane potential and increase reactive oxygen species (ROS) generation, thus promoting apoptosis [32,33].

Some effects of resveratrol have been attributed to its estrogenic activity resulting from its structural similarity to diethylstilbestrol, a synthetic estrogen. Resveratrol can bind to estrogen receptors and may function as an agonist or antagonist, leading to opposite responses depending on the concentration, competition and expression of estrogen receptors in the cells. In MCF-7 human breast cancer cells, for example, which express these receptors, resveratrol may act as a superagonist [34].

Resveratrol is recognized as a potent tumor growth inhibitor in diverse experimental models. Previous studies have established its anticancer effects in a variety of cultured tumor cell lines, including colon [35–37], breast [19,38,39], prostate [40,41], pancreatic [42–44], lung [19,45,46], melanoma [47,48], glioma [49,50], and leukemia cells [51,52], among others. It has been suggested that the anti-proliferative properties of this microcomponent are related to its capacity to block DNA synthesis and to interfere with various stages of cell progression by regulating the machinery of proteins involved in cell cycle control. Hsieh and colleagues [53] first reported the ability of resveratrol to control growth and cell cycle transitions in human breast carcinoma cell lines with different metastatic potentials: highly invasive MDA-MB-435 and the minimally invasive MCF-7 cells. Resveratrol exerts

a greater inhibitory effect on MDA-MB-435 cells, in which a reduction of the fraction of cells in the G1 phase and a corresponding accumulation of cells in S phase was observed. Since then, many other studies have reported that resveratrol at micromolar concentrations arrests the cell cycle of a variety of human cancer cell lines at the G1/S transition, in S phase or in the G2/M phase [5,54,55]. Although several studies have found that the induction of cell cycle arrest by resveratrol is a reversible process and does not trigger apoptosis, many other authors have reported that this process is often followed by apoptotic cell death [56].

Investigations regarding the effects of resveratrol on apoptosis induction in tumor cells have revealed that this compound can stimulate cell death by modulating proteins involved in both the intrinsic and extrinsic apoptotic pathways [56]. Numerous lines of evidence suggest that resveratrol affects the activation of pro-apoptotic and the inhibition of anti-apoptotic molecules. These mechanisms have been described in culture cells and in vivo in tumor models that have been chemically induced in animals and xenographic models in nude mice [55]. Studies suggest that resveratrol-mediated apoptosis includes the activation of death receptors such as Fas and TRAIL; the activation of Bax, Bak, PUMA, Noxa and Bim; and the inhibition of Bcl-2 and Bcl-XL. Some of these responses are mediated by the activation of the p53 tumor suppressor protein by resveratrol, as will be later discussed.

In addition, resveratrol demonstrates important anti-angiogenic effects, thus contributing to the reduction of the metastatic potential of tumor cells. The following mechanisms are involved in this process: the inhibition of extracellular matrix metalloproteinases gene expression, such as MMP-2 and MMP-9, which are involved in tumor invasiveness; and the inhibition of the expression of HIF-1α and VEGF, factors that are directly related to new blood vessel formation [54,55].

Although some of the anti-carcinogenic effects of resveratrol have already been shown to be triggered by the activation of extracellular receptors [57], there is evidence that its internalization by cells is necessary for the activation of some specific intracellular targets. It has been demonstrated by multiphoton microscopy (two-photon excitation) that resveratrol, but not its glycosylated or sulfated metabolites, is efficiently captured by neuroblastoma cells, allowing this molecule to exert its antitumor effects, unlike metabolites [58]. Additionally, the importance of cell membrane microdomains in the early biochemical events triggered by resveratrol resulting in cancer cell death, as well as in its absorption and distribution, has been previously described. Resveratrol accumulates in lipid rafts and is then taken up by cells through raft-dependent endocytosis. These events allow for the activation of kinase pathways and redistribution of cell death receptors within lipid microdomains, ultimately leading to apoptotic cell death [17].

Although diverse in vitro and cell culture experiments have described the pro-apoptotic potential of resveratrol, a study conducted in a xenograft model has shown that this compound can inhibit tumor growth in vivo when administered orally, but there is no evidence of apoptosis induction. This finding may be due to the low bioavailability of resveratrol in animals. However, the tumor growth inhibition can be explained by other mechanisms that are independent of a pro-apoptotic effect, such as the anti-proliferative and anti-angiogenic activity of resveratrol [59]. The systemic administration of resveratrol inhibits the initiation and growth of tumors in a variety of cancer models in rodents. The efficacy of low daily doses of resveratrol (200 μg/kg body weight, for example) in animals with induced colon carcinogenesis suggests that even low concentrations of the compound, such as those obtained by the ingestion of red wine, could be therapeutic in some cases. However, the protective effects of resveratrol are observed more frequently when higher and pharmacologically achievable concentrations are used [60]. There are also studies showing that resveratrol, when administered in animals through peritumoral and intratumoral injections, presents a bioavailability in plasma and tumor significantly higher than that found after oral administration, thus improving the responses of tumor cells to this compound [58].

In addition, studies regarding the effects of resveratrol on in vivo models of spontaneous carcinogenesis are limited and contradictory. Resveratrol supplementation in these experimental models has shown positive, neutral as well as negative outcomes, depending on resveratrol route of

administration, dose, tumor model, species, and molecular properties intrinsic to the cancer cell type as already described by others [21,61,62].

Because of these pleiotropic effects, scientists consider resveratrol to have potential as an anticancer drug, and efforts have focused on obtaining a thorough understanding of its mechanisms of action.

3. The p53 Tumor Suppressor Protein as a Chemotherapeutic Target of Resveratrol

p53 is a critical tumor suppressor protein that has an essential role in cancer prevention. Wild-type p53 blocks tumor development through the induction of cell cycle arrest and/or apoptosis. As a transcription factor, p53 regulates the transcription of specific target genes involved in these processes, thus triggering growth arrest, senescence, cell differentiation and/or cell death under different stress conditions, such as DNA damage, oncogene activation, hypoxia, and telomere erosion, among others [63–65].

The p53 pathway is extremely sensitive to small amounts of DNA molecule damage, which is crucial for the early detection of genetic lesions in tumors [66]. In response to this damage, the activity of specific proteins that are responsible for p53 activation is stimulated, such as checkpoint 2 protein (Chk2). Chk2 is a serine/threonine kinase and, when phosphorylated by ATM, may activate p53, thus promoting its stabilization through phosphorylation at the serine 20 residue, which prevents MDM2-mediated p53 degradation [67]. Consequently, p53 stabilization leads to the activation of *cip1*, one of its main target genes. The *cip1* product is the p21 protein, which acts as an inhibitor of cyclin-dependent kinases (CDKs) in G1 phase of the cell cycle. CDKs allow the transition from G1 to S phase and from G2 to M phase, thus promoting the synthesis and replication of DNA and cell division. Inhibition occurs by preventing the phosphorylation of several regulatory proteins, blocking cell cycle progression [68].

p53 also modulates cell death pathways by mechanisms that are dependent or independent of its activity as a transcription factor. p53-mediated apoptosis occurs through the induction of the transcription of its target gene, whereas p53-independent apoptosis occurs mainly by the interaction of p53 with anti-apoptotic or pro-apoptotic proteins. Numerous studies have shown that p53 can induce the expression of proteins involved in both intrinsic and extrinsic apoptotic pathways [69]. Some mitochondrial proteins show increased expression in response to p53, such as NOXA, PUMA and p53AIP1. Additionally, p53 stimulates the transcription of members of the pro-apoptotic Bcl2 gene family, such as Bax and Bak, allowing the release of cytochrome c into the cytoplasm and their binding to protease activating factor apoptotic (Apaf-1), which leads to the oligomerization of Apaf-1 complex/caspase 9 (apoptosome). This complex recruits pro-caspase 9 and activates effector caspases. p53 can also promote apoptosis through the activation of death receptors, including Fas, DR4 and DR5 [70].

The ability of wild-type p53 overexpression to induce apoptosis may be a major reason why cancer cells frequently exhibit disabled p53 or p53-mediated pathways during the oncogenic process. The same genetic changes that cause the loss of apoptosis during tumor development may also result in tumor chemoresistance to anticancer therapies that kill tumor cells by apoptosis. Elucidation of the mechanisms involved in these cellular responses may provide insights into strategies to induce cell death and suggest new targets for improving cancer treatment.

Consistent evidence suggests that resveratrol can induce p53-dependent cell death in a variety of cancer cell lines [19,71–75]. Previous studies have demonstrated that this microcomponent promotes the activation and stabilization of cellular levels of p53 protein in tumor cell cultures by inducing post-translational modifications, such as phosphorylation and acetylation. Such modifications are required for the transcriptional activation of p53-responsive genes [76]. Interestingly, resveratrol and other polyphenolic compounds can also trigger apoptosis independently of the p53 cellular status. Alternative mechanisms, including the role of p73, a p53-related tumor suppressor, have been reported in recent studies [77].

Resveratrol-induced p53 activation and apoptosis pathways have been shown to be mediated by MAP kinases (mitogen-activated protein kinases). She and colleagues [72] demonstrated for the first time that resveratrol could increase endogenous levels of p53, especially in the phosphorylated state, in epidermal JB6 cells, which constitute a well-developed model of cell culture to study tumor progression. Likewise, levels of phosphorylated protein kinases (ERKs, p38 kinase and JNKs) increase in the presence of resveratrol in a time-dependent manner [73]. In MCF-7 human breast cancer cells, a mechanism has been suggested in which resveratrol, when binding to an integrin located in the plasma membrane, triggers the activation of ERKs, which in turn phosphorylate p53 protein [57].

In MCF-7 cells, the mechanisms by which resveratrol induces cell death appear to involve the activation of MAP kinases (MAPK and ERKs 1 and 2), which is associated with the phosphorylation and acetylation of p53 at serine residues [78]. Other authors have demonstrated that in these cells, resveratrol treatment resulted in a dose-dependent inhibition of cell growth and an accumulation of cells in S phase of the cell cycle. In addition, the anti-proliferative effects of resveratrol are associated with inhibition of cyclin D and CDKs and induction of p53 and the inhibitor of CDKs, p21. In these cells, resveratrol-induced apoptosis involves the activation of caspase 9, reduced expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL and increased levels of the pro-apoptotic Bax protein [79].

Another study identified the role of p53-dependent and -independent pathways in resveratrol-induced apoptosis in breast cancer cells and showed that this compound could induce cell death in cells expressing wild-type p53 but not in those expressing a mutant form of the protein [71]. In cancer cells derived from lung (A549), liver (HepG2), thyroid (FTC 236 and FTC 238), and osteosarcoma cells (SJSA1), among others, the anti-proliferative and pro-apoptotic effects of resveratrol have been shown to be mediated by p53 [80–82]. In prostate cancer cells, resveratrol increased the expression of p53-p(ser15) and/or p53-ac(lys382) and total p53 protein without a change in p53 mRNA. This compound also induced p53 translocation to mitochondria and promoted cell cycle alterations, as well as the induction of apoptosis [83–87].

A study by our group highlighted that, despite the p53-independent apoptosis reported for some cancers, a functional wild-type p53 is required to increase the sensitivity of tumor cells to resveratrol. We showed that transient transfection of a wild-type p53 gene caused H1299 lung cancer cells ($p53^{-/-}$) to become more responsive to the pro-apoptotic properties of resveratrol, similar to the findings in p53-positive MCF-7 cells. These findings suggest a possible therapeutic strategy based on the use of resveratrol for the treatment of tumors that are typically unresponsive to conventional therapies due to the loss of normal p53 function [19].

In the last 15 years, our group has also evaluated the stability and aggregation properties of wild-type and mutant p53. We have previously demonstrated the formation of different types of aggregates after the physical induction of p53 core domain (p53C) unfolding [88] and the ability of a small cognate double-stranded DNA to stabilize both p53C and full-length p53, thus rescuing aggregated and misfolded species of the protein [89]. In addition to these in vitro studies, we used co-localization assays to detect p53 aggregates in archived samples of breast cancer tissues expressing the p53 mutant R248Q and other p53 hot-spot mutants [90]. We have also shown that hot-spot mutants have a greater tendency to aggregate than wild-type p53. Using different techniques, we demonstrated the amyloid nature of the aggregates. It has been demonstrated that seeding p53C R248Q mutant oligomers and fibrils triggers the aggregation of wild-type p53, a behavior typical of a prion. We also observed the co-localization of full-length p53 and aggregates in breast cancer cell lines. In MDA-MB 231 cells, R280K p53 mutant expression revealed a massive accumulation of p53 aggregates in the cell nucleus [91]. Co-aggregation of mutant p53 with other proteins has also been described and may lead to a gain-of-function phenotype. Mutant p53 aggregation appears to occur together with its paralogs p63 and p73 [92–94]. Amyloid aggregates of mutant p53 have also been discovered in other types of malignant tumors, such as malignant skin tumors [95] and ovarian cancer [96]. These findings may reveal the biological significance of the prion-like behavior of oncogenic p53 mutants and help with the development of new strategies to disrupt the formation of aggregates [91,97–99].

Resveratrol has been shown to inhibit amyloid aggregation by binding to several amyloidogenic proteins such as transthyretin [100–102], the islet amyloid polypeptide (IAPP) [103], amyloid beta peptide [104] and alpha-synuclein [105]. Our group has studied the interaction of resveratrol with p53 and evaluated its effect on p53 amyloid aggregation [106,107]. The results have shown that part of the anti-tumoral effects of resveratrol might be related to the inhibition of p53 aggregation.

All together, these data demonstrate the involvement of the p53 pathway in the effects triggered by resveratrol in cancer cells, as summarized in Figure 1.

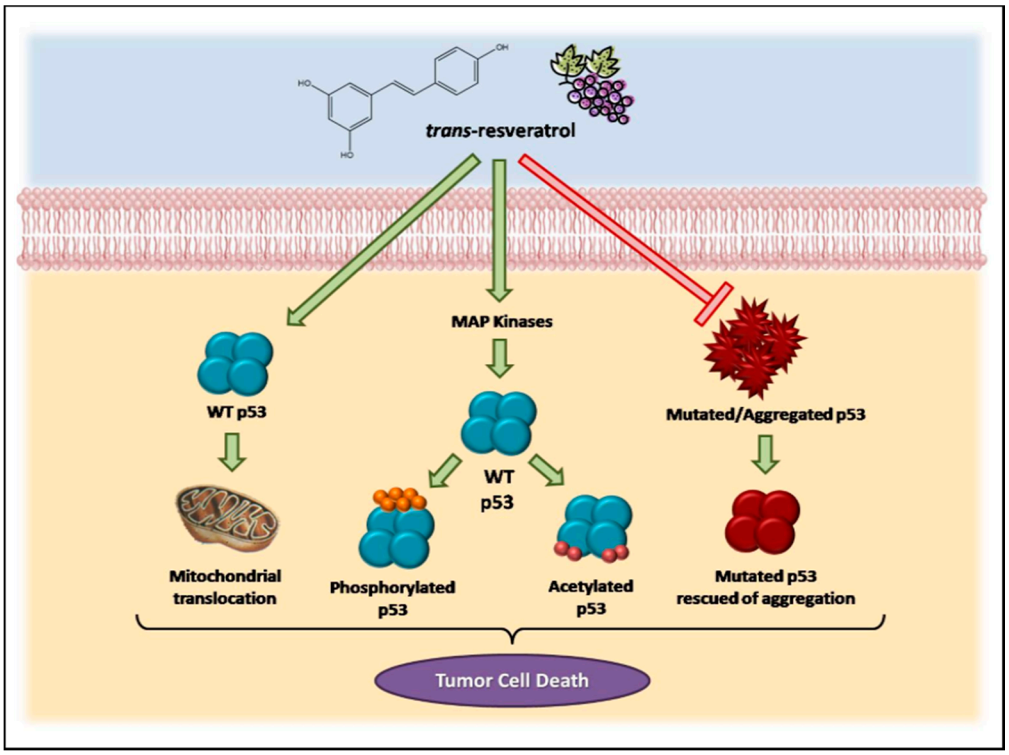

Figure 1. The involvement of the p53 pathway in the effects triggered by resveratrol in cancer cells.

4. Bioavailability of Resveratrol

The low bioavailability and extensive metabolism of resveratrol are a constant source of concern regarding whether the concentrations used for most in vitro studies are even relevant in vivo [108]. One of the biggest challenges for resveratrol in therapy is its poor bioavailability. Due to its rapid phase II metabolism in the liver and intestine [108,109], the bioavailability of ingested and intravenous doses of resveratrol is unable to achieve pharmacologically active concentrations in plasma [110], but the enterohepatic recirculation may contribute to a delayed elimination of the drug from the body and introduce a prolonged effect. By its binding to plasmatic proteins, resveratrol also exhibits a prolonged effect [11].

It is clear that only a small fraction of ingested resveratrol reaches the body as the parent compound. Furthermore, the amount of resveratrol ingested from dietary sources, such as red wine and juices, among others, would very rarely exceed 5 mg, resulting in plasma levels that are either not detectable or are orders of magnitude below the micromolar concentrations employed in vitro. The administration of approximately 25 mg of resveratrol results in plasma concentrations of the free form ranging from 1 to 5 ng/mL [111]; the administration of higher doses (up to 5 g) produced concentrations of free resveratrol up to approximately 500 ng/mL, or just over 2 μM [112].

The actual plasma level and absorption kinetics are highly dependent on other compounds present in the dietary matrices. Therefore, a higher degree of bioavailability has been observed when resveratrol is obtained through wine and/or ingestion via the diet as a single pure compound [113].

Pignatelli et al. [114] observed a mean increase of 1 μM resveratrol in the plasma upon ingestion of 300 mL of red wine a day for 15 days. The actual resveratrol level in the wine was not known in these experiments, but assuming a mean level of resveratrol in red wine of 8.2 μM indicates a relatively high bioavailability [115]. Similarly, a comparison of the metabolic profile of resveratrol as a component of red wine and of grape extract revealed reduced resveratrol absorption and an extended presence in the gut when resveratrol was a component of grape extract [116].

Approximately 20–30% of resveratrol is not recovered in urine or feces [108], causing one to question whether resveratrol is still present in cells throughout the body. It is possible that resveratrol is associated with lipid compartments and released slowly [117].

Some studies have emphasized that other polyphenols present in red wine, such as quercetin, catechin, and gallic acid, could function as potential chemopreventive agents [118–120]. From this perspective, the research group led by Dr. Latruffe has previously shown that a mixture of polyphenolic extracts from grape vine shoots exhibits superior anti-proliferative activity in colon cancer cells to resveratrol alone due to a synergistic interaction between polyphenols [121,122].

The combination of resveratrol with various stilbenes did show synergism (with pterostilbene and polydatin) when analyzing the antioxidant as well as the cytostatic effects [123,124]. The combination of curcumin with resveratrol has been tested in various in vitro models, with the antioxidant capacity, cytostatic effects, and induction of apoptosis being evaluated [125]. Various flavonoids have been tested in combination with resveratrol, including chrysin, quercetin, catechin, genistein, and combinations of several flavonoids. The effects varied depending on the measured activity and combinations of the tested compounds. In general, the same response, but at lower concentrations, was most often observed by using the combined compounds [117]. The role of ethanol is also potentially important in improving the solubility of polyphenols and altering the cell membrane fluidity for cellular uptake, while a high level of ethanol could counteract the beneficial effects of polyphenols [122].

The metabolism of resveratrol by the human microbiota is another confusing factor in relation to the level of resveratrol and metabolites in the body. A changed metabolite profile was observed when using human fecal microbiota [126], which is why metabolism by the gastrointestinal microbiota is also relevant. Aires et al., 2013 [127] have provided significant new insights into the molecular mechanism of resveratrol, and their data support the notion that, despite low bioavailability in vivo, the biological effects of resveratrol could be mediated by its metabolites.

Modifications of resveratrol stability, chemical structure, and metabolism could alter its cellular and molecular targets and could be crucial for improving or decreasing the efficiency of its chemopreventive properties [25]. Interest in the bioproduction and chemical synthesis of stilbenes has also emerged to identify highly active molecules that could be used for medical applications, especially for cell proliferation inhibition [128]. Modification of the hydroxylation and methoxylation patterns of resveratrol had inhibitory effects on the human colorectal tumor SW480 cell line and no effects on non-tumor cells (IEC18 intestinal epithelial cells), demonstrating the selectivity of these molecules for cancer cells [129].

Cisplatin, carboplatin, and oxaliplatin are commonly used chemotherapy drugs that crosslink DNA in rapidly growing cells. In most experiments, an additive effect and sometimes a synergistic effect has been observed on the reduction of the cell viability of various cancer cell lines [130,131]. Similar effects were observed with DNA intercalating drugs such as doxorubicin and docetaxel [132], topoisomerase inhibitors (such as etoposide) [133], and nucleotide analogs (fluorouracil, fludarabine, cladribine, gemcitabine, clofarabine, and decitabine) [134]. Additionally, the combination of resveratrol with DNA-alkylating substances (cyclophosphamide, temozolomide, melphalan, and carmustine) resulted in a potentiation compared with the effect of the drug alone [135]. Reduced or counteracting effects of resveratrol have also been observed when resveratrol is combined with inhibitors of microtubules (vinblastine and paclitaxel), depending on the order of treatment [130]. Similarly, the cytotoxic effect of the proteasome inhibitor MG132 is reduced by resveratrol [51]. In several cases, a synergistic effect has been postulated.

Resveratrol may act in an additive or synergistic manner with other polyphenols and may influence the metabolism or activity of other drugs. The synergism of various polyphenols with resveratrol has been observed experimentally [123,136–138] and underlies the effects of many nutraceutical formulations.

This challenge, the use of natural or synthetic analogs with improved bioavailability or more potency than resveratrol, as well as the combinations of drugs that provide a synergistic effect or improved bioavailability are promising strategies, as in the case of quercetin and other flavonoids [139]. This last method is very attractive as an anticancer drug therapy because the combination of drugs might result in the use of lower doses of individual compounds, leading to better pharmacological action due to additive or synergistic effects, and less collateral effects on the organism [140,141].

Additionally, nanoencapsulated resveratrol has shown enhanced bioavailability relative to the parent compound [142,143]. Encapsulation and the use of alternative routes of uptake have also been explored [144,145].

5. Clinical Trials and Therapeutic Perspectives for the Use of Resveratrol

It is important to note that often there are discrepancies between the doses of resveratrol used in cells and levels obtained in vivo. For example, many studies showing an effect of resveratrol on signaling used concentrations ranging from 10 μM to 100 μM [146]. In contrast, a single 25 mg dose of resveratrol, corresponding to high red wine consumption, resulted in marginal levels of plasma resveratrol in human subjects, and a 5 g dose produced a transient peak of only 2.4 μM [147]. When lower concentrations corresponding to plasma levels are used on cells, the outcomes are variable, and often, no effects are detected [148]. Almeida et al. [111] showed that repeated dosing can increase the plasma half-life of resveratrol by more than two-fold.

The duration of the trials varies from acute exposure [149,150], to a few days [7,151–153] of exposure [154,155], to up to one year [156]. In most trials, the subjects are exposed to resveratrol in time frames of 1–3 months. The relatively short duration of the trials is a challenge because they permit analysis of therapeutic but not preventive potential. Without taking the practical and economical aspects of such studies into consideration, a trial to show a preventive effect of resveratrol should be performed for a minimum of one year. It is obvious that such trials are expensive and not easily funded, but they will be necessary to obtain relevant information regarding the preventive/therapeutic potential of resveratrol [148].

Notably, the resveratrol doses available in supplements and used in many clinical trials are 2–3 orders of magnitude beyond what can be obtained from the diet [157]. It is difficult to estimate normal human consumption of resveratrol because the intake of red wine differs greatly in the population and the content of resveratrol varies (mean, 1.9 ± 1.7 mg/L), but the dose may reach 4 mg/person/day [115]. A single dose of resveratrol results in plasma levels of 2–18 μμM [149]. Ingestion of 3 g daily in obese individuals for eight weeks caused rapid and extensive conjugation of resveratrol [158].

Resveratrol trials in humans after single [108,147] or multiple daily doses of up to 600 mg/day administered over two or three days [111,159] showed that resveratrol is safe under the tested conditions and that the main metabolites found in the circulation are R3S, R4G, and R3G, with particularly high levels in the case of the sulfo-conjugate [160]. A few phase 1 clinical trials focusing on the pharmacokinetics of resveratrol were published before 2010, but since then, the number of clinical trials exploring the biological effects of resveratrol has increased significantly.

In recent years, resveratrol has been shown to possess a fascinating spectrum of pharmacologic properties that could be useful in human medicine [157,161]. To search for clinical trials with resveratrol to write this review, the clinicaltrials.gov site was accessed in April 2017, and 129 studies were identified. After excluding non-cancer studies, only 20 trials remained. Oral resveratrol is used as a pure compound or in resveratrol-rich products (grapes, grape juice or a mixture of supplements),

and the doses and durations of the interventions differed, as shown in Table S1. The following are the 20 clinical trials that were identified.

Clinical trial 1: "**Resveratrol for Patients with Colon Cancer**". A trial conducted by Nguyen et al. [162], with biomarker endpoints evaluating the expression of multiple components and target genes of the Wnt pathways, represents the first reported clinical trial of resveratrol in patients with cancer. The first two patients receiving resveratrol will be treated at a dose of 20 mg/day, the third and fourth patients at a dose of 80 mg/day, and the fifth and sixth patients with a dose of 160 mg/day. All patients receiving grape extract will receive 125 mg/day that will have to be mixed with one 8 oz glass of water. There will be no dose adjustments. If a patient has any side effects which are attributed to the resveratrol, it will be discontinued. The conclusion of the study was that grape powder containing low doses of resveratrol in combination with other bioactive components suppressed the expression of Wnt target genes, cyclin D1 and axin in normal colonic mucosa, suggesting that Wnt pathway inhibition might contribute to resveratrol-mediated colon cancer prevention.

Clinical trial 2: "**A Biological Study of Resveratrol's Effects o**n Notch-1 Signaling in Subjects with Low Grade Gastrointestinal Tumors" is the work of Emily R. Winslow from the University of Wisconsin, Madison. Resveratrol has been shown to activate Notch-1, the signaling of which prevents tumor cell growth. This trial examines the effects of resveratrol and Notch-1 on neuroendocrine tumor tissue and the tolerance of people with neuroendocrine tumors who take resveratrol for up to three months (5 g per day orally administered in two divided doses of 2.5 g each with minimal dose-limiting toxicities according to the National Cancer Institute (NCI) Common Toxicity Criteria). The levels of tumor markers (e.g., chromogranin, 5-HIAA, gastrin, and others) pretreatment will be compared with the post-treatment levels (collected every three months) as a measure of the tumor response. In addition, serial axial imaging will be used to document tumor response rates according to standard Response Evaluation Criteria in Solid Tumors (RECIST).

Clinical trial 3: "**Resveratrol and Human Hepatocyte Function in Cancer**" is a trial coordinated by Dr. Brian G. Harbrecht from the University of Louisville. Resveratrol has shown a beneficial effect on the cellular function of normal and cancer liver cells in samples of liver tissue collected during elective liver surgery. Outcomes based on three measures will test the hypothesis that resveratrol used as a nutritional supplement (1 g pill daily) for 10 days before surgery will have the following effects: (1) improve metabolic function in liver cells, as assessed by the expression of signaling proteins, such as Akt, p38, MAPK, AMPK and PEPCK; (2) reduce cellular growth and proliferation of cancer cells, based on the expression of genes and proteins such as cyclin and p53 and the apoptosis proteins Bcl-2 and Bcl-xL; and (3) decrease inflammation in the liver, as determined by evaluating different levels of genes and proteins for nitric oxide synthase, cytokines such as interleukin-6, and nuclear factor-kappa B signaling proteins. Unfortunately, this trial was withdrawn prior to enrollment.

Clinical trial 4: "**Phase I Biomarker Study of Dietary Grape-Derived Low Dose Resveratrol for Colon Cancer Prevention**" from the University of California, Irvine, was conducted by Randall F. Holcombe. The purpose of this study was to determine the minimum amount of resveratrol-rich fresh red grapes needed to exhibit signs of colon cancer prevention. The grape-supplemented diet provided a low dose of resveratrol in conjunction with other potentially active components contained within the grapes. Colon tissue was obtained by limited flexible sigmoidoscopy before and after ingestion of the red grape-containing diet. Different dosages of grapes (1 or 2/3 or 1/3 lb/day fresh red grapes) were utilized. This study showed the effects of dietary grape-derived low-dose resveratrol on biomarkers related to the Wnt pathway and provided critical information regarding the utility of this nutritional approach for the prevention of colon cancer.

Clinical trial 5. The phase 1 trial, "**Resveratrol in Treating Patients with Colorectal Cancer That Can Be Removed by Surgery**", conducted by the research group of Dr. Brenner D., coordinated by

the National Cancer Institute, addressed the side effects and the optimal dose of resveratrol for the treatment of patients with colorectal cancer that can be removed by surgery. Resveratrol was offered from eight days before colectomy and potentially halted tumor cell growth by blocking M-1G adducts and levels of cyclooxygenase-2 protein/Ki67.

Clinical trial 6 is a phase 1 trial, "**UMCC 2003-064 Resveratrol in Preventing Cancer in Healthy Participants (IRB 2004-535)**", led by Dean Brenner from the University of Michigan Cancer Center to study the side effects and optimal dose of resveratrol for the prevention of cancer in healthy participants. The concentration of resveratrol and its metabolites were analyzed in the plasma, urine, and feces. Beginning five days before study drug administration, participants were placed on a controlled diet (avoiding all resveratrol-containing food or drink) for washout. The participants received oral resveratrol once on Day 1 and were followed at two and seven days. To assess safety, pharmacokinetics and the insulin-like growth factor axis after repeated doses of resveratrol, Brown and colleagues [4] from Dr. Dean Brenner research group recruited forty volunteers to ingest resveratrol at dosages of 0.5 g, 1.0 g, 2.5 g or 5.0 g daily for 29 days and only sixteen participants are treated at the maximum tolerable dose. The higher doses (2.5 g and 5.0 g) generated micromolar concentrations of the parent and substantially higher levels of glucuronide and sulfate conjugates in the plasma and caused gastrointestinal symptoms of mild to moderate severity. The observed decrease in circulating IGF-1 and IGFBP-3 may contribute to cancer chemopreventive activity.

Clinical trial 7: "**A Clinical Study to Assess the Safety, Pharmacokinetics, and Pharmacodynamics of SRT501 in Subjects with Colorectal Cancer and Hepatic Metastases**" is a randomized, double-blind, placebo-controlled, inpatient/outpatient study. Micronized resveratrol (SRT501) was given at a dosage of 5 g daily for 14 days to patients with colorectal cancer with hepatic metastases scheduled to undergo hepatectomy. Micronization allows increased resveratrol absorption, thus increasing its availability, and SRT501 was well tolerated. Mean plasma resveratrol levels following a single dose of SRT501 were 1.9 $\pm$ 1.4 ng/mL, exceeding those published for equivalent doses of non-micronized resveratrol by 2- to 3.5-fold [4,147]. Resveratrol was detectable in hepatic tissue following SRT501 administration. Cleaved caspase-3, a marker of apoptosis, was significantly increased by 39% in malignant hepatic tissue following SRT501 treatment compared with tissue from placebo-treated patients [163].

Clinical trial 8: "**Resveratrol in Healthy Adult Participants**" is led by the principal investigator, Dr. Hsiao-Hui (Sherry) Chow, from Arizona Cancer Center, Tucson. Samples of blood and urine from healthy adult participants who are taking resveratrol once daily for four weeks are being studied. This phase 1 trial is studying the side effects of resveratrol and assessing its effects on healthy adult participants. This trial will compare the CYP enzyme activities from baseline to the end of the resveratrol intervention. CYP1A2, 2D6, 2C9, and 3A4 activity will be assessed according to the plasma paraxanthine/caffeine ratio, urinary dextromethorphan/dextrorphan ratio, urinary losartan/losartan metabolite ratio, and area under the plasma buspirone concentration-time curve, respectively. Additionally, GST activity and GST-pi level in blood lymphocytes and serum bilirubin levels will be used to assess phase II enzyme activity. Finally, a safety evaluation using the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 will be conducted, and any adverse events will be described.

Clinical trial 9: "**Resveratrol in Postmenopausal Women with High Body Mass Index**" is a pilot phase 1 trial examining resveratrol in postmenopausal women with a high body mass index to determine the ability of resveratrol to modulate circulating sex steroid hormones and estrogen metabolites to evaluate its potential for breast cancer prevention. Forty subjects initiated the resveratrol intervention (1 g daily for 12 weeks), and six withdrew early due to adverse events. The resveratrol intervention did not result in significant changes in serum concentrations of estradiol, estrone, or testosterone, but it led to, on average, a 10% increase in the concentrations of sex steroid hormone

binding globulin (SHBG). It also resulted in, on average, a 73% increase in urinary 2-hydroxyestrone (2-OHE1) levels, leading to a favorable change in the urinary 2-OHE1/16α-OHE1 ratio. One participant had a symptomatic grade 4 elevation of liver enzymes at the end of the study intervention. Two subjects had grade 3 skin rashes. The most common adverse events were diarrhea and increased total cholesterol, which were reported in 30% and 27.5% of the subjects, respectively. The authors concluded that among overweight and obese postmenopausal women, a 1 g dose of resveratrol daily has favorable effects on estrogen metabolism and SHBG [164]. Clinical trial reports indicate that the research group of Dr. Chow intends to evaluate the effects of resveratrol on serum levels of C-peptide, serum C-reactive protein (CRP), and adipocytokine expression and secretion as measured by serum leptin and adiponectin. Urinary 8-isoprostaglandin F2 alpha (8-iso-PGF2 alpha) and 8-hydroxydeoxyguanosine (8OHdG) will be assessed to evaluate oxidative stress, and the safety of resveratrol intervention will be measured by the reported adverse events, complete blood count with differential (CBC/diff), comprehensive metabolic panel (CMP), and lipid profile.

Clinical trial 10: "**Resveratrol with or without Piperine to Enhance Plasma Levels of Resveratrol**". There is some evidence that resveratrol in combination with piperine (an alkaloid found in pepper) is more effective for fighting cancer, and therefore, the purpose of this study is to determine whether this combination is more effective than resveratrol alone. Because the investigators do not know the dose of piperine to use in combination with resveratrol, two different doses of piperine will be studied. Twenty-four participants, comprising equal numbers of males and females, will receive, for 30 days, a single dose of resveratrol (2.5 g) without piperine, resveratrol (2.5 g) with piperine at 5 mg, or resveratrol (2.5 g) with piperine at 25 mg. Blood levels of resveratrol and piperine will be measured, and adverse events and side effects will be analyzed. This trial is led by Howard H Bailey from the University of Wisconsin, Madison and has collaborators at the National Institutes of Health (NIH) and the NCI.

Clinical trial 11: "Pilot Study of Resveratrol in Older Adults with Impaired Glucose Tolerance". This proposed pilot study will examine resveratrol treatment (500 mg capsules, three capsules (1500 mg) orally twice a day for six weeks) in older adults with impaired glucose tolerance (IGT) to explore its effects on post-meal blood glucose metabolism.

Clinical trial 12: "Resveratrol and Cardiovascular Health in the Elderly". The objectives of this study are to test the effects of different doses of resveratrol (75 mg or 150 mg, 2×/day, orally) on heart and blood vessel health. This phase 1/2 trial of resVida (an oral preparation of resveratrol) in 90 overweight/obese people over the age of 50 (30 in each group) will be conducted for 12 months.

Clinical trial 13: "Resveratrol and the Metabolic Syndrome". The investigators propose to validate that resveratrol, administered to subjects with the metabolic syndrome under controlled conditions of weight stability, common diet, and strict compliance with the study drug, will improve the symptoms of the metabolic syndrome, thereby decreasing the chance of developing diabetes or heart disease.

Clinical trial 14: "**Dietary Intervention in Follicular Lymphoma (KLYMF)**" from Oslo University Hospital by Dr. Harald Holte, Jr. The recruitment status of this study is unknown. The completion date has passed, and the status has not been verified for more than two years. This open study projecting to include 45 patients seeks to perform a dietary intervention for 16 weeks and compare the apoptosis rate, proliferation rate and immune cell infiltrate before and after the intervention period. A dietary intervention study in patients with follicular lymphoma (FL) Stage III/IV consists of omega 3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) 1000 mg × 5 daily, selenium (L-Seleno methionine) 100 mcg × 2 daily, garlic extract (Allicin) 6 garlic pearls daily; and 100% pomegranate juice (ellagic acid), grape juice (resveratrol, quercetin), and green tea (epigallocatechin gallate), in a volume of two cups daily for 16 weeks.

Clinical trial 15: A phase 1 study for metastatic colorectal patients [163] did not report nephrotoxicity. As SRT501 is extensively metabolized, renal failure appeared to be specific to multiple myeloma

patients [165] due to multiple causes, and hence all patients are at risk. **"A Clinical Study to Assess the Safety and Activity of SRT501 Alone or in Combination with Bortezomib in Patients with Multiple Myeloma"**. Twenty-four patients were enrolled in a phase 2 clinical trial of 5 g of SRT501 with and without bortezomib in patients with multiple myeloma who had relapsed or were refractory to at least one prior therapy. Disease stabilization by bortezomib may have prevented renal failure, whereas low efficacy of SRT501 with nausea and vomiting may have resulted in disease progression and dehydration, leading to renal failure. Renal toxicity was observed in five of the twenty-four patients. Renal failure is one of the specific clinical features of multiple myeloma and can be observed in nearly half of patients throughout the disease [166]. This study demonstrated an unacceptable safety profile and minimal efficacy in patients with relapsed/refractory multiple myeloma, highlighting the risks of novel drug development in such populations [167].

Clinical trial 16: "Anti-inflammatory and Antioxidant Effects of Resveratrol on Healthy Adults", is a clinical trial phase 3 that investigated the hypothesis that resveratrol (500 mg) administered orally to healthy adult smokers induces a decrease in inflammatory and oxidative mediators characterizing the low-grade systemic inflammatory state and the oxidant-antioxidant imbalance of tobacco users. Markers of total antioxidant/oxidative stress (C-reactive protein, 4-hydroxynonenal, nitrotyrosine, endothelial nitric oxide synthase (eNOS)-polymorphism, superoxide dismutase (SOD2)-polymorphism, catalase-polymorphism, pentraxin 3, interleukin-6, tumor necrosis factor-α) will be analyzed at baseline and every 30 days for three months. This trial will be conducted by Dr. Simona Bo from the University of Turin, Italy.

Clinical trial 17: "Study of Supplement's Antioxidant Properties That Contains Natural Extracts". The possibility has been raised that the complex mixture of phytochemicals in foods may contribute to their protective effects. In this view, it is possible that multiple compounds act through complimentary or synergistic mechanisms to provide a greater biologic effect than can be achieved by any individual component/nutrient. To investigate this hypothesis, a double-blind, randomized, and placebo-controlled clinical trial was conducted by Dr. Elizabeth Fragopoulou, from Harokopio University to investigate the effects of a multi-micronutrient supplement against oxidative stress (isoprostane, DNA/RNA damage, protein carbonyl levels, oxLDL, TBARS, resistance to serum oxidation, antioxidant enzymes) in apparently healthy adults for four or eight weeks. The supplement contained the following per 80 mL (a daily dose): *Aloe barbadensis* miller gel (USA/Mexico 36%); grape juice, P*olygonum cuspidatum* extract (containing 10% resveratrol); green tea extract; 1.1 mg of vitamin B1 (100% RDA); 2.5 μg of vitamin B12 (100% RDA); 12 mg of vitamin E (α-TE) (100% RDA); coenzyme Q10; 200 μg of folic acid (100% RDA); ascorbic acid; 27.5 μg of selenium (100% RDA); and 4.2 mg of iron (100% RDA).

Clinical trial 18: "Effects of Micronized Trans-resveratrol Treatment on Polycystic Ovary Syndrome (PCOS) Patients". The purpose of this study, conducted by Dr. Beata Banaszewska from Poznan University of Medical Sciences, Poland with the University of California as a collaborator, is to determine whether three months of therapy with micronized trans-resveratrol (500 mg) can improve clinical (excessive hair, menstrual cycle), endocrine (androgens) and metabolic (lipids, markers of systemic inflammation) profiles in women with PCOS.

Clinical trial 19: Resveratrol is able to potentiate simvastatin-induced inhibition of cell proliferation in a concentration-dependent manner and inhibit the mevalonate pathway, suggesting a novel mechanism of action of resveratrol and underscoring the potential translational/clinical relevance of the interaction of this microcomponent with simvastatin [150]. The phase 4 clinical trial "**Effects of Simvastatin and Micronized Trans-resveratrol Treatment on Polycystic Ovary Syndrome (PCOS) Patients"** is being conducted by Beata Banaszewska, Poznan University of Medical Sciences, Poland. This study is designed to evaluate the endocrine and metabolic effects of simvastatin (20 mg daily) and micronized trans-resveratrol (500 mg daily) on PCOS. Evaluations are performed at baseline and repeated after

three and six months of treatment, and the main outcome is a change in serum total testosterone and fasting insulin levels.

Clinical trial 20: "Evaluation of Oral Lipid Ingestion in Relation to Ovarian Androgen Secretion in Polycystic Ovary Syndrome (PCOS) (ELI-ROAS)". The purpose of this phase 1 trial is to determine the relationship between lipid-induced inflammation and ovarian androgen secretion in women with PCOS, and to examine the effect of the drug salsalate (2 g twice daily) and *Polygonum cuspidatum* extract (PCE) (200 mg containing 20% of resveratrol twice daily) for 12 weeks on lipid-induced inflammation, ovarian androgen secretion, body composition and ovulation in a subset of normal weight women with PCOS. The principal investigator, Dr. Frank González, Director of the Division of Reproductive Endocrinology and Infertility, from Indiana University, hypothesizes that in women with PCOS, HCG administration will stimulate an exaggerated ovarian androgen response, dairy cream ingestion will stimulate white blood cells to generate an inflammatory response and that there is a relationship between HCG-stimulated ovarian androgen secretion and the inflammatory response to dairy cream ingestion regardless of body fat status. Thirty (30) women with PCOS (10 normal weight with normal abdominal adiposity, 10 normal weight with increased abdominal adiposity and 10 obese) and 30 ovulatory control women (10 normal weight with normal abdominal adiposity, 10 normal weight with increased abdominal adiposity and 10 obese) will participate over a three-year period. The investigator also hypothesizes that both salsalate and PCE administration for 12 weeks will attenuate the ovarian androgen response to HCG administration and the inflammatory response to dairy cream ingestion, reduce abdominal adiposity, increase insulin sensitivity and induce ovulation in normal weight women with PCOS. In a subset of 16 women with PCOS, eight will receive salsalate (four normal weight with normal abdominal adiposity and four normal weight with increased abdominal adiposity) and eight will receive PCE (four normal weight with normal abdominal adiposity and four normal weight with increased abdominal adiposity) over a three-year period. This pilot project will help determine the feasibility of conducting a larger double-blind, randomized trial in women with PCOS to further test the latter hypothesis.

A summary of clinical evidence for the preventive effects of resveratrol in human health, especially in cancer, is presented in Figure 2.

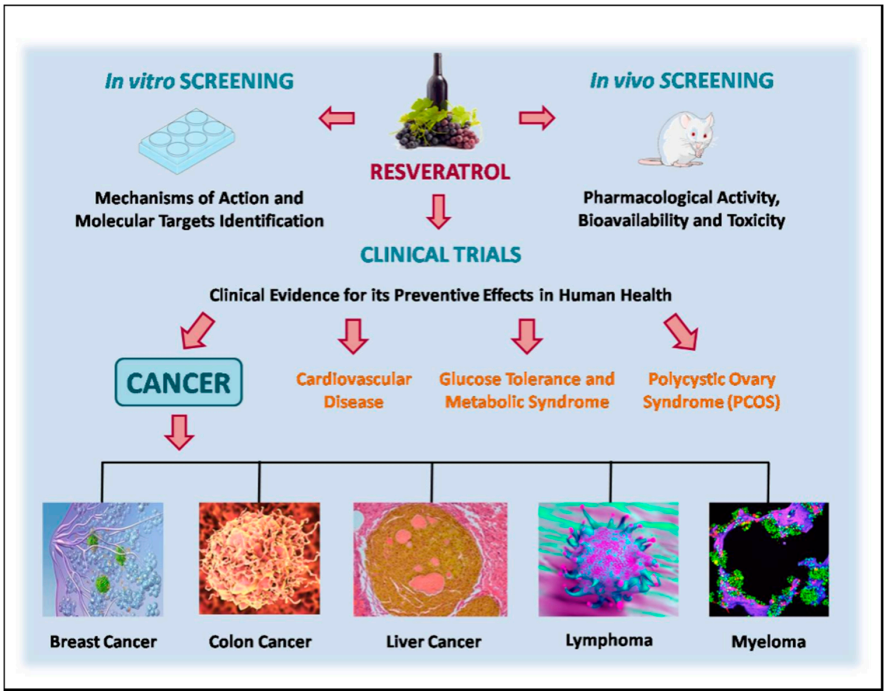

Figure 2. In vitro, in vivo and clinical evidence for the preventive effects of resveratrol in human health.

Considering the twenty studies shown above, only ten (50% of total) were completed. Most of them are phase 1 clinical trials with the aim to look at doses and side effects. Eight of the clinical studies are associated to different types of cancer, showing that until now resveratrol is associated to preventive effects. We further suggest that the quality of clinical trials in future should be improved through an increased number of participants, appropriate study designs, new formulations and/or routes of administration, and biomarkers relevant to healthy participants. Unfortunately, at this moment, the preventive and therapeutic effects of resveratrol in humans are still only supported by in vitro and in vivo model organism studies.

6. Adverse Effects

Based on animal studies, resveratrol is generally well tolerated, and very few short-term or acute exposure experiments in humans have been performed. When eight healthy subjects were exposed to 2 g of resveratrol twice daily for eight days, six of eight subjects had mild episodic diarrhea/loose stool, typically at the beginning of the eight-day treatment period, and one of the subjects developed a temporary rash and headache [168]. In a double-blinded, randomized, placebo-controlled study, up to 975 mg/day were given to healthy volunteers, among which two adult subjects (male and female) in each group were subjected to 25 mg, 50 mg, 100 or 150 mg six times/day for a total of two days. Adverse effects were mild in severity and similar among all groups. Repeated administration of resveratrol was well tolerated but produced relatively low plasma concentrations of resveratrol, despite the high doses and short dosing interval used [111]. Exposure of up to 270 mg resveratrol to nineteen volunteers for one week did not cause any discomfort [150].

According to Elliott et al. [169], healthy volunteers tolerated resveratrol well in a seven-day exposure study, but experimental details were not provided, making evaluation of the results challenging. The same article describes very briefly a study that included daily exposure to 2.5 g or 5 g resveratrol for 28 days. The authors reported that adverse events were generally mild in nature and reversible, but no experimental details were provided, precluding a closer evaluation [169]. Twenty colon cancer patients who received 0.5 g or 1.0 g resveratrol daily for eight days before surgical resection showed good tolerance [154].

7. Recommendations

The first international conference on resveratrol and health, Resveratrol 2010, was held in September 2010 in Denmark, with the purpose of assessing the current state of knowledge in the field and making recommendations for human use and future studies of resveratrol. A clear theme of the conference, which was attended by all of the authors, was that multiple mechanisms are likely to contribute to the beneficial effects of resveratrol, making it difficult to agree on a specific dose, biomarker, or outcome that can define the molecule [157,170].

The second international conference on resveratrol and health, Resveratrol 2012, occurred at the University of Leicester in England and concluded that the published evidence from human trials is not sufficiently strong to justify the recommendation of chronic resveratrol consumption by humans for any given indication. New animal data and recent short-term clinical trials are promising and indicate the need for further long-term human clinical trials, and the use of resveratrol is not an alternative to maintaining a healthy lifestyle.

During the third International Conference on Resveratrol and Health, Resveratrol 2014 (Hawaii), longer exposure times to resveratrol in new trials were recommended. To date, no real clinical trials exploring resveratrol in relation to cancer have been published [171]. At the fourth International Conference on Resveratrol and Health, Resveratrol 2016 (Taiwan), the highlights were focused on the interesting effects of resveratrol on hemorrhagic and septic shock models, its promising neuroprotective abilities and positive influence in psoriasis, and its role in bacterial shift.

8. Conclusions

This review describes recent evidence regarding resveratrol as a chemopreventive agent and a conceptual framework for a new approach to cancer prevention and therapeutics, which is in high demand. A broad-spectrum approach involves synergistic combinations of multiple low-toxicity agents as an association of microcomponents (e.g., resveratrol plus another bioactive compound) or with chemotherapeutic drugs that can collectively impact many pathways that are known to be important for carcinogenic processes, in addition to angiogenesis and metastases. The potential targets of the effect of resveratrol were presented, and p53 as an important protein involved in carcinogenesis could represent a new focus for exploration in translational studies.

Supplementary Materials: The following are available online: Table S1. Synthesis of clinical trials with resveratrol and cancer presented in the site of www.clinicaltrials.gov (accessed in April 2017).

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References

1. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* **2015**, *136*, E359–E386. [CrossRef] [PubMed]
2. World Health Organization Cancer Fact Sheet N297. Available online: http://www.who.int/mediacentre/factsheets/fs297/en/ (accessed on 30 April 2017).
3. Palumbo, M.O.; Kavan, P.; Miller, W.H.; Panasci, L.; Assouline, S.; Johnson, N.; Cohen, V.; Patenaude, F.; Pollak, M.; Jagoe, R.T.; et al. Systemic cancer therapy: Achievements and challenges that lie ahead. *Front. Pharmacol.* **2013**, *4*, 57. [CrossRef] [PubMed]
4. Brown, V.A.; Patel, K.R.; Viskaduraki, M. Repeat Dose Study of the Cancer Chemopreventive Agent Resveratrol in Healthy Volunteers: Safety, Pharmacokinetics, and Effect on the Insulin-like Growth Factor Axis. *Cancer Res.* **2010**, *70*, 9003–9011. [CrossRef] [PubMed]
5. Signorelli, P.; Ghidoni, R. Resveratrol as an anticancer nutrient: Molecular basis, open questions and promises. *J. Nutr. Biochem.* **2005**, *16*, 449–466. [CrossRef] [PubMed]
6. Ali, K.; Maltese, F.; Choi, Y.H.; Verpoorte, R. Metabolic constituents of grapevine and grape-derived products. *Phytochem. Rev.* **2010**, *9*, 357–378. [CrossRef] [PubMed]
7. Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.; Fong, H.H.; Farnsworth, N.R.; Kinghorn, A.D.; Mehta, R.G.; et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218–220. [CrossRef] [PubMed]
8. Shukla, Y.; Singh, R. Resveratrol and cellular mechanisms of cancer prevention. *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 1–8. [CrossRef] [PubMed]
9. Ndiaye, M.; Kumar, R.; Ahmad, N. Resveratrol in cancer management: Where are we and where we go from here? *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 144–149. [CrossRef] [PubMed]
10. Varoni, E.M.; Lo Faro, A.F.; Sharifi-Rad, J.; Iriti, M. Anticancer Molecular Mechanisms of Resveratrol. *Front. Nutr.* **2016**, *3*, 8. [CrossRef] [PubMed]
11. Delmas, D.; Lançon, A.; Colin, D.; Jannin, B.; Latruffe, N. Resveratrol as a chemopreventive agent: A promising molecule for fighting cancer. *Curr. Drug Targets* **2006**, *7*, 423–442. [CrossRef] [PubMed]
12. Ciolino, H.P.; Daschner, P.J.; Yeh, G.C. Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. *Cancer Res.* **1998**, *58*, 5707–5712. [PubMed]
13. Frombaum, M.; Le Clanche, S.; Bonnefont-Rousselot, D.; Borderie, D. Antioxidant effects of resveratrol and other stilbene derivatives on oxidative stress and NO bioavailability: Potential benefits to cardiovascular diseases. *Biochimie* **2012**, *94*, 269–276. [CrossRef] [PubMed]

14. Poulsen, M.M.; Fjeldborg, K.; Ornstrup, M.J.; Kjær, T.N.; Nøhr, M.K.; Pedersen, S.B. Resveratrol and inflammation: Challenges in translating pre-clinical findings to improved patient outcomes. *Biochim. Biophys. Acta Mol. Basis Dis.* **2015**, *1852*, 1124–1136. [CrossRef] [PubMed]
15. Bernhard, D.; Tinhofer, I.; Tonko, M.; Hübl, H.; Ausserlechner, M.J.; Greil, R.; Kofler, R. Csordas, a Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acute leukemia cells. *Cell Death Differ.* **2000**, *7*, 834–842. [CrossRef] [PubMed]
16. Dörrie, J.; Gerauer, H.; Wachter, Y.; Zunino, S.J. Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. *Cancer Res.* **2001**, *61*, 4731–4739. [PubMed]
17. Delmas, D.; Rébé, C.; Lacour, S.; Filomenko, R.; Athias, A.; Gambert, P.; Cherkaoui-Malki, M.; Jannin, B.; Dubrez-Daloz, L.; Latruffe, N.; et al. Resveratrol-induced Apoptosis Is Associated with Fas Redistribution in the Rafts and the Formation of a Death-inducing Signaling Complex in Colon Cancer Cells. *J. Biol. Chem.* **2003**, *278*, 41482–41490. [CrossRef] [PubMed]
18. Reis-Sobreiro, M.; Gajate, C.; Mollinedo, F. Involvement of mitochondria and recruitment of Fas/CD95 signaling in lipid rafts in resveratrol-mediated antimyeloma and antileukemia actions. *Oncogene* **2009**, *28*, 3221–3234. [CrossRef] [PubMed]
19. Ferraz da Costa, D.C.; Casanova, F.A.; Quarti, J.; Malheiros, M.S.; Sanches, D.; dos Santos, P.S.; Fialho, E.; Silva, J.L. Transient Transfection of a Wild-Type p53 Gene Triggers Resveratrol-Induced Apoptosis in Cancer Cells. *PLoS ONE* **2012**, *7*, e48746. [CrossRef] [PubMed]
20. Chin, Y.-T.; Hsieh, M.-T.; Yang, S.-H.; Tsai, P.-W.; Wang, S.-H.; Wang, C.-C.; Lee, Y.-S.; Cheng, G.-Y.; HuangFu, W.-C.; London, D.; et al. Anti-proliferative and gene expression actions of resveratrol in breast cancer cells in vitro. *Oncotarget* **2014**, *5*, 12891–12907. [CrossRef] [PubMed]
21. Carter, L.G.; D'Orazio, J.A.; Pearson, K.J. Resveratrol and cancer: Focus on in vivo evidence. *Endocr. Relat. Cancer* **2014**, *21*, R209–R225. [CrossRef] [PubMed]
22. Hsieh, T.; Wu, J.M. Resveratrol: Biological and pharmaceutical properties as anticancer molecule. *BioFactors* **2010**, *36*, 360–369. [CrossRef] [PubMed]
23. Bishayee, A. Cancer prevention and treatment with resveratrol: From rodent studies to clinical trials. *Cancer Prev. Res. (Phila)* **2009**, *2*, 409–418. [CrossRef] [PubMed]
24. Delmas, D.; Solary, E.; Latruffe, N. Resveratrol, a phytochemical inducer of multiple cell death pathways: Apoptosis, autophagy and mitotic catastrophe. *Curr. Med. Chem.* **2011**, *18*, 1100–1121. [CrossRef] [PubMed]
25. Delmas, D.; Aires, V.; Limagne, E.; Dutartre, P.; Mazué, F.; Ghiringhelli, F.; Latruffe, N. Transport, stability, and biological activity of resveratrol. *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 48–59. [CrossRef] [PubMed]
26. Casanova, F.; Quarti, J.; Ferraz Da Costa, D.C.; Ramos, C.A.; Da Silva, J.L.; Fialho, E. Resveratrol chemosensitizes breast cancer cells to melphalan by cell cycle arrest. *J. Cell. Biochem.* **2012**, *113*, 2586–2596. [CrossRef] [PubMed]
27. Ciolino, H.P.; Yeh, G.C. Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and CYP1A1 expression by resveratrol. *Mol. Pharmacol.* **1999**, *56*, 760–767. [PubMed]
28. Chang, T.K.; Chen, J.; Lee, W.B. Differential inhibition and inactivation of human CYP1 enzymes by trans-resveratrol: Evidence for mechanism-based inactivation of CYP1A2. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 874–882. [PubMed]
29. Li, Y.; Cao, Z.; Zhu, H. Upregulation of endogenous antioxidants and phase 2 enzymes by the red wine polyphenol, resveratrol in cultured aortic smooth muscle cells leads to cytoprotection against oxidative and electrophilic stress. *Pharmacol. Res.* **2006**, *53*, 6–15. [CrossRef] [PubMed]
30. Chow, H.H.S.; Garland, L.L.; Hsu, C.H.; Vining, D.R.; Chew, W.M.; Miller, J.A.; Perloff, M.; Crowell, J.A.; Alberts, D.S. Resveratrol modulates drug- and carcinogen-metabolizing enzymes in a healthy volunteer study. *Cancer Prev. Res.* **2010**, *3*, 1168–1175. [CrossRef] [PubMed]
31. Lançon, A.; Hanet, N.; Jannin, B.; Delmas, D.; Heydel, J.M.; Lizard, G.; Chagnon, M.C.; Artur, Y.; Latruffe, N. Resveratrol in human hepatoma HepG2 cells: Metabolism and inducibility of detoxifying enzymes. *Drug Metab. Dispos.* **2007**, *35*, 699–703. [CrossRef] [PubMed]
32. Hadi, S.M.; Ullah, M.F.; Azmi, A.S.; Ahmad, A.; Shamim, U.; Zubair, H.; Khan, H.Y. Resveratrol mobilizes endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: A putative mechanism for chemoprevention of cancer. *Pharm. Res.* **2010**, *27*, 979–988. [CrossRef] [PubMed]

33. De la Lastra, C.A.; Villegas, I. Resveratrol as an antioxidant and pro-oxidant agent: Mechanisms and clinical implications. *Biochem. Soc. Trans.* **2007**, *35*, 1156–1160. [CrossRef] [PubMed]
34. Gehm, B.D.; Levenson, A.S.; Liu, H.; Lee, E.J.; Amundsen, B.M.; Cushman, M.; Jordan, V.C.; Jameson, J.L. Estrogenic effects of resveratrol in breast cancer cells expressing mutant and wild-type estrogen receptors: Role of AF-1 and AF-2. *J. Steroid Biochem. Mol. Biol.* **2004**, *88*, 223–234. [CrossRef] [PubMed]
35. Hope, C.; Planutis, K.; Planutiene, M.; Moyer, M.P.; Johal, K.S.; Woo, J.; Santoso, C.; Hanson, J.A.; Holcombe, R.F. Low concentrations of resveratrol inhibit Wnt signal throughput in colon-derived cells: Implications for colon cancer prevention. *Mol. Nutr. Food Res.* **2008**, *52*, S52–S61. [CrossRef] [PubMed]
36. Cui, X.; Jin, Y.; Hofseth, A.B.; Pena, E.; Habiger, J.; Chumanevich, A.; Poudyal, D.; Nagarkatti, M.; Nagarkatti, P.S.; Singh, U.P.; et al. Resveratrol suppresses colitis and colon cancer associated with colitis. *Cancer Prev. Res.* **2010**, *3*, 549–559. [CrossRef] [PubMed]
37. Aires, V.; Brassart, B.; Carlier, A.; Scagliarini, A.; Mandard, S.; Limagne, E.; Solary, E.; Martiny, L.; Tarpin, M.; Delmas, D. A role for peroxisome proliferator-activated receptor gamma in resveratrol-induced colon cancer cell apoptosis. *Mol. Nutr. Food Res.* **2014**, *58*, 1785–1794. [CrossRef] [PubMed]
38. Le Corre, L.; Chalabi, N.; Delort, L.; Bignon, Y.-J.; Bernard-Gallon, D.J. Resveratrol and breast cancer chemoprevention: Molecular mechanisms. *Mol. Nutr. Food Res.* **2005**, *49*, 462–471. [CrossRef] [PubMed]
39. Levi, F.; Pasche, C.; Lucchini, F.; Ghidoni, R.; Ferraroni, M.; La Vecchia, C. Resveratrol and breast cancer risk. *Eur. J. Cancer Prev.* **2005**, *14*, 139–142. [CrossRef] [PubMed]
40. Harper, C.E.; Patel, B.B.; Wang, J.; Arabshahi, A.; Eltoum, I.A.; Lamartiniere, C.A. Resveratrol suppresses prostate cancer progression in transgenic mice. *Carcinogenesis* **2007**, *28*, 1946–1953. [CrossRef] [PubMed]
41. Jasiński, M.; Jasińska, L.; Ogrodowczyk, M. Resveratrol in prostate diseases-A short review. *Cent. Eur. J. Urol.* **2013**, *66*, 144–149.
42. Golkar, L.; Ding, X.Z.; Ujiki, M.B.; Salabat, M.R.; Kelly, D.L.; Scholtens, D.; Fought, A.J.; Bentrem, D.J.; Talamonti, M.S.; Bell, R.H.; et al. Resveratrol Inhibits Pancreatic Cancer Cell Proliferation Through Transcriptional Induction of Macrophage Inhibitory Cytokine-1. *J. Surg. Res.* **2007**, *138*, 163–169. [CrossRef] [PubMed]
43. Zhou, J.H.; Cheng, H.Y.; Yu, Z.Q.; He, D.W.; Pan, Z.; Yang, D.T. Resveratrol induces apoptosis in pancreatic cancer cells. *Chin. Med. J.* **2011**, *124*, 1695–1699. [PubMed]
44. Cui, J.; Sun, R.; Yu, Y.; Gou, S.; Zhao, G.; Wang, C. Antiproliferative effect of resveratrol in pancreatic cancer cells. *Phytother. Res.* **2010**, *24*, 1637–1644. [CrossRef] [PubMed]
45. Berge, G.; Øvrebø, S.; Eilertsen, E.; Haugen, A.; Mollerup, S. Analysis of resveratrol as a lung cancer chemopreventive agent in A/J mice exposed to benzoapyrene. *Br. J. Cancer* **2004**, *91*, 1380–1383. [CrossRef] [PubMed]
46. Whyte, L.; Huang, Y.-Y.; Torres, K.; Mehta, R.G. Molecular mechanisms of resveratrol action in lung cancer cells using dual protein and microarray analyses. *Cancer Res.* **2007**, *67*, 12007–12017. [CrossRef] [PubMed]
47. Niles, R.M.; McFarland, M.; Weimer, M.B.; Redkar, A.; Fu, Y.M.; Meadows, G.G. Resveratrol is a potent inducer of apoptosis in human melanoma cells. *Cancer Lett.* **2003**, *190*, 157–163. [CrossRef]
48. Bhattacharya, S.; Darjatmoko, S.R.; Polans, A.S. Resveratrol modulates the malignant properties of cutaneous melanoma through changes in the activation and attenuation of the antiapoptotic protooncogenic protein Akt/PKB. *Melanoma Res.* **2011**, *21*, 180–187. [CrossRef] [PubMed]
49. Jiang, H.; Zhang, L.; Kuo, J.; Kuo, K.; Gautam, S.C.; Groc, L.; Rodriguez, A.I.; Koubi, D.; Hunter, T.J.; Corcoran, G.B.; et al. Resveratrol-induced apoptotic death in human U251 glioma cells. *Mol. Cancer Ther.* **2005**, *4*, 554–561. [CrossRef] [PubMed]
50. Li, J.; Qin, Z.; Liang, Z. The prosurvival role of autophagy in Resveratrol-induced cytotoxicity in human U251 glioma cells. *BMC Cancer* **2009**, *9*, 215. [CrossRef] [PubMed]
51. Niu, X.-F.; Liu, B.-Q.; Du, Z.-X.; Gao, Y.-Y.; Li, C.; Li, N.; Guan, Y.; Wang, H.-Q. Resveratrol protects leukemic cells against cytotoxicity induced by proteasome inhibitors via induction of FOXO1 and p27Kip1. *BMC Cancer* **2011**, *11*, 99. [CrossRef] [PubMed]
52. Jeong, S.H.; Jo, W.S.; Song, S.; Suh, H.; Seol, S.Y.; Leem, S.H.; Kwon, T.K.; Yoo, Y.H. A novel resveratrol derivative, HS1793, overcomes the resistance conferred by Bcl-2 in human leukemic U937 cells. *Biochem. Pharmacol.* **2009**, *77*, 1337–1347. [CrossRef] [PubMed]

53. Hsieh, T.C.; Burfeind, P.; Laud, K.; Backer, J.M.; Traganos, F.; Darzynkiewicz, Z.; Wu, J.M. Cell cycle effects and control of gene expression by resveratrol in human breast carcinoma cell lines with different metastatic potentials. *Int. J. Oncol.* **1999**, *15*, 245–252. [CrossRef] [PubMed]
54. Kundu, J.K.; Surh, Y.J. Cancer chemopreventive and therapeutic potential of resveratrol: Mechanistic perspectives. *Cancer Lett.* **2008**, *269*, 243–261. [CrossRef] [PubMed]
55. Athar, M.; Back, J.H.; Kopelovich, L.; Bickers, D.R.; Kim, A.L. Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms. *Arch. Biochem. Biophys.* **2009**, *486*, 95–102. [CrossRef] [PubMed]
56. Lin, H.Y.; Tang, H.Y.; Davis, F.B.; Davis, P.J. Resveratrol and apoptosis. *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 79–88. [CrossRef] [PubMed]
57. Lin, H.Y.; Lansing, L.; Merillon, J.M.; Davis, F.B.; Tang, H.Y.; Shih, A.; Vitrac, X.; Krisa, S.; Keating, T.; Cao, H.J.; et al. Integrin alphaVbeta3 contains a receptor site for resveratrol. *FASEB J.* **2006**, *20*, 1742–1744. [CrossRef] [PubMed]
58. Kenealey, J.D.; Subramanian, L.; Van Ginkel, P.R.; Darjatmoko, S.; Lindstrom, M.J.; Somoza, V.; Ghosh, S.K.; Song, Z.; Hsung, R.P.; Kwon, G.S.; et al. Resveratrol metabolites do not elicit early pro-apoptotic mechanisms in neuroblastoma cells. *J. Agric. Food Chem.* **2011**, *59*, 4979–4986. [CrossRef] [PubMed]
59. Gupta, S.C.; Kannappan, R.; Reuter, S.; Kim, J.H.; Aggarwal, B.B. Chemosensitization of tumors by resveratrol. *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 150–160. [CrossRef] [PubMed]
60. Baur, J.A.; Sinclair, D.A. Therapeutic potential of resveratrol: The in vivo evidence. *Nat. Rev. Drug Discov.* **2006**, *5*, 493–506. [CrossRef] [PubMed]
61. Cristina, A.; Caterina, B.; Kathleen, W.; Rita, C.; Marzia, B.; Mauro, M.; Albana, H.; Manuela, I.; Augusto, A.; Cristina, M. Resveratrol fuels HER2 and ERα-positive breast cancer behaving as proteasome inhibitor. *Aging (Albany. N. Y.)* **2017**, *9*, 508–523. [CrossRef] [PubMed]
62. Bartolacci, C.; Andreani, C.; Amici, A.; Marchini, C. Walking a Tightrope: A Perspective of Resveratrol Effects on Breast Cancer. *Curr. Protein Pept. Sci.* **2017**.
63. Vousden, K.H.; Lane, D.P. p53 in health and disease. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 275–283. [CrossRef] [PubMed]
64. Hainaut, P.; Wiman, K.G. (Eds.) *25 Years of p53 Research*; Springer: Berlin, Germany, 2005.
65. Vogelstein, B.; Lane, D.; Levine, A.J. Surfing the p53 network. *Nature* **2000**, *408*, 307–310. [CrossRef] [PubMed]
66. Meek, D.W. Tumour suppression by p53: A role for the DNA damage response? *Nat. Rev. Cancer* **2009**, *9*, 714–723. [CrossRef] [PubMed]
67. Caspari, T. Checkpoints: How to activate p53. *Curr. Biol.* **2000**, *10*, R315–R317. [CrossRef]
68. Bunz, F.; Dutriaux, A.; Lengauer, C.; Waldman, T.; Zhou, S.; Brown, J.P.; Sedivy, J.M.; Kinzler, K.W.; Vogelstein, B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **1998**, *282*, 1497–1501. [CrossRef] [PubMed]
69. Zuckerman, V.; Wolyniec, K.; Sionov, R.V.; Haupt, S.; Haupt, Y. Tumour suppression by p53: The importance of apoptosis and cellular senescence. *J. Pathol.* **2009**, *219*, 3–15. [CrossRef] [PubMed]
70. Fuster, J.J.; Sanz-González, S.M.; Moll, U.M.; Andrés, V. Classic and novel roles of p53: Prospects for anticancer therapy. *Trends Mol. Med.* **2007**, *13*, 192–199. [CrossRef] [PubMed]
71. Huang, C.; Ma, W.Y.; Goranson, A.; Dong, Z. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* **1999**, *20*, 237–242. [CrossRef] [PubMed]
72. Liao, P.-C.; Ng, L.-T.; Lin, L.-T.; Richardson, C.D.; Wang, G.-H.; Lin, C.-C. Resveratrol arrests cell cycle and induces apoptosis in human hepatocellular carcinoma Huh-7 cells. *J. Med. Food* **2010**, *13*, 1415–1423. [CrossRef] [PubMed]
73. She, Q.B.; Bode, A.M.; Ma, W.Y.; Chen, N.Y.; Dong, Z. Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res.* **2001**, *61*, 1604–1610. [PubMed]
74. Hsieh, T.C.; Wong, C.; John Bennett, D.; Wu, J.M. Regulation of p53 and cell proliferation by resveratrol and its derivatives in breast cancer cells: An in silico and biochemical approach targeting integrin αvβ3. *Int. J. Cancer* **2011**, *129*, 2732–2743. [CrossRef] [PubMed]
75. Dong, Z. Molecular mechanism of the chemopreventive effect of resveratrol. In *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*; Elsevier: Amsterdam, The Netherlands, 2003; Volume 523–524, pp. 145–150.

76. Bode, A.M.; Dong, Z. Post-translational modification of p53 in tumorigenesis. *Nat. Rev. Cancer* **2004**, *4*, 793–805. [CrossRef] [PubMed]
77. Etienne-Selloum, N.; Dandache, I. Polyphenolic compounds targeting p53-family tumor suppressors: Current progress and challenges. *Futur. Asp. Tumor Suppressor Gene* **2013**.
78. Zhang, S.; Cao, H.J.; Davis, F.B.; Tang, H.-Y.; Davis, P.J.; Lin, H.-Y. Oestrogen inhibits resveratrol-induced post-translational modification of p53 and apoptosis in breast cancer cells. *Br. J. Cancer* **2004**, *91*, 178–185. [CrossRef] [PubMed]
79. Kim, Y.-A.; Choi, B.T.; Lee, Y.T.; Park, D.I.; Rhee, S.-H.; Park, K.-Y.; Choi, Y.H. Resveratrol inhibits cell proliferation and induces apoptosis of human breast carcinoma MCF-7 cells. *Oncol. Rep.* **2004**, *11*, 441–446. [CrossRef] [PubMed]
80. Kuo, P.L.; Chiang, L.C.; Lin, C.C. Resveratrol-induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. *Life Sci.* **2002**, *72*, 23–34. [CrossRef]
81. Shih, A.; Davis, F.B.; Lin, H.Y.; Davis, P.J. Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 1223–1232. [CrossRef] [PubMed]
82. Alkhalaf, M.; Jaffal, S. Potent antiproliferative effects of resveratrol on human osteosarcoma SJSA1 cells: Novel cellular mechanisms involving the ERKs/p53 cascade. *Free Radic. Biol. Med.* **2006**, *41*, 318–325. [CrossRef] [PubMed]
83. Kai, L.; Samuel, S.K.; Levenson, A.S. Resveratrol enhances p53 acetylation and apoptosis in prostate cancer by inhibiting MTA1/NuRD complex. *Int. J. Cancer* **2010**, *126*, 1538–1548. [CrossRef] [PubMed]
84. Shankar, S.; Chen, Q.; Siddiqui, I.; Sarva, K.; Srivastava, R.K. Sensitization of TRAIL-resistant LNCaP cells by resveratrol (3,4′,5 tri-hydroxystilbene): Molecular mechanisms and therapeutic potential. *J. Mol. Signal.* **2007**, *2*, 7. [CrossRef] [PubMed]
85. Fang, Y.; Demarco, V.G.; Nicholl, M.B. Resveratrol enhances radiation sensitivity in prostate cancer by inhibiting cell proliferation and promoting cell senescence and apoptosis. *Cancer Sci.* **2012**, *103*, 1090–1098. [CrossRef] [PubMed]
86. Hsieh, T.C.; Huang, Y.C.; Wu, J.M. Control of prostate cell growth, DNA damage and repair and gene expression by resveratrol analogues, in vitro. *Carcinogenesis* **2011**, *32*, 93–101. [CrossRef] [PubMed]
87. Rashid, A.; Liu, C.; Sanli, T.; Tsiani, E.; Singh, G.; Bristow, R.G.; Dayes, I.; Lukka, H.; Wright, J.; Tsakiridis, T. Resveratrol enhances prostate cancer cell response to ionizing radiation. Modulation of the AMPK, Akt and mTOR pathways. *Radiat. Oncol.* **2011**, *6*, 144. [CrossRef] [PubMed]
88. Ishimaru, D.; Andrade, L.R.; Teixeira, L.S.P.; Quesado, P.A.; Maiolino, L.M.; Lopez, P.M.; Cordeiro, Y.; Costa, L.T.; Heckl, W.M.; Weissmüller, G.; et al. Fibrillar aggregates of the tumor suppressor p53 core domain. *Biochemistry* **2003**, *42*, 9022–9027. [CrossRef] [PubMed]
89. Ishimaru, D.; Ano Bom, A.P.D.; Lima, L.M.T.R.; Quesado, P.A.; Oyama, M.F.C.; De Moura Gallo, C.V.; Cordeiro, Y.; Silva, J.L. Cognate DNA stabilizes the tumor suppressor p53 and prevents misfolding and aggregation. *Biochemistry* **2009**, *48*, 6126–6135. [CrossRef] [PubMed]
90. Levy, C.B.; Stumbo, A.C.; Ano Bom, A.P.D.; Portari, E.A.; Carneiro, Y.; Silva, J.L.; De Moura-Gallo, C.V. Co-localization of mutant p53 and amyloid-like protein aggregates in breast tumors. *Int. J. Biochem. Cell Biol.* **2011**, *43*, 60–64. [CrossRef] [PubMed]
91. Ano Bom, A.P.D.; Rangel, L.P.; Costa, D.C.F.; De Oliveira, G.A.P.; Sanches, D.; Braga, C.A.; Gava, L.M.; Ramos, C.H.I.; Cepeda, A.O.T.; Stumbo, A.C.; et al. Mutant p53 aggregates into prion-like amyloid oligomers and fibrils: Implications for cancer. *J. Biol. Chem.* **2012**, *287*, 28152–28162. [CrossRef] [PubMed]
92. Xu, J.; Reumers, J.; Couceiro, J.R.; De Smet, F.; Gallardo, R.; Rudyak, S.; Cornelis, A.; Rozenski, J.; Zwolinska, A.; Marine, J.-C.C.; et al. De Gain of function of mutant p53 by coaggregation with multiple tumor suppressors. *Nat. Chem. Biol.* **2011**, *7*, 285–295. [CrossRef] [PubMed]
93. Wang, G.; Fersht, A.R. Multisite aggregation of p53 and implications for drug rescue. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 201700308. [CrossRef] [PubMed]
94. Cino, E.A.; Soares, I.N.; Pedrote, M.M.; De Oliveira, G.A.P.; Silva, J.L. Aggregation tendencies in the p53 family are modulated by backbone hydrogen bonds. *Sci. Rep.* **2016**, *6*, 32535. [CrossRef] [PubMed]
95. Lasagna-Reeves, C.A.; Clos, A.L.; Castillo-Carranza, D.; Sengupta, U.; Guerrero-Muñoz, M.; Kelly, B.; Wagner, R.; Kayed, R. Dual role of p53 amyloid formation in cancer; loss of function and gain of toxicity. *Biochem. Biophys. Res. Commun.* **2013**, *430*, 963–968. [PubMed]

96. Soragni, A.; Janzen, D.M.; Johnson, L.M.; Pellegrini, M.; Memarzadeh, S.; Eisenberg, D.S.; Soragni, A.; Janzen, D.M.; Johnson, L.M.; Lindgren, A.G.; et al. Article A Designed Inhibitor of p53 Aggregation Rescues p53 Tumor Suppression in Ovarian Carcinomas. *Cancer Cell* **2016**, *29*, 1–14. [CrossRef] [PubMed]
97. Ishimaru, D.; Maia, L.F.; Maiolino, L.M.; Quesado, P.A.; Lopez, P.C.M.; Almeida, F.C.L.; Valente, A.P.; Silva, J.L. Conversion of wild-type p53 core domain into a conformation that mimics a hot-spot mutant. *J. Mol. Biol.* **2003**, *333*, 443–451. [CrossRef] [PubMed]
98. Ano Bom, A.P.; Freitas, M.S.; Moreira, F.S.; Ferraz, D.; Sanches, D.; Gomes, A.M.O.; Valente, A.P.; Cordeiro, Y.; Silva, J.L. The p53 Core Domain Is a Molten Globule at Low pH. *J. Biol. Chem.* **2010**, *285*, 2857–2866.
99. Silva, J.L.; Gallo, C.V.D.M.; Costa, D.C.F.; Rangel, L.P. Prion-like aggregation of mutant p53 in cancer. *Trends Biochem. Sci.* **2014**, *39*, 260–267. [CrossRef] [PubMed]
100. Klabunde, T.; Petrassi, H.M.; Oza, V.B.; Raman, P.; Kelly, J.W.; Sacchettini, J.C. Rational design of potent human transthyretin amyloid disease inhibitors. *Nat. Struct. Biol.* **2000**, *7*, 312–321. [PubMed]
101. Bourgault, S.; Choi, S.; Buxbaum, J.N.; Kelly, J.W.; Price, J.L.; Reixach, N. Mechanisms of transthyretin cardiomyocyte toxicity inhibition by resveratrol analogs. *Biochem. Biophys. Res. Commun.* **2011**, *410*, 707–713. [CrossRef] [PubMed]
102. Florio, P.; Folli, C.; Cianci, M.; Del Rio, D.; Zanotti, G.; Berni, R. Transthyretin binding heterogeneity and antiamyloidogenic activity of natural polyphenols and their metabolites. *J. Biol. Chem.* **2015**, *290*, 29769–29780. [CrossRef] [PubMed]
103. Evers, F.; Jeworrek, C.; Tiemeyer, S.; Weise, K.; Sellin, D.; Paulus, M.; Struth, B.; Tolan, M.; Winter, R. Elucidating the Mechanism of Lipid Membrane-Induced IAPP Fibrillogenesis and Its Inhibition by the Red Wine Compound Resveratrol: A Synchrotron X-ray Reflectivity Study. *J. Am. Chem. Soc.* **2009**, *131*, 9516–9521. [CrossRef] [PubMed]
104. Feng, Y.; Wang, X.P.; Yang, S.G.; Wang, Y.J.; Zhang, X.; Du, X.T.; Sun, X.X.; Zhao, M.; Huang, L.; Liu, R.T. Resveratrol inhibits beta-amyloid oligomeric cytotoxicity but does not prevent oligomer formation. *Neurotoxicology* **2009**, *30*, 986–995. [CrossRef] [PubMed]
105. Gautam, S.; Karmakar, S.; Batra, R.; Sharma, P.; Pradhan, P.; Singh, J.; Kundu, B.; Chowdhury, P.K. Polyphenols in combination with β-cyclodextrin can inhibit and disaggregate α-synuclein amyloids under cell mimicking conditions: A promising therapeutic alternative. *Biochim. Biophys. Acta Proteins Proteom.* **2017**, *1865*, 589–603. [CrossRef] [PubMed]
106. Costa, D.; Campos, N.; Rangel, L.; Zanphorlin, L.; Ramos, C.; Silva, J. Federation of American Societies for Experimental Biology. *FASEB J.* **2014**, *28* (Suppl. 1), 754.1.
107. Costa, D. *Aspectos Celulares e Estruturais da Modulação da Proteína Supressora de Tumor p53 por Resveratrol*; Federal University of Rio de Janeiro: Rio de Janeiro, Brazil, 2011.
108. Walle, T.; Hsieh, F.; DeLegge, M.H.; Oatis, J.E.; Walle, U.K. High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* **2004**, *32*, 1377–1382. [CrossRef] [PubMed]
109. Vitaglione, P.; Sforza, S.; Galaverna, G.; Ghidini, C.; Caporaso, N.; Vescovi, P.P.; Fogliano, V.; Marchelli, R. Bioavailability of trans-resveratrol from red wine in humans. *Mol. Nutr. Food Res.* **2005**, *49*, 495–504. [CrossRef] [PubMed]
110. Gambini, J.; Inglés, M.; Olaso, G.; Lopez-Grueso, R.; Bonet-Costa, V.; Gimeno-Mallench, L.; Mas-Bargues, C.; Abdelaziz, K.M.; Gomez-Cabrera, M.C.; Vina, J.; et al. Properties of Resveratrol: In Vitro and In Vivo Studies about Metabolism, Bioavailability, and Biological Effects in Animal Models and Humans. *Oxid. Med. Cell. Longev.* **2015**, *2015*, 837042. [CrossRef] [PubMed]
111. Almeida, L.; Vaz-da-Silva, M.; Falcão, A.; Soares, E.; Costa, R.; Loureiro, A.I.; Fernandes-Lopes, C.; Rocha, J.F.; Nunes, T.; Wright, L.; et al. Pharmacokinetic and safety profile of trans-resveratrol in a rising multiple-dose study in healthy volunteers. *Mol. Nutr. Food Res.* **2009**, *53*, S7–S15. [CrossRef] [PubMed]
112. Boocock, D.J.; Patel, K.R.; Faust, G.E.S.; Normolle, D.P.; Marczylo, T.H.; Crowell, J.A.; Brenner, D.E.; Booth, T.D.; Gescher, A.; Steward, W.P. Quantitation of trans-resveratrol and detection of its metabolites in human plasma and urine by high performance liquid chromatography. *J. Chromatogr. B* **2007**, *848*, 182–187. [CrossRef] [PubMed]
113. Vang, O. Resveratrol: Challenges in analyzing its biological effects. *Resveratrol Health* **2015**, *1348*, 161–170. [CrossRef] [PubMed]

114. Pignatelli, P.; Ghiselli, A.; Buchetti, B.; Carnevale, R.; Natella, F.; Germanò, G.; Fimognari, F.; Di Santo, S.; Lenti, L.; Violi, F. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. *Atherosclerosis* **2006**, *188*, 77–83. [CrossRef] [PubMed]
115. Stervbo, U.; Vang, O.; Bonnesen, C. A review of the content of the putative chemopreventive phytoalexin resveratrol in red wine. *Food Chem.* **2007**, *101*, 449–457. [CrossRef]
116. Rotches-Ribalta, M.; Andres-Lacueva, C.; Estruch, R.; Escribano, E.; Urpi-Sarda, M. Pharmacokinetics of resveratrol metabolic profile in healthy humans after moderate consumption of red wine and grape extract tablets. *Pharmacol. Res.* **2012**, *66*, 375–382. [CrossRef] [PubMed]
117. Erdogan, C.S.; Vang, O. Challenges in analyzing the biological effects of resveratrol. *Nutrients* **2016**, *8*, e353. [CrossRef] [PubMed]
118. Ebeler, S.E.; Brenneman, C.A.; Kim, G.S.; Jewell, W.T.; Webb, M.R.; Chacon-Rodriguez, L.; MacDonald, E.A.; Cramer, A.C.; Levi, A.; Ebeler, J.D.; et al. Dietary catechin delays tumor onset in a transgenic mouse model. *Am. J. Clin. Nutr.* **2002**, *76*, 865–872. [PubMed]
119. Wang, P.; Zhang, K.; Zhang, Q.; Mei, J.; Chen, C.J.; Feng, Z.Z.; Yu, D.H. Effects of quercetin on the apoptosis of the human gastric carcinoma cells. *Toxicol. In Vitro* **2012**, *26*, 221–228. [CrossRef] [PubMed]
120. Angel-Morales, G.; Noratto, G.; Mertens-Talcott, S. Red wine polyphenolics reduce the expression of inflammation markers in human colon-derived CCD-18Co myofibroblast cells: Potential role of microRNA-126. *Food Funct.* **2012**, *3*, 745–752. [CrossRef] [PubMed]
121. Colin, D.; Gimazane, A.; Lizard, G.; Izard, J.C.; Solary, E.; Latruffe, N.; Delmas, D. Effects of resveratrol analogs on cell cycle progression, cell cycle associated proteins and 5fluoro-uracil sensitivity in human derived colon cancer cells. *Int. J. Cancer* **2009**, *124*, 2780–2788. [CrossRef] [PubMed]
122. Mazué, F.; Delmas, D.; Murillo, G.; Saleiro, D.; Limagne, E.; Latruffe, N. Differential protective effects of red wine polyphenol extracts (RWEs) on colon carcinogenesis. *Food Funct.* **2014**, *5*, 663–670. [CrossRef] [PubMed]
123. Mikstacka, R.; Rimando, A.M.; Ignatowicz, E. Antioxidant effect of trans-Resveratrol, pterostilbene, quercetin and their combinations in human erythrocytes In vitro. *Plant Foods Hum. Nutr.* **2010**, *65*, 57–63. [CrossRef] [PubMed]
124. De Maria, S.; Scognamiglio, I.; Lombardi, A.; Amodio, N.; Caraglia, M.; Cartenì, M.; Ravagnan, G.; Stiuso, P. Polydatin, a natural precursor of resveratrol, induces cell cycle arrest and differentiation of human colorectal Caco-2 cell. *J. Transl. Med.* **2013**, *11*, 264. [CrossRef] [PubMed]
125. Du, Q.; Hu, B.; An, H.M.; Shen, K.E.P.; Xu, L.; Deng, S.; Wei, M.M. Synergistic anticancer effects of curcumin and resveratrol in Hepa1-6 hepatocellular carcinoma cells. *Oncol. Rep.* **2013**, *29*, 1851–1858. [PubMed]
126. Bode, L.M.; Bunzel, D.; Huch, M.; Cho, G.-S.; Ruhland, D.; Bunzel, M.; Bub, A.; Franz, C.M.A.P.; Kulling, S.E. In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. *Am. J. Clin. Nutr.* **2013**, *97*, 295–309. [CrossRef] [PubMed]
127. Aires, V.; Limagne, E.; Cotte, A.K.; Latruffe, N.; Ghiringhelli, F.; Delmas, D. Resveratrol metabolites inhibit human metastatic colon cancer cells progression and synergize with chemotherapeutic drugs to induce cell death. *Mol. Nutr. Food Res.* **2013**, *57*, 1170–1181. [CrossRef] [PubMed]
128. Mazué, F.; Colin, D.; Gobbo, J.; Wegner, M.; Rescifina, A.; Spatafora, C.; Fasseur, D.; Delmas, D.; Meunier, P.; Tringali, C.; et al. Structural determinants of resveratrol for cell proliferation inhibition potency: Experimental and docking studies of new analogs. *Eur. J. Med. Chem.* **2010**, *45*, 2972–2980. [CrossRef] [PubMed]
129. Chalal, M.; Delmas, D.; Meunier, P.; Latruffe, N.; Vervandier-Fasseur, D. Inhibition of cancer derived cell lines proliferation by synthesized hydroxylated stilbenes and new ferrocenyl-stilbene analogs. Comparison with resveratrol. *Molecules* **2014**, *19*, 7850–7868. [CrossRef] [PubMed]
130. Mao, Q.-Q.; Bai, Y.; Lin, Y.-W.; Zheng, X.-Y.; Qin, J.; Yang, K.; Xie, L.-P. Resveratrol confers resistance against taxol via induction of cell cycle arrest in human cancer cell lines. *Mol. Nutr. Food Res.* **2010**, *54*, 1574–1584. [CrossRef] [PubMed]
131. Nessa, M.U.; Beale, P.; Chan, C.; Yu, J.Q.; Huq, F. Combinations of resveratrol, cisplatin and oxaliplatin applied to human ovarian cancer cells. *Anticancer Res.* **2012**, *32*, 53–59. [PubMed]
132. Díaz-Chávez, J.; Fonseca-Sánchez, M.A.; Arechaga-Ocampo, E.; Flores-Pérez, A.; Palacios-Rodríguez, Y.; Domínguez-Gómez, G.; Marchat, L.A.; Fuentes-Mera, L.; Mendoza-Hernández, G.; Gariglio, P.; et al. Proteomic Profiling Reveals That Resveratrol Inhibits HSP27 Expression and Sensitizes Breast Cancer Cells to Doxorubicin Therapy. *PLoS ONE* **2013**, *8*, e64378. [CrossRef] [PubMed]

133. Heiduschka, G.; Lill, C.; Seemann, R.; Brunner, M.; Schmid, R.; Houben, R.; Bigenzahn, J.; Thurnher, D. The effect of resveratrol in combination with irradiation and chemotherapy: Study using Merkel cell carcinoma cell lines. *Strahlenther. Onkol.* **2014**, *190*, 75–80. [CrossRef] [PubMed]
134. Podhorecka, M.; Halicka, D.; Klimek, P.; Kowal, M.; Chocholska, S.; Dmoszynska, A. Resveratrol increases rate of apoptosis caused by purine analogues in malignant lymphocytes of chronic lymphocytic leukemia. *Ann. Hematol.* **2011**, *90*, 173–183. [CrossRef] [PubMed]
135. Lin, C.J.; Lee, C.C.; Shih, Y.L.; Lin, T.Y.; Wang, S.H.; Lin, Y.F.; Shih, C.M. Resveratrol enhances the therapeutic effect of temozolomide against malignant glioma in vitro and in vivo by inhibiting autophagy. *Free Radic. Biol. Med.* **2012**, *52*, 377–391. [CrossRef] [PubMed]
136. Park, H.J.; Yang, J.-Y.; Ambati, S.; Della-Fera, M.A.; Hausman, D.B.; Rayalam, S.; Baile, C.A. Combined Effects of Genistein, Quercetin, and Resveratrol in Human and 3T3-L1 Adipocytes. *J. Med. Food* **2008**, *11*, 773–783. [CrossRef] [PubMed]
137. Zamin, L.L.; Filippi-Chiela, E.C.; Dillenburg-Pilla, P.; Horn, F.; Salbego, C.; Lenz, G. Resveratrol and quercetin cooperate to induce senescence-like growth arrest in C6 rat glioma cells. *Cancer Sci.* **2009**, *100*, 1655–1662. [CrossRef] [PubMed]
138. Kowalczyk, M.C.; Kowalczyk, P.; Tolstykh, O.; Hanausek, M.; Walaszek, Z.; Slaga, T.J. Synergistic effects of combined phytochemicals and skin cancer prevention in SENCAR mice. *Cancer Prev. Res.* **2010**, *3*, 170–178. [CrossRef] [PubMed]
139. De Santi, C.; Pietrabissa, A.; Spisni, R.; Mosca, F.; Pacifici, G.M. Sulphation of resveratrol, a natural product present in grapes and wine, in the human liver and duodenum. *Xenobiotica* **2000**, *30*, 609–617. [CrossRef] [PubMed]
140. Niedzwiecki, A.; Roomi, M.W.; Kalinovsky, T.; Rath, M. Anticancer efficacy of polyphenols and their combinations. *Nutrients* **2016**, *8*, e552. [CrossRef] [PubMed]
141. Lee, Y.J.; Lee, G.J.; Yi, S.S.; Heo, S.H.; Park, C.R.; Nam, H.S.; Cho, M.K.; Lee, S.H. Cisplatin and resveratrol induce apoptosis and autophagy following oxidative stress in malignant mesothelioma cells. *Food Chem. Toxicol.* **2016**, *97*, 96–107. [CrossRef] [PubMed]
142. Siddiqui, I.A.; Sanna, V.; Ahmad, N.; Sechi, M.; Mukhtar, H. Resveratrol nanoformulation for cancer prevention and therapy. *Ann. N. Y. Acad. Sci.* **2015**, *1348*, 20–31. [CrossRef] [PubMed]
143. Chauhan, A.S. Dendrimer nanotechnology for enhanced formulation and controlled delivery of resveratrol. *Ann. N. Y. Acad. Sci.* **2015**, *1348*, 134–140. [CrossRef] [PubMed]
144. Venuti, V.; Cannavà, C.; Cristiano, M.C.; Fresta, M.; Majolino, D.; Paolino, D.; Stancanelli, R.; Tommasini, S.; Ventura, C.A. A characterization study of resveratrol/sulfobutyl ether-β-cyclodextrin inclusion complex and in vitro anticancer activity. *Colloids Surf. B Biointerfaces* **2014**, *115*, 22–28. [CrossRef] [PubMed]
145. Blanchard, O.L.; Friesenhahn, G.; Javors, M.A.; Smoliga, J.M. Development of a lozenge for oral transmucosal delivery of trans-resveratrol in humans: Proof of concept. *PLoS ONE* **2014**, *9*, e90131. [CrossRef] [PubMed]
146. Widlund, A.L.; Baur, J.A.; Vang, O. mTOR: More targets of resveratrol? *Expert Rev. Mol. Med.* **2013**, *15*, e10. [CrossRef] [PubMed]
147. Boocock, D.J.; Faust, G.E.S.; Patel, K.R.; Schinas, A.M.; Brown, V.A.; Ducharme, M.P.; Booth, T.D.; Crowell, J.A.; Perloff, M.; Gescher, A.J.; et al. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. *Cancer Epidemiol. Biomark. Prev.* **2007**, *16*, 1246–1252. [CrossRef] [PubMed]
148. Vang, O. What is new for resveratrol? Is a new set of recommendations necessary? *Ann. N. Y. Acad. Sci.* **2013**, *1290*, 1–11. [CrossRef] [PubMed]
149. Kennedy, D.O.; Wightman, E.L.; Reay, J.L.; Lietz, G.; Okello, E.J.; Wilde, A.; Haskell, C.F. Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: A double-blind, placebo-controlled, crossover investigation. *Am. J. Clin. Nutr.* **2010**, *91*, 1590–1597. [CrossRef] [PubMed]
150. Wong, R.H.X.; Howe, P.R.C.; Buckley, J.D.; Coates, A.M.; Kunz, I.; Berry, N.M. Acute resveratrol supplementation improves flow-mediated dilatation in overweight/obese individuals with mildly elevated blood pressure. *Nutr. Metab. Cardiovasc. Dis.* **2011**, *21*, 851–856. [CrossRef] [PubMed]
151. Howitz, K.; Bitterman, J.; Cohen, H. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* **2003**, *425*, 191–196. [CrossRef] [PubMed]

152. Baur, J.A.; Pearson, K.J.; Price, N.L.; Jamieson, H.A.; Lerin, C.; Kalra, A.; Prabhu, V.V.; Allard, J.S.; Lopez-Lluch, G.; Lewis, K.; et al. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **2006**, *444*, 337–342. [CrossRef] [PubMed]
153. Lagouge, M.; Argmann, C.; Gerhart-Hines, Z.; Meziane, H.; Lerin, C.; Daussin, F.; Messadeq, N.; Milne, J.; Lambert, P.; Elliott, P.; et al. Resveratrol Improves Mitochondrial Function and Protects against Metabolic Disease by Activating SIRT1 and PGC-1α. *Cell* **2006**, *127*, 1109–1122. [CrossRef] [PubMed]
154. Patel, K.R.; Brown, V.A.; Jones, D.J.L.; Britton, R.G.; Hemingway, D.; Miller, A.S.; West, K.P.; Booth, T.D.; Perloff, M.; Crowell, J.A.; et al. Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. *Cancer Res.* **2010**, *70*, 7392–7399. [CrossRef] [PubMed]
155. Heger, A.; Ferk, F.; Nersesyan, A.; Szekeres, T.; Kundi, M.; Wagner, K.H.; Haidinger, G.; Mišík, M.; Knasmüller, S. Intake of a resveratrol-containing dietary supplement has no impact on DNA stability in healthy subjects. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **2012**, *749*, 82–86. [CrossRef] [PubMed]
156. Tomé-Carneiro, J.; Gonzálvez, M.; Larrosa, M.; García-Almagro, F.J.; Avilés-Plaza, F.; Parra, S.; Yáñez-Gascón, M.J.; Ruiz-Ros, J.A.; García-Conesa, M.T.; Tomás-Barberán, F.A.; et al. Consumption of a grape extract supplement containing resveratrol decreases oxidized LDL and ApoB in patients undergoing primary prevention of cardiovascular disease: A triple-blind, 6-month follow-up, placebo-controlled, randomized trial. *Mol. Nutr. Food Res.* **2012**, *56*, 810–821. [CrossRef] [PubMed]
157. Vang, O.; Ahmad, N.; Baile, C.A.; Baur, J.A.; Brown, K.; Csiszar, A.; Das, D.K.; Delmas, D.; Gottfried, C.; Lin, H.Y.; et al. What is new for an old molecule? Systematic review and recommendations on the use of resveratrol. *PLoS ONE* **2011**, *6*, e19881. [CrossRef] [PubMed]
158. Chachay, V.S.; Macdonald, G.A.; Martin, J.H.; Whitehead, J.P.; O'Moore-Sullivan, T.M.; Lee, P.; Franklin, M.; Klein, K.; Taylor, P.J.; Ferguson, M.; et al. Resveratrol Does Not Benefit Patients With Nonalcoholic Fatty Liver Disease. *Clin. Gastroenterol. Hepatol.* **2014**, *12*, 2092–2103. [CrossRef] [PubMed]
159. Nunes, T.; Almeida, L.; Rocha, J.-F.; Falcão, A.; Fernandes-Lopes, C.; Loureiro, A.I.; Wright, L.; Vaz-da-Silva, M.; Soares-da-Silva, P. Pharmacokinetics of trans-resveratrol following repeated administration in healthy elderly and young subjects. *J. Clin. Pharmacol.* **2009**, *49*, 1477–1482. [CrossRef] [PubMed]
160. Patel, K.R.; Scott, E.; Brown, V.A.; Gescher, A.J.; Steward, W.P.; Brown, K. Clinical trials of resveratrol. *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 161–169. [CrossRef] [PubMed]
161. Smoliga, J.M.; Vang, O.; Baur, J.A. Challenges of translating basic research into therapeutics: Resveratrol as an example. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* **2012**, *67*, 158–167. [CrossRef] [PubMed]
162. Nguyen, A.V.; Martinez, M.; Stamos, M.J.; Moyer, M.P.; Planutis, K.; Hope, C.; Holcombe, R.F. Results of a phase I pilot clinical trial examining the effect of plant-derived resveratrol and grape powder on Wnt pathway target gene expression in colonic mucosa and colon cancer. *Cancer Manag. Res.* **2009**, *1*, 25–37. [PubMed]
163. Howells, L.M.; Berry, D.P.; Elliott, P.J.; Jacobson, E.W.; Hoffmann, E.; Hegarty, B.; Brown, K.; Steward, W.P.; Gescher, A.J. Phase I randomized, double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases-Safety, pharmacokinetics, and pharmacodynamics. *Cancer Prev. Res.* **2011**, *4*, 1419–1425. [CrossRef] [PubMed]
164. Chow, H.-H.S.; Garland, L.L.; Heckman-Stoddard, B.M.; Hsu, C.-H.; Butler, V.D.; Cordova, C.A.; Chew, W.M.; Cornelison, T.L. A pilot clinical study of resveratrol in postmenopausal women with high body mass index: Effects on systemic sex steroid hormones. *J. Transl. Med.* **2014**, *12*, 223. [CrossRef] [PubMed]
165. Bird, J.M.; Owen, R.G.; D'Sa, S.; Snowden, J.A.; Pratt, G.; Ashcroft, J.; Yong, K.; Cook, G.; Feyler, S.; Davies, F.; et al. Guidelines for the diagnosis and management of multiple myeloma 2011. *Br. J. Haematol.* **2011**, *154*, 32–75. [CrossRef] [PubMed]
166. Grzasko, N.; Morawska, M.; Hus, M. Optimizing the treatment of patients with multiple myeloma and renal impairment. *Clin. Lymphoma Myeloma Leuk.* **2015**, *15*, 187–198. [CrossRef] [PubMed]
167. Iggo, N.; Winearls, C.G.; Davies, D.R. The development of cast nephropathy in multiple myeloma. *QJM* **1997**, *90*, 653–656. [CrossRef] [PubMed]
168. La Porte, C.; Voduc, N.; Zhang, G.; Seguin, I.; Tardiff, D.; Singhal, N.; Cameron, D.W. Steady-state pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (Ethanol) in healthy human subjects. *Clin. Pharmacokinet.* **2010**, *49*, 449–454. [CrossRef] [PubMed]

169. Elliott, P.J.; Walpole, S.M.; Morelli, L.; Lambert, P.D.; Lunsmann, W.; Westphal, C.H.; Lavu, S. Resveratrol/SRT501. Sirtuin SIRT1 activator treatment of type 2 diabetes. *Drug Future* **2009**, *34*, 291–295. [CrossRef]
170. Vang, O.; Das, D.K. Resveratrol and Health. *Ann. Rev. N. Y. Acad. Sci.* **2011**, *1215*, 1–170.
171. Vang, O.; Pezzuto, J.M. Introduction to Resveratrol and Health: 3rd International Conference. *Ann. N. Y. Acad. Sci.* **2015**, *1348*, v–vi. [CrossRef] [PubMed]

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Review

Differential Methylation and Acetylation as the Epigenetic Basis of Resveratrol's Anticancer Activity

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Abstract: Numerous studies support the potent anticancer activity of resveratrol and its regulation of key oncogenic signaling pathways. Additionally, the activation of sirtuin 1, a deacetylase, by resveratrol has been known for many years, making resveratrol perhaps one of the earliest nutraceuticals with associated epigenetic activity. Such epigenetic regulation by resveratrol, and the mechanism thereof, has attracted much attention in the past decade. Focusing on methylation and acetylation, the two classical epigenetic regulations, we showcase the potential of resveratrol as an effective anticancer agent by virtue of its ability to induce differential epigenetic changes. We discuss the de-repression of tumor suppressors such as BRCA-1, nuclear factor erythroid 2-related factor 2 (NRF2) and Ras Associated Domain family-1α (RASSF-1α) by methylation, PAX1 by acetylation and the phosphatase and tensin homologue (PTEN) by both methylation and acetylation, in addition to the epigenetic regulation of oncogenic NF-κB and STAT3 signaling by resveratrol. Further, we evaluate the literature supporting the potentiation of HDAC inhibitors and the inhibition of DNMTs by resveratrol in different human cancers. This discussion underlines a robust epigenetic activity of resveratrol that warrants further evaluation, particularly in clinical settings.

Keywords: resveratrol; epigenetic; methylation; acetylation

1. Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) (Figure 1) is a naturally occurring polyphenol found in peanuts and the skin of grapes and berries. It is a phytoalexin produced in response to injury, ultraviolet radiation or a pathogen attack. The initial interest in resveratrol was because of its antioxidant properties [1], which led to the recognition of its chemopreventive ability [2]. Resveratrol was also found to generate reactive oxygen species, leading to effective anticancer activity through a prooxidant mechanism [3,4] which also happens to be a hallmark of several other polyphenols [5–7]. It is now believed that resveratrol exhibits both antioxidant and prooxidant

properties [8–10], which depend largely on the tumor microenvironment and the presence of transition metal ions, particularly copper ions. Besides its anticancer properties, resveratrol has also been investigated to explain the 'French paradox' [11], a phenomenon of the relatively low incidence of coronary heart diseases in the French population despite a diet rich in saturated fats, perhaps due to the consumption of red wine with high resveratrol content.

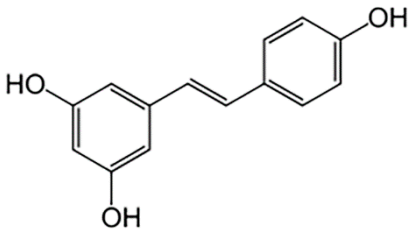

Figure 1. Chemical structure of resveratrol.

The realization of the potent anticancer properties of resveratrol was followed by numerous investigations into the various signaling pathways that it affects, leading to the observed effects [12–17]. In recent years, the focus of cancer research has shifted to stem cells, epithelial-to-mesenchymal transition (EMT) and epigenetic regulation, even in the context of lead compounds such as resveratrol that have a natural origin. Accordingly, a number of studies have documented the ability of resveratrol to affect stem cell populations [18,19] and EMT [20,21]. This article focuses on epigenetic regulation by resveratrol, which is increasingly being proposed as contributing to its anticancer properties.

2. Epigenetic Regulation by Resveratrol

Even though 'epigenetics' was originally intended to define heritable changes in phenotypes that were independent of changes in the DNA sequence, the meaning of this word has now considerably broadened to describe the alterations in human chromatin that influence DNA-templated processes [22,23]. Epigenetic changes do not result in any changes in the DNA sequence, but can still have lasting effects on gene expression. Epigenetic changes involve at least four known modifications of DNA and sixteen classes of histone modifications [22–24].

A number of studies have recognized the effects of nutraceuticals, including polyphenol resveratrol, on the epigenetic machinery in humans [25–35]. Resveratrol can modulate epigenetic patterns by altering the levels of S-adenosylmethionine and S-adenosylhomocysteine or by directing the enzymes that catalyze DNA methylation and histone modifications [27]. It also activates the deacetylase sirtuin and regulates oncogenic and tumor suppressor micro-RNAs [36]. Methylation and acetylation are two classical epigenetic modifications, and these will be the subject of our discussion in this article as we showcase the epigenetic basis of the anticancer action of resveratrol.

3. Methylation

The DNA methylation of gene promoters has a profound effect on their eventual expression and function. Differential DNA methylation can lead to a disease condition in healthy phenotypes. This is particularly true for cancer wherein the differential DNA methylation of the promoters of oncogenes and tumor suppressor genes helps maintain a delicate balance. The increased (hyper) methylation of promoter CpG islands causes gene silencing, while the decreased (hypo) methylation of promoter CpG islands leads to the expression of the gene.

3.1. Breast Cancer

Breast cancer is a widely studied cancer when it comes to epigenetic regulation, particularly differential methylation, by resveratrol. Studies have been conducted to elucidate the genome-wide methylation patterns after resveratrol treatment, and there have also been efforts to understand the

epigenetic basis of the de-repression of tumor suppressors by resveratrol. The subsection to follow will focus on this activity of resveratrol.

3.1.1. Genome-Wide Analyses

A study on the genome-wide methylation-modifying effects of resveratrol, which involved exposing MCF10CA1h and MCF10CA1a cells to resveratrol for 9 days followed by Illumina 450K array analysis, revealed a profound effect of resveratrol on genome-wide DNA methylation with approximately 75% differentially methylated genes being hypermethylated [37]. This study evaluated the epigenetic activity of not only resveratrol but also pterostilbene, an analog of resveratrol. While treatment with resveratrol was done at a dose of 15 μM, pterostilbene treatment was at a 7 μM dose. The compounds targeted genes that are over-expressed in tumors because of DNA hypomethylation. Since increased DNA methylation results in the silencing of genes, it makes sense that the resveratrol-induced hypermethylated genes have predominantly oncogenic functions [37], such as the Notch signaling pathway. However, in another study that also looked at genome-wide DNA methylation after resveratrol treatment (in this case, 24 h and 48 h treatment), only about 12.5% of CpG loci were found to be differentially methylated by resveratrol [38]. This study used MDA-MB-231 breast cancer cells and the concentration of resveratrol was 100 μM. Additionally, this study predominantly found DNA hypomethylation by resveratrol. It is possible that the differences in genome-wide DNA methylation (75% vs. 12.5%) in these two studies might be reflective of the different experimental setups, in particular, the time-durations of the resveratrol treatment as well as the doses used, with longer treatment leading to substantially more methylation.

3.1.2. Effects on Tumor Suppressors

Tumor suppressor genes are typically inactivated at the onset of tumorigenesis and, therefore, their reactivation by anticancer therapy is a mechanism through which tumor progression can be controlled. Several studies have documented the epigenetic reactivation of the tumor suppressor genes by resveratrol in breast cancer (Figure 2), as discussed below.

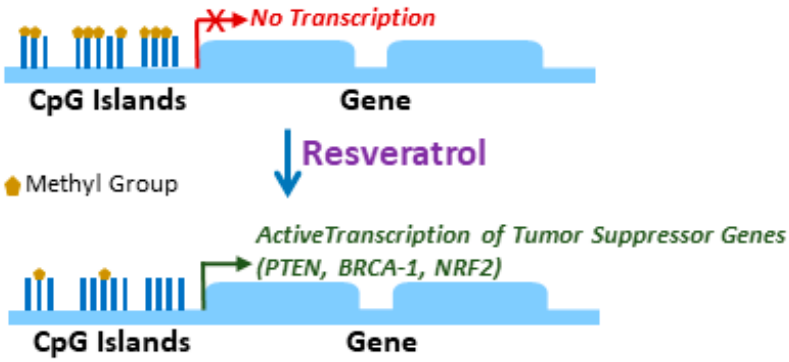

Figure 2. Activation of tumor suppressor genes by resveratrol through promoter DNA hypomethylation. The CpG islands in the DNA promoter regions of the tumor suppressor genes are hypermethylated resulting in their silencing. De-methylation of these CpG islands by resveratrol results in the activation of transcription and the eventual expression of tumor suppressor genes such as the phosphatase and tensin homologue (PTEN), BRCA-1 and nuclear factor erythroid 2-related factor 2 (NRF2).

Phosphatase and tensin homologue (PTEN) is a well-characterized tumor suppressor in breast cancer [39]. In one of the earlier studies describing an effect of resveratrol on promoter DNA methylation, it was reported that resveratrol was highly efficient in reducing PTEN promoter DNA methylation in MCF7 breast cancer cells [40]. Since reduced DNA methylation leads to gene expression, this action of resveratrol induced the expression of PTEN which could explain its anticancer effects. Further, it was reported that the epigenetic effects were complex as not only PTEN was induced but

the cell cycle regulator p21 was up-regulated as well, in addition to the down-regulation of DNMT (DNA methyltransferase). This is interesting because DNA methyltransferases increase methylation. Thus, it appears that resveratrol is able to reduce DNA methylation possibly through two different ways—by reducing methylation and by inducing de-methylation.

BRCA-1 (breast cancer type 1) is another tumor suppressor gene. Its expression is known to be regulated by epigenetic mechanisms. In a study that demonstrated the effects of resveratrol on BRCA-1 methylation [41], it was shown that exposure of breast cancer cells MCF7 to tumor promoter TCDD (2,3,7,8 tetrachlorodibenzo-p-dioxin) resulted in the hypermethylation of BRCA-1 promoter CpG island concomitant with an increased association of trimethylated histone H3K9 and DNMTs, namely DNMT1, DNMT3a and DNMT3b, with BRCA-1 promoter. Resveratrol, at physiologically relevant doses, was able to repress these TCDD effects. In a follow-up study, the research group evaluated the effects of TCDD alone, and in combination with resveratrol, on pregnant Sprague–Dawley rats [42]. It was found that, similar to in vitro observations in MCF7 cells, gestational exposure to TCDD led to the reduced DNA CpG island methylation of BRCA-1 promoter in the mammary tissues of the offspring, which was preceded by the occupation of BRCA-1 promoter by DNMT-1. Also, confirming a possible therapeutic role, resveratrol was found to partially attenuate TCDD effects [42].

Nuclear factor erythroid 2-related factor 2 (NRF2) is also a tumor suppressor that is epigenetically regulated by resveratrol. In a study that looked at the effects of resveratrol on E2 (17β-estradiol)-induced carcinogenesis [43], resveratrol alone up-regulated NRF2 in mammary tissues of rats, and attenuated the repressive effects of E2 on NRF2. E2 suppressed NRF2 through DNA methylation, an activity that was inhibited by resveratrol, thus providing further evidence in support of its regulation of promoter DNA methylation in breast cancer.

3.1.3. DNMTs as Mediators of Resveratrol Effects in Breast Cancer

There is much evidence supporting an inhibitory effect of resveratrol against DNMTs [40,41,44–46]. However, most of the evidence comes from in vitro studies. To validate these findings, a pilot study was conducted that enrolled 39 women with increased breast cancer risk [47]. The subjects were divided into three groups—placebo and those receiving 5 or 50 mg resveratrol. Resveratrol was administered twice daily for twelve weeks and the focus was on the DNA methylation of four cancer-related genes—p16, Ras Associated Domain family-1α (RASSF-1α), Adenomatous Polyposis Coli (APC) and Cyclin D2 (CCND2). An inverse relationship between serum resveratrol levels and RASSF-1α methylation was observed, i.e., when resveratrol levels increased, the methylation of RASSF-1α decreased, thus leading to the expression of this tumor suppressor gene. In another study by this group [48], an in vivo effect of resveratrol on DNMT expression was examined in normal vs. tumor tissues. The model evaluated was ACI rats, an inbred line derived from the August and Copenhagen strains. An interesting observation was that resveratrol affected DNMT3b, but not DNMT1. Two different doses of resveratrol were tested and while DNMT3b differed in normal vs. tumor tissues of rats treated with low resveratrol, a high resveratrol dose resulted in decreased DNMT3b in tumor tissues with increased DNMT3b in the normal tissues [48]. This observation is a little different from the one performed in vitro in immortalized breast cancer epithelial cells, MCF10A [49], where resveratrol, at a non-cytotoxic dose, only induced subtle changes in the DNA methylation of eight pre-determined genes. However, the involvement of DNMT3b in resveratrol activity was also identified in a genome-wide DNA methylation study in breast cancer cells [37].

Further confirming the importance of targeting DNMTs in breast cancer patients, the elevated expression of DNMT transcripts was reported in a study that evaluated breast cancer tissues from 40 breast cancer patients and compared those with tissues from 10 paired normal breast tissues [50]. This study further confirmed the down-regulation of DNMT transcripts in vitro in breast cancer cells.

3.2. Glioma

Glioblastoma multiforme was the other malignancy where evidence of the effect of resveratrol on methylation was initially presented. It was shown that resveratrol could sensitize

resistant glioblastoma T98G cells to temozolomide, inhibiting temozolomide IC-50 with increased apoptosis [51]. Interestingly, temozolomide-resistant cells have increased MGMT (O(6)-methylguanine-DNA-methyltransferase) activity and the protein expression of MGMT is an important determinant of temozolomide-resistance [51]. These results are suggestive of yet another DNA methylation-suppressing activity of resveratrol. In a recent report, inhibition of Wnt signaling has been identified as the mechanism by which resveratrol induces cell death in T98G cells [52]. Epigenetic regulation has been identified as one of the bases of perturbed Wnt signaling [53] and it is plausible that Wnt signaling might be another piece in the puzzle.

3.3. Lung Cancer

Lung cancer, the majority of which is non-small cell lung cancer (NSCLC), is the leading cause of cancer-related deaths in the United States, as well as worldwide. A number of studies have described the inhibitory effects of resveratrol against various lung cancer models [54–56], albeit, a majority of the studies have focused on NSCLC with little information on the other lung cancer types. In recent years, efforts have been made towards the personalized management of lung cancer [57], with a focus on epigenetics in such personalized therapy [58]. Further, it has recently been demonstrated that resveratrol can epigenetically regulate the expression of zinc finger protein36 (ZFP36) through differential DNA methylation [59]. Specifically, resveratrol reduced the methylation of ZFP36, resulting in its up-regulation in A549 NSCLC cells. A role of ZFP36 in human malignancies is increasingly being realized, making it an attractive target for therapy [60]. Thus, its epigenetic targeting by resveratrol further underlines the anticancer potential of resveratrol, particularly in lung cancer.

4. Acetylation

Even before the realization of the methylation potential of resveratrol, it has been known to modulate acetylation within the cellular microenvironment. A quarter century back, resveratrol was reported to be an activator of sirtuin-1 (SIRT1), the NAD-dependent deacetylase, marking its influence on acetylation, and thereby epigenetic regulation [61]. While the activity of resveratrol against Class III HDACs sirtuins is well characterized, it has been suggested that perhaps resveratrol possesses a pan-HDAC inhibitory property and can inhibit HDACs representing class I, class II as well as class IV [62]. Recently, resveratrol has been identified as an inhibitor of bromodomains [63] and the bromodomain and extraterminal (BET) family [64]. Bromodomains affect epigenetic machinery by recognizing lysine acetylation on histones, thereby functioning as epigenetic readers. The interactions of resveratrol with bromodomains open yet another mechanism of epigenetic regulation by resveratrol which is not yet fully explored. The next few subsections discuss the reported effects of resveratrol on the acetylation of different genes in various human cancers.

4.1. Breast Cancer

Acetylation and its impact on breast cancer progression is well appreciated. It is because of this knowledge that HDAC inhibitors still remain an attractive therapeutic strategy against breast cancers [65–67]. It is not only the HDAC inhibitors but also the inhibitors of HATs (histone acetyltransferases) that are being evaluated [68], which provides a good example of how dynamic the process of acetylation and deacetylation is, and how an imbalance can lead to tumor onset and progression. In a study [69] that is indicative of an intricate connection between methylation and acetylation, the two classical epigenetic modifications, it was reported that lysine acetylation within the signal transducer and activator of transcription 3 (STAT3) can impact the interaction of DNMT1 with STAT3 and is accompanied by the demethylation and, thereby, the re-expression of tumor suppressor genes. This study used resveratrol as an acetylation inhibitor and the observations in triple negative breast cancer (TNBC) were further confirmed in melanoma. TNBCs are characterized by the absence of the estrogen receptor (ER) and progesterone receptor (PR), as well as human epidermal growth factor receptor 2 (HER2), and it has been reported that the absence of the *ERα* gene in tumor cells is

often a result of methylation [70]. With the observation that STAT3 is acetylated and, therefore, highly expressed in TNBCs, it was evaluated whether inhibiting STAT3 acetylation could reactivate *ERα* [69]. The TNBC cell line MDA-MB-468 was used as the model and it was observed that treatment with resveratrol significantly reduced STAT3 acetylation as well as *ERα* gene promoter DNA methylation (Figure 3). This resulted in the increased expression of *ERα* and the sensitization of otherwise resistant cells to the ER-targeted therapy, tamoxifen. Further, growth of in vivo tumors in mice was not significantly reduced by tamoxifen alone, as expected, but by the combinational treatment comprising resveratrol and tamoxifen, thus validating the in vitro findings [69]. As further evidence of the effect of resveratrol on acetylation in breast cancer, in MCF-7 breast cancer cells, resveratrol has been shown to induce H3 acetylation [71]. Thus, there seems to be evidence suggesting a modulatory effect of resveratrol on protein as well as histone acetylation.

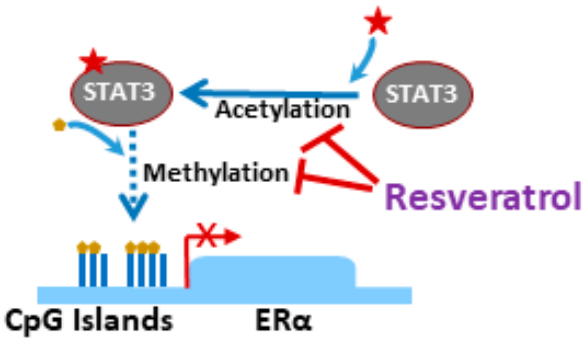

Figure 3. Epigenetic regulation in triple negative breast cancers (TNBCs). TNBCs are characterized by activated STAT3 signaling, involving acetylated STAT3. ERα signaling in TNBCs is silenced through promoter DNA hypermethylation which might be related to STAT3 acetylation but the mechanisms remain unclear (and are therefore shown with a dotted line). Resveratrol is an effective inhibitor of STAT3 acetylation as well as ERα promoter DNA methylation. Restoration of ER-signaling makes TNBC cells sensitive to the ER-targeting therapy, tamoxifen.

4.2. Cervical Cancer

In cervical cancer models, paired box gene1 (PAX1) is a tumor suppressor whose expression is repressed during tumorigenesis by DNA hypermethylation. In a study that evaluated the effect of nutraceuticals, including resveratrol, on the inhibition of cervical cancer through the reactivation of PAX1, it was reported that resveratrol was capable of reactivating PAX1 expression in Caski cells [72]. However, surprisingly, the reactivation of PAX1 was not found to be due to the effect of resveratrol on the DNA methylation of PAX1 promoter. Rather, it possibly involved the acetylation modulating ability of resveratrol through its regulation of HDAC activity. Similar to an effect of resveratrol on histone H3 acetylation in breast cancer cells above, resveratrol has been reported to induce H3 acetylation in HeLa cervical cancer cells as well [71]. Such an effect of resveratrol on H3 acetylation assumes significance given the proposed role of histone H3 acetylation as a prognostic marker for cervical cancer patients [73].

4.3. Colon Cancer

NF-κB signaling is known to be important to the progression of colon cancer [74], especially in resistance to cisplatin [75]. An increase in the protein acetylation of the NF-κB p65 subunit leads to the activation of the NF-κB pathway and its nuclear accumulation. Therefore, the inhibition of the acetylation of p65 can potentially be an effective strategy to check the growth of colon cancer as well as to overcome resistance to cisplatin. In an in vitro study performed in HK2 cells, resveratrol was found to decrease the protein acetylation of the p65 subunit, thus reversing the cell viability-inducing activity of cisplatin [76]. Such down-regulation of NF-κB protein acetylation by resveratrol in colorectal cells was confirmed in another study [77] and this resulted in reduced tumor invasion and metastasis because of the down regulation of NF-κB-regulated factors, such as MMP-9 and CXCR4.

4.4. Leukemia and Lymphoma

In leukemia, resveratrol can potentiate the activity of HDAC inhibitors [78], while in a Hodgkin lymphoma represented by L-428 cells, resveratrol can effectively induce apoptosis as well as cell cycle arrest [79]. As the mechanism, it was observed that resveratrol induced the tumor suppressor p53 through an increase in the p53 K373-acetylation (Table 1). Additionally, resveratrol treatment was found to induce the lysine acetylation of FOXO3a [79]. In leukemia U937 cells, resveratrol potentiates reactive oxygen species production by retinoic acid, particularly the production of superoxide anions, primarily through the up-regulation of the gp91-phox gene that is part of the membrane-bound cytochrome b_{558} [80]. As a mechanism, it was elucidated that resveratrol promoted acetylation within the promoter region of the gp91-phox gene, particularly the Lys-9 residues and Lys-14 residues of histone H3 within the chromatin surrounding the gene promoter.

Table 1. Epigenetic effects of resveratrol on tumor suppressors: mechanisms of their re-activation.

Tumor Suppressor	Cancer Type	Effect of Resveratrol	Reference
BRCA-1	Breast	Reduced promotor DNA methylation in vitro Reduced promotor DNA methylation in vivo	[36] [37]
NRF2	Breast	Reduced promotor DNA methylation	[38]
p53	Lymphoma Prostate	Induced acetylation	[73] [75]
PAX1	Cervical	Regulation of histone acetylation	[66]
PTEN	Breast Prostate	Reduced promoter DNA methylation Acetylation and activation	[35] [77]
RASSF-1α	Breast	Reduced DNA methylation	[42]

4.5. Prostate Cancer

In prostate cancer, metastasis-associated protein 1 (MTA1) is oncogenic with its expression correlating with tumor progression. It is itself involved in the transcriptional repression of target genes through the post-translational modifications of histones as well as non-histones by virtue of it being a part of the nucleosome remodeling deacetylation corepressor complex, the 'NuRD complex'. Resveratrol was shown to down-regulate MTA1, leading to acetylation and the activation of the tumor suppressor p53 through the destabilization of the corepressor complex [81]. The NuRD complex plays a role in maintaining chromatin conformation, which it achieves through the deacetylation of histone proteins [82]. HDAC1 and HDA2 are components of the NuRD complex, with the MTA1-HDAC1 subunit responsible for the deacetylation of histones by NuRD. With a direct regulation of MTA1, and the observation that HDAC1 was decreased in resveratrol-treated MTA1 immunoprecipitates, it is evident that resveratrol has a profound effect on histone acetylation. Further, the effects of resveratrol were similar to those of the HDAC inhibitor SAHA, thus underlying the acetylation-affecting epigenetic activity of resveratrol. The results were further confirmed in vivo in a follow-up study [83], and it was shown that the MTA1-mediated tumor progression was, in part, due to PTEN inactivation and that resveratrol could acetylate and reactivate PTEN [84] (Table 1).

Signaling through the androgen receptor (AR) is important in prostate cancer, even in the advanced castrate-resistant prostate cancers. In a study that specifically looked at the regulation of AR signaling by resveratrol, it was observed that treatment with 10 μM resveratrol for just 3 h inhibited the acetylation of AR and affected the binding of AR to the enhancer region of prostate-specific antigen (PSA) [85]. At a slightly longer treatment of 24 h, resveratrol inhibited the nuclear accumulation of AR as well. Given the important role that AR plays in prostate cancer, such epigenetic regulation of its activity and the effect on down-stream signaling by resveratrol is an encouraging finding that gives hope for its possible use as a therapy against prostate cancer.

5. Conclusions and Perspectives

While various cellular signaling pathways and genes (oncogenes as well as tumor suppressor genes) are still being evaluated as therapeutic targets of anticancer agents such as resveratrol, in recent years, attention has also turned to epigenetic regulation. In fact, epigenetic regulation of classical signaling pathways is increasingly being realized. For example, two of the very well characterized signaling pathways, NF-κB and STAT3, are epigenetically regulated by resveratrol [69,76,77,86]. This represents a fundamental evolution in our understanding with regards to the intricate regulation of oncogenic pathways. Our discussion on the topic, as presented in this article, detailed many studies that provided evidence supporting the epigenetic activity of resveratrol. However, resveratrol does not regulate gene expression only through epigenetic mechanisms. For example, in a study in acute lymphoblastic leukemia [87] that looked at the possible effect of resveratrol on the methylation of MDR1 (multidrug resistance gene 1), no evidence of the differential DNA methylation of MDR1 promoter by resveratrol was found. While resveratrol had a visible suppressive effect on MDR1, there did not seem to be any epigenetic perspective. This is a perfect reminder that not all regulation of gene expression and function by resveratrol has an epigenetic basis. Additionally, regulation through microRNAs (miRNAs) is within the realm of epigenetic regulation, but we decided to cover just the classical epigenetic mechanisms with regards to methylation and acetylation so as to keep the discussion more focused. Finally, the bioavailability of resveratrol still remains a concern, but that should not deter us from fully elucidating its mechanism of action and its potential as a viable anticancer lead. Several groups are working hard on improving the bioavailability through novel approaches and once they achieve success, resveratrol should be ready for further evaluations in clinical settings as an anticancer agent with multifaceted epigenetic activity.

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References

1. Miller, N.J.; Rice-Evans, C.A. Antioxidant activity of resveratrol in red wine. *Clin. Chem.* **1995**, *41*, 1789. [PubMed]
2. Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.; Fong, H.H.; Farnsworth, N.R.; Kinghorn, A.D.; Mehta, R.G.; et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218–220. [CrossRef] [PubMed]
3. Ahmad, A.; Farhan Asad, S.; Singh, S.; Hadi, S.M. DNA breakage by resveratrol and cu(ii): Reaction mechanism and bacteriophage inactivation. *Cancer Lett.* **2000**, *154*, 29–37. [CrossRef]
4. Ahmad, A.; Syed, F.A.; Singh, S.; Hadi, S.M. Prooxidant activity of resveratrol in the presence of copper ions: Mutagenicity in plasmid DNA. *Toxicol. Lett.* **2005**, *159*, 1–12. [CrossRef]
5. Khan, H.Y.; Zubair, H.; Faisal, M.; Ullah, M.F.; Farhan, M.; Sarkar, F.H.; Ahmad, A.; Hadi, S.M. Plant polyphenol induced cell death in human cancer cells involves mobilization of intracellular copper ions and reactive oxygen species generation: A mechanism for cancer chemopreventive action. *Mol. Nutr. Food Res.* **2014**, *58*, 437–446. [CrossRef] [PubMed]
6. Khan, H.Y.; Zubair, H.; Ullah, M.F.; Ahmad, A.; Hadi, S.M. A prooxidant mechanism for the anticancer and chemopreventive properties of plant polyphenols. *Curr. Drug Targets* **2012**, *13*, 1738–1749. [CrossRef] [PubMed]
7. Farhan, M.; Oves, M.; Chibber, S.; Hadi, S.M.; Ahmad, A. Mobilization of nuclear copper by green tea polyphenol epicatechin-3-gallate and subsequent prooxidant breakage of cellular DNA: Implications for cancer chemotherapy. *Int. J. Mol. Sci.* **2016**, *18*, 34. [CrossRef] [PubMed]
8. de la Lastra, C.A.; Villegas, I. Resveratrol as an antioxidant and pro-oxidant agent: Mechanisms and clinical implications. *Biochem. Soc. Trans.* **2007**, *35*, 1156–1160. [CrossRef] [PubMed]

9. Casey, S.C.; Amedei, A.; Aquilano, K.; Azmi, A.S.; Benencia, F.; Bhakta, D.; Bilsland, A.E.; Boosani, C.S.; Chen, S.; Ciriolo, M.R.; et al. Cancer prevention and therapy through the modulation of the tumor microenvironment. *Semin. Cancer Biol.* **2015**, *35*, S199–S223. [CrossRef] [PubMed]
10. Chen, C.Y.; Kao, C.L.; Liu, C.M. The cancer prevention, anti-inflammatory and anti-oxidation of bioactive phytochemicals targeting the tlr4 signaling pathway. *Int. J. Mol. Sci.* **2018**, *19*, 2729. [CrossRef]
11. Kopp, P. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'french paradox'? *Eur. J. Endocrinol.* **1998**, *138*, 619–620. [CrossRef] [PubMed]
12. Shukla, Y.; Singh, R. Resveratrol and cellular mechanisms of cancer prevention. *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 1–8. [CrossRef] [PubMed]
13. Yang, X.; Li, X.; Ren, J. From french paradox to cancer treatment: Anti-cancer activities and mechanisms of resveratrol. *Anti-Cancer Agents Med. Chem.* **2014**, *14*, 806–825. [CrossRef]
14. McCalley, A.E.; Kaja, S.; Payne, A.J.; Koulen, P. Resveratrol and calcium signaling: Molecular mechanisms and clinical relevance. *Molecules* **2014**, *19*, 7327–7340. [CrossRef] [PubMed]
15. Farooqi, A.A.; Wu, S.J.; Chang, Y.T.; Tang, J.Y.; Li, K.T.; Ismail, M.; Liaw, C.C.; Li, R.N.; Chang, H.W. Activation and inhibition of atm by phytochemicals: Awakening and sleeping the guardian angel naturally. *Arch. Immunol. Ther. Exp.* **2015**, *63*, 357–366. [CrossRef] [PubMed]
16. de Oliveira, M.R.; Nabavi, S.F.; Manayi, A.; Daglia, M.; Hajheydari, Z.; Nabavi, S.M. Resveratrol and the mitochondria: From triggering the intrinsic apoptotic pathway to inducing mitochondrial biogenesis, a mechanistic view. *Biochimi. Biophys. Acta* **2016**, *1860*, 727–745. [CrossRef] [PubMed]
17. Zhang, L.; Wen, X.; Li, M.; Li, S.; Zhao, H. Targeting cancer stem cells and signaling pathways by resveratrol and pterostilbene. *BioFactors* **2018**, *44*, 61–68. [CrossRef]
18. Sarkar, F.H.; Li, Y.; Wang, Z.; Kong, D. The role of nutraceuticals in the regulation of wnt and hedgehog signaling in cancer. *Cancer Metast. Rev.* **2010**, *29*, 383–394. [CrossRef]
19. McCubrey, J.A.; Lertpiriyapong, K.; Steelman, L.S.; Abrams, S.L.; Yang, L.V.; Murata, R.M.; Rosalen, P.L.; Scalisi, A.; Neri, L.M.; Cocco, L.; et al. Effects of resveratrol, curcumin, berberine and other nutraceuticals on aging, cancer development, cancer stem cells and microrna. *Aging* **2017**, *9*, 1477–1536. [CrossRef]
20. Karimi Dermani, F.; Saidijam, M.; Amini, R.; Mahdavinezhad, A.; Heydari, K.; Najafi, R. Resveratrol inhibits proliferation, invasion, and epithelial-mesenchymal transition by increasing mir-200c expression in hct-116 colorectal cancer cells. *J. Cell. Biochem.* **2017**, *118*, 1547–1555. [CrossRef]
21. Xu, J.; Liu, D.; Niu, H.; Zhu, G.; Xu, Y.; Ye, D.; Li, J.; Zhang, Q. Resveratrol reverses doxorubicin resistance by inhibiting epithelial-mesenchymal transition (emt) through modulating pten/akt signaling pathway in gastric cancer. *J. Exp. Clin. Cancer Res.* **2017**, *36*, 19. [CrossRef] [PubMed]
22. Dawson, M.A.; Kouzarides, T. Cancer epigenetics: From mechanism to therapy. *Cell* **2012**, *150*, 12–27. [CrossRef] [PubMed]
23. Ahmad, A.; Li, Y.; Bao, B.; Kong, D.; Sarkar, F.H. Epigenetic regulation of mirna-cancer stem cells nexus by nutraceuticals. *Mol. Nutr. Food Res.* **2014**, *58*, 79–86. [CrossRef] [PubMed]
24. Baylin, S.B.; Jones, P.A. A decade of exploring the cancer epigenome—biological and translational implications. *Nat. Rev. Cancer* **2011**, *11*, 726–734. [CrossRef] [PubMed]
25. Vanden Berghe, W. Epigenetic impact of dietary polyphenols in cancer chemoprevention: Lifelong remodeling of our epigenomes. *Pharmacol. Res.* **2012**, *65*, 565–576. [CrossRef] [PubMed]
26. Hardy, T.M.; Tollefsbol, T.O. Epigenetic diet: Impact on the epigenome and cancer. *Epigenomics* **2011**, *3*, 503–518. [CrossRef] [PubMed]
27. Park, L.K.; Friso, S.; Choi, S.W. Nutritional influences on epigenetics and age-related disease. *Proc. Nutr. Soc.* **2012**, *71*, 75–83. [CrossRef] [PubMed]
28. Gerhauser, C. Cancer chemoprevention and nutriepigenetics: State of the art and future challenges. *Top. Curr. Chem.* **2013**, *329*, 73–132. [PubMed]
29. Wang, Y.; Li, Y.; Liu, X.; Cho, W.C. Genetic and epigenetic studies for determining molecular targets of natural product anticancer agents. *Curr. Cancer Drug Targets* **2013**, *13*, 506–518. [CrossRef] [PubMed]
30. Vahid, F.; Zand, H.; Nosrat-Mirshekarlou, E.; Najafi, R.; Hekmatdoost, A. The role dietary of bioactive compounds on the regulation of histone acetylases and deacetylases: A review. *Gene* **2015**, *562*, 8–15. [CrossRef] [PubMed]
31. Gao, Y.; Tollefsbol, T.O. Impact of epigenetic dietary components on cancer through histone modifications. *Curr. Med. Chem.* **2015**, *22*, 2051–2064. [CrossRef] [PubMed]

32. Kala, R.; Tollefsbol, T.O. A novel combinatorial epigenetic therapy using resveratrol and pterostilbene for restoring estrogen receptor-alpha (eralpha) expression in eralpha-negative breast cancer cells. *PLoS ONE* **2016**, *11*, e0155057. [CrossRef] [PubMed]
33. Fernandes, G.F.S.; Silva, G.D.B.; Pavan, A.R.; Chiba, D.E.; Chin, C.M.; Dos Santos, J.L. Epigenetic regulatory mechanisms induced by resveratrol. *Nutrients* **2017**, *9*, 1201. [CrossRef] [PubMed]
34. Lee, P.S.; Chiou, Y.S.; Ho, C.T.; Pan, M.H. Chemoprevention by resveratrol and pterostilbene: Targeting on epigenetic regulation. *BioFactors* **2018**, *44*, 26–35. [CrossRef] [PubMed]
35. Singh, A.K.; Bishayee, A.; Pandey, A.K. Targeting histone deacetylases with natural and synthetic agents: An emerging anticancer strategy. *Nutrients* **2018**, *10*, 731. [CrossRef]
36. Stefanska, B.; Karlic, H.; Varga, F.; Fabianowska-Majewska, K.; Haslberger, A. Epigenetic mechanisms in anti-cancer actions of bioactive food components–the implications in cancer prevention. *Br. J. Pharmacol.* **2012**, *167*, 279–297.
37. Lubecka, K.; Kurzava, L.; Flower, K.; Buvala, H.; Zhang, H.; Teegarden, D.; Camarillo, I.; Suderman, M.; Kuang, S.; Andrisani, O.; et al. Stilbenoids remodel the DNA methylation patterns in breast cancer cells and inhibit oncogenic notch signaling through epigenetic regulation of maml2 transcriptional activity. *Carcinogenesis* **2016**, *37*, 656–668. [CrossRef]
38. Medina-Aguilar, R.; Perez-Plasencia, C.; Marchat, L.A.; Gariglio, P.; Garcia Mena, J.; Rodriguez Cuevas, S.; Ruiz-Garcia, E.; Astudillo-de la Vega, H.; Hernandez Juarez, J.; Flores-Perez, A.; et al. Methylation landscape of human breast cancer cells in response to dietary compound resveratrol. *PLoS ONE* **2016**, *11*, e0157866. [CrossRef]
39. Ramaiah, M.J.; Vaishnave, S. Bmi1 and pten are key determinants of breast cancer therapy: A plausible therapeutic target in breast cancer. *Gene* **2018**, *678*, 302–311.
40. Stefanska, B.; Salame, P.; Bednarek, A.; Fabianowska-Majewska, K. Comparative effects of retinoic acid, vitamin d and resveratrol alone and in combination with adenosine analogues on methylation and expression of phosphatase and tensin homologue tumour suppressor gene in breast cancer cells. *Br. J. Nutr.* **2012**, *107*, 781–790. [CrossRef]
41. Papoutsis, A.J.; Borg, J.L.; Selmin, O.I.; Romagnolo, D.F. Brca-1 promoter hypermethylation and silencing induced by the aromatic hydrocarbon receptor-ligand tcdd are prevented by resveratrol in mcf-7 cells. *J. Nutr. Biochem.* **2012**, *23*, 1324–1332. [CrossRef] [PubMed]
42. Papoutsis, A.J.; Selmin, O.I.; Borg, J.L.; Romagnolo, D.F. Gestational exposure to the ahr agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin induces brca-1 promoter hypermethylation and reduces brca-1 expression in mammary tissue of rat offspring: Preventive effects of resveratrol. *Mol. Carcinog.* **2015**, *54*, 261–269. [CrossRef] [PubMed]
43. Singh, B.; Shoulson, R.; Chatterjee, A.; Ronghe, A.; Bhat, N.K.; Dim, D.C.; Bhat, H.K. Resveratrol inhibits estrogen-induced breast carcinogenesis through induction of nrf2-mediated protective pathways. *Carcinogenesis* **2014**, *35*, 1872–1880. [CrossRef] [PubMed]
44. Singh, V.; Sharma, P.; Capalash, N. DNA methyltransferase-1 inhibitors as epigenetic therapy for cancer. *Curr. Cancer Drug Targets* **2013**, *13*, 379–399. [CrossRef] [PubMed]
45. Kala, R.; Shah, H.N.; Martin, S.L.; Tollefsbol, T.O. Epigenetic-based combinatorial resveratrol and pterostilbene alters DNA damage response by affecting sirt1 and dnmt enzyme expression, including sirt1-dependent gamma-h2ax and telomerase regulation in triple-negative breast cancer. *BMC Cancer* **2015**, *15*, 672. [CrossRef] [PubMed]
46. Gao, Y.; Tollefsbol, T.O. Combinational proanthocyanidins and resveratrol synergistically inhibit human breast cancer cells and impact epigenetic(-)mediating machinery. *Int. J. Mol. Sci.* **2018**, *19*, 2204. [CrossRef] [PubMed]
47. Zhu, W.; Qin, W.; Zhang, K.; Rottinghaus, G.E.; Chen, Y.C.; Kliethermes, B.; Sauter, E.R. Trans-resveratrol alters mammary promoter hypermethylation in women at increased risk for breast cancer. *Nutr. Cancer* **2012**, *64*, 393–400. [CrossRef]
48. Qin, W.; Zhang, K.; Clarke, K.; Weiland, T.; Sauter, E.R. Methylation and mirna effects of resveratrol on mammary tumors vs. Normal tissue. *Nutr. Cancer* **2014**, *66*, 270–277. [CrossRef]
49. Beetch, M.; Lubecka, K.; Kristofzski, H.; Suderman, M.; Stefanska, B. Subtle alterations in DNA methylation patterns in normal cells in response to dietary stilbenoids. *Mol. Nutr. Food Res.* **2018**, *2018*, e1800193. [CrossRef]

50. Mirza, S.; Sharma, G.; Parshad, R.; Gupta, S.D.; Pandya, P.; Ralhan, R. Expression of DNA methyltransferases in breast cancer patients and to analyze the effect of natural compounds on DNA methyltransferases and associated proteins. *J. Breast Cancer* **2013**, *16*, 23–31. [CrossRef]
51. Huang, H.; Lin, H.; Zhang, X.; Li, J. Resveratrol reverses temozolomide resistance by downregulation of mgmt in t98g glioblastoma cells by the nf-kappab-dependent pathway. *Oncol. Rep.* **2012**, *27*, 2050–2056. [PubMed]
52. Yang, H.C.; Wang, J.Y.; Bu, X.Y.; Yang, B.; Wang, B.Q.; Hu, S.; Yan, Z.Y.; Gao, Y.S.; Han, S.Y.; Qu, M.Q. Resveratrol restores sensitivity of glioma cells to temozolamide through inhibiting the activation of wnt signaling pathway. *J. Cell. Physiol.* **2018**, *2018*. [CrossRef]
53. Koval, A.; Katanaev, V.L. Dramatic dysbalancing of the wnt pathway in breast cancers. *Sci. Rep.* **2018**, *8*, 7329. [CrossRef] [PubMed]
54. Zhu, Y.; He, W.; Gao, X.; Li, B.; Mei, C.; Xu, R.; Chen, H. Resveratrol overcomes gefitinib resistance by increasing the intracellular gefitinib concentration and triggering apoptosis, autophagy and senescence in pc9/g nsclc cells. *Sci. Rep* **2015**, *5*, 17730. [CrossRef] [PubMed]
55. Li, W.; Ma, X.; Li, N.; Liu, H.; Dong, Q.; Zhang, J.; Yang, C.; Liu, Y.; Liang, Q.; Zhang, S.; et al. Resveratrol inhibits hexokinases ii mediated glycolysis in non-small cell lung cancer via targeting akt signaling pathway. *Exp. Cell Res.* **2016**, *349*, 320–327. [CrossRef]
56. Wang, J.; Li, J.; Cao, N.; Li, Z.; Han, J.; Li, L. Resveratrol, an activator of sirt1, induces protective autophagy in non-small-cell lung cancer via inhibiting akt/mtor and activating p38-mapk. *OncoTargets Ther.* **2018**, *11*, 7777–7786. [CrossRef] [PubMed]
57. Ahmad, A.; Gadgeel, S.M. Lung cancer and personalized medicine: Novel therapies and clinical management. Preface. *Adv. Exp. Med. Biol.* **2016**, *890*, v–vi.
58. Ahmad, A. Epigenetics in personalized management of lung cancer. *Adv Exp Med Biol* **2016**, *890*, 111–122.
59. Fudhaili, A.; Yoon, N.A.; Kang, S.; Ryu, J.; Jeong, J.Y.; Lee, D.H.; Kang, S.S. Resveratrol epigenetically regulates the expression of zinc finger protein 36 in nonsmall cell lung cancer cell lines. *Oncol. Rep.* **2019**, *41*, 1377–1386.
60. Gupta, G.; Bebawy, M.; Pinto, T.J.A.; Chellappan, D.K.; Mishra, A.; Dua, K. Role of the tristetraprolin (zinc finger protein 36 homolog) gene in cancer. *Crit. Rev. Eukaryot. Gene. Expr.* **2018**, *28*, 217–221. [CrossRef]
61. Howitz, K.T.; Bitterman, K.J.; Cohen, H.Y.; Lamming, D.W.; Lavu, S.; Wood, J.G.; Zipkin, R.E.; Chung, P.; Kisielewski, A.; Zhang, L.L.; et al. Small molecule activators of sirtuins extend saccharomyces cerevisiae lifespan. *Nature* **2003**, *425*, 191–196. [CrossRef] [PubMed]
62. Venturelli, S.; Berger, A.; Bocker, A.; Busch, C.; Weiland, T.; Noor, S.; Leischner, C.; Schleicher, S.; Mayer, M.; Weiss, T.S.; et al. Resveratrol as a pan-hdac inhibitor alters the acetylation status of histone [corrected] proteins in human-derived hepatoblastoma cells. *PLoS ONE* **2013**, *8*, e73097.
63. Dutra, L.A.; Heidenreich, D.; Silva, G.; Man Chin, C.; Knapp, S.; Santos, J.L.D. Dietary compound resveratrol is a pan-bet bromodomain inhibitor. *Nutrients* **2017**, *9*, 1172. [CrossRef] [PubMed]
64. Sahni, J.M.; Keri, R.A. Targeting bromodomain and extraterminal proteins in breast cancer. *Pharmacol. Res.* **2018**, *129*, 156–176. [CrossRef] [PubMed]
65. Garmpis, N.; Damaskos, C.; Garmpi, A.; Kalampokas, E.; Kalampokas, T.; Spartalis, E.; Daskalopoulou, A.; Valsami, S.; Kontos, M.; Nonni, A.; et al. Histone deacetylases as new therapeutic targets in triple-negative breast cancer: Progress and promises. *Cancer Genom. Proteom.* **2017**, *14*, 299–313.
66. Damaskos, C.; Garmpis, N.; Valsami, S.; Kontos, M.; Spartalis, E.; Kalampokas, T.; Kalampokas, E.; Athanasiou, A.; Moris, D.; Daskalopoulou, A.; et al. Histone deacetylase inhibitors: An attractive therapeutic strategy against breast cancer. *Anticancer Res.* **2017**, *37*, 35–46. [CrossRef]
67. Zucchetti, B.; Shimada, A.K.; Katz, A.; Curigliano, G. The role of histone deacetylase inhibitors in metastatic breast cancer. *The Breast* **2018**, *43*, 130–134. [CrossRef] [PubMed]
68. Guo, P.; Chen, W.; Li, H.; Li, M.; Li, L. The histone acetylation modifications of breast cancer and their therapeutic implications. *Pathol. Oncol. Res.* **2018**, *24*, 807–813. [CrossRef]
69. Lee, H.; Zhang, P.; Herrmann, A.; Yang, C.; Xin, H.; Wang, Z.; Hoon, D.S.; Forman, S.J.; Jove, R.; Riggs, A.D.; et al. Acetylated stat3 is crucial for methylation of tumor-suppressor gene promoters and inhibition by resveratrol results in demethylation. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 7765–7769. [CrossRef]
70. Lapidus, R.G.; Nass, S.J.; Butash, K.A.; Parl, F.F.; Weitzman, S.A.; Graff, J.G.; Herman, J.G.; Davidson, N.E. Mapping of er gene cpg island methylation-specific polymerase chain reaction. *Cancer Res.* **1998**, *58*, 2515–2519.

71. Saenglee, S.; Jogloy, S.; Patanothai, A.; Leid, M.; Senawong, T. Cytotoxic effects of peanut phenolics possessing histone deacetylase inhibitory activity in breast and cervical cancer cell lines. *Pharmacol. Rep.* **2016**, *68*, 1102–1110. [PubMed]
72. Parashar, G.; Capalash, N. Promoter methylation-independent reactivation of pax1 by curcumin and resveratrol is mediated by uhrf1. *Clin. Exp. Med.* **2016**, *16*, 471–478. [CrossRef] [PubMed]
73. Beyer, S.; Zhu, J.; Mayr, D.; Kuhn, C.; Schulze, S.; Hofmann, S.; Dannecker, C.; Jeschke, U.; Kost, B.P. Histone h3 acetyl k9 and histone h3 tri methyl k4 as prognostic markers for patients with cervical cancer. *Int. J. Mol. Sci.* **2017**, *18*, 477. [CrossRef] [PubMed]
74. Plewka, D.; Plewka, A.; Miskiewicz, A.; Morek, M.; Bogunia, E. Nuclear factor-kappa b as potential therapeutic target in human colon cancer. *J. Cancer Res. Ther.* **2018**, *14*, 516–520. [CrossRef]
75. Pu, J.; Bai, D.; Yang, X.; Lu, X.; Xu, L.; Lu, J. Adrenaline promotes cell proliferation and increases chemoresistance in colon cancer ht29 cells through induction of mir-155. *Biochem. Biophys. Res. Commun.* **2012**, *428*, 210–215. [CrossRef] [PubMed]
76. Jung, Y.J.; Lee, J.E.; Lee, A.S.; Kang, K.P.; Lee, S.; Park, S.K.; Lee, S.Y.; Han, M.K.; Kim, D.H.; Kim, W. Sirt1 overexpression decreases cisplatin-induced acetylation of nf-kappab p65 subunit and cytotoxicity in renal proximal tubule cells. *Biochem. Biophys. Res. Commun.* **2012**, *419*, 206–210. [CrossRef] [PubMed]
77. Buhrmann, C.; Shayan, P.; Popper, B.; Goel, A.; Shakibaei, M. Sirt1 is required for resveratrol-mediated chemopreventive effects in colorectal cancer cells. *Nutrients* **2016**, *8*, 145. [CrossRef]
78. Yaseen, A.; Chen, S.; Hock, S.; Rosato, R.; Dent, P.; Dai, Y.; Grant, S. Resveratrol sensitizes acute myelogenous leukemia cells to histone deacetylase inhibitors through reactive oxygen species-mediated activation of the extrinsic apoptotic pathway. *Mol. Pharmacol.* **2012**, *82*, 1030–1041. [CrossRef]
79. Frazzi, R.; Valli, R.; Tamagnini, I.; Casali, B.; Latruffe, N.; Merli, F. Resveratrol-mediated apoptosis of hodgkin lymphoma cells involves sirt1 inhibition and foxo3a hyperacetylation. *Int. J. Cancer* **2013**, *132*, 1013–1021. [CrossRef]
80. Kikuchi, H.; Mimuro, H.; Kuribayashi, F. Resveratrol strongly enhances the retinoic acid-induced superoxide generating activity via up-regulation of gp91-phox gene expression in u937 cells. *Biochem. Biophys. Res. Commun.* **2018**, *495*, 1195–1200. [CrossRef]
81. Kai, L.; Samuel, S.K.; Levenson, A.S. Resveratrol enhances p53 acetylation and apoptosis in prostate cancer by inhibiting mta1/nurd complex. *Int. J. Cancer* **2010**, *126*, 1538–1548. [CrossRef] [PubMed]
82. Xue, Y.; Wong, J.; Moreno, G.T.; Young, M.K.; Cote, J.; Wang, W. Nurd, a novel complex with both atp-dependent chromatin-remodeling and histone deacetylase activities. *Mol. Cell* **1998**, *2*, 851–861. [CrossRef]
83. Li, K.; Dias, S.J.; Rimando, A.M.; Dhar, S.; Mizuno, C.S.; Penman, A.D.; Lewin, J.R.; Levenson, A.S. Pterostilbene acts through metastasis-associated protein 1 to inhibit tumor growth, progression and metastasis in prostate cancer. *PLoS ONE* **2013**, *8*, e57542. [CrossRef] [PubMed]
84. Dhar, S.; Kumar, A.; Li, K.; Tzivion, G.; Levenson, A.S. Resveratrol regulates pten/akt pathway through inhibition of mta1/hdac unit of the nurd complex in prostate cancer. *Biochim. Biophys. Acta* **2015**, *1853*, 265–275. [CrossRef] [PubMed]
85. Harada, N.; Atarashi, K.; Murata, Y.; Yamaji, R.; Nakano, Y.; Inui, H. Inhibitory mechanisms of the transcriptional activity of androgen receptor by resveratrol: Implication of DNA binding and acetylation of the receptor. *J. Steroid Biochem. Mol. Biol.* **2011**, *123*, 65–70. [CrossRef]
86. Chao, S.C.; Chen, Y.J.; Huang, K.H.; Kuo, K.L.; Yang, T.H.; Huang, K.Y.; Wang, C.C.; Tang, C.H.; Yang, R.S.; Liu, S.H. Induction of sirtuin-1 signaling by resveratrol induces human chondrosarcoma cell apoptosis and exhibits antitumor activity. *Sci. Rep.* **2017**, *7*, 3180. [CrossRef]
87. Zadi Heydarabad, M.; Nikasa, M.; Vatanmakanian, M.; Azimi, A.; Farshdousti Hagh, M. Regulatory effect of resveratrol and prednisolone on mdr1 gene expression in acute lymphoblastic leukemia cell line (ccrf-cem): An epigenetic perspective. *J. Cell Biochem.* **2018**, *119*, 4890–4896. [CrossRef]

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REVIEW
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Mucosal and Cutaneous Human Papillomavirus Infections and Cancer Biology

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Papillomaviridae is a family of small non-enveloped icosahedral viruses with double-stranded circular DNA. More than 200 different human papillomaviruses (HPVs) have been listed so far. Based on epidemiological data, a subgroup of alphapapillomaviruses (alpha HPVs) was referred to as high-risk (HR) HPV types. HR HPVs are the etiological agents of anogenital cancer and a subset of head and neck cancers. The cutaneous HPV types, mainly from beta and gamma genera, are widely present on the surface of the skin in the general population. However, there is growing evidence of an etiological role of betapapillomaviruses (beta HPVs) in non-melanoma skin cancer (NMSC), together with ultraviolet (UV) radiation. Studies performed on mucosal HR HPV types, such as 16 and 18, showed that both oncoproteins E6 and E7 play a key role in cervical cancer by altering pathways involved in the host immune response to establish a persistent infection and by promoting cellular transformation. Continuous expression of E6 and E7 of mucosal HR HPV types is essential to initiate and to maintain the cellular transformation process, whereas expression of E6 and E7 of cutaneous HPV types is not required for the maintenance of the skin cancer phenotype. Beta HPV types appear to play a role in the initiation of skin carcinogenesis, by exacerbating the accumulation of UV radiation-induced DNA breaks and somatic mutations (the hit-and-run mechanism), and they would therefore act as facilitators rather than direct actors in NMSC. In this review, the natural history of HPV infection and the transforming properties of various HPV genera will be described, with a particular focus on describing the state of knowledge about the role of cutaneous HPV types in NMSC.

Keywords: Papillomavirus, infection and cancer, transformation, anogenital cancer, skin cancer

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INTRODUCTION

Papillomaviridae is a family of small non-enveloped icosahedral viruses with double-stranded circular DNA, which range in length from 5,748 bp for *Sparus aurata* papillomavirus 1 (SaPV1) to 8,607 bp for canine papillomavirus type 1 (CPV1). Papillomaviruses (PVs) infect basal keratinocytes of the mucosal and cutaneous epithelia of both animals (reptiles, birds, marsupials, and others) and humans (1). In 2016, the first characterization of a PV in fish (SaPV1) rendered this family of viruses much older than expected, with an emergence 450 million years ago (2). PVs are considered to be host-restricted; however, in rare cases cross-species transmission may also occur (3, 4).

Based on the nucleotide sequences of the major capsid protein L1, the PV study group within the International Committee on Taxonomy of Viruses (http://ictv.global/report/papillomaviridae) has classified the PVs into 53 genera, among which only 5 genera include PVs that infect humans (HPVs). To be classified as a novel PV type, the nucleotide sequence of L1 must share <90% similarity with other PVs (5). During the past decade, there has been an exponential increase in the identification of new betapapillomaviruses (beta HPVs) and gammapapillomaviruses (gamma HPVs) that have been discovered with the advent of new technologies such as next-generation sequencing. More than 200 different HPV types have been listed by the International HPV Reference Center (www.hpvcenter.se) (6), and this number continues to expand (7).

HPV types are organized into five major genera: alpha, beta, gamma, mu, and nu (5). The genus gamma includes the majority of the known HPVs, with 99 types, followed by the genera alpha ($n = 65$) and beta ($n = 54$). The genera mu and nu include only 3 and 1 types, respectively (www.hpvcenter.se, on 2019-01-30). A recent study reported the identification of the complete genome of 83 previously unknown HPV types, among which 69 were classified as gamma (8).

Based on epidemiological and biological data, a subgroup of 12 mucosal HPV types classified in the genus *Alphapapillomavirus* (alpha HPV), i.e., HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, referred to as high-risk (HR) HPV types, has been classified as carcinogenic (IARC Group 1), and eight other HPV types, i.e., HPV26, 53, 66, 67, 68, 70, 73, and 82, have been classified as probably or possibly carcinogenic (IARC Groups 2A and 2B) (9, 10). The HR HPV types are the etiological agents of several cancers, such as those of the cervix, vagina, vulva, anus, penis, and a subset of head and neck cancers (HNCs), particularly oropharyngeal cancer (11). In 2012, 15.4% of cancers worldwide were attributed to carcinogenic infections (12). Together, the HPV-related cancers represent 630,000 new cancer cases per year, which account for ∼30% of all cancers induced by infectious agents (10). Epidemiological and biological studies showed that HPV16 is the most oncogenic of the HR HPVs. The genus alpha also includes low-risk (LR) HPV types that cause benign lesions. HPV6 and 11, the most studied LR HPV types, induce benign genital warts or condylomata acuminata (13). These HPV types are also found in recurrent respiratory papillomatosis (14), in children. The genus alpha also includes a few cutaneous HPV types (HPV2, 3, 7, 10, 27, 28, and 57), which cause common and plantar warts (15–18).

The genera beta, gamma, mu, and nu contain HPVs that infect cutaneous epithelia. The genus alpha also contains some HPV types, such as HPV2, 3, and 10, that colonize the human skin. The mu and nu genera include only a few HPV types, whereas the genus beta includes more than 54 HPV types, which are subdivided into five species (β1–5), and the genus gamma includes 98 types subdivided into 27 species (6). In the immunocompetent population, these so-called cutaneous HPV types induce asymptomatic chronic infections. However, members of the genera gamma, mu, and nu can induce benign skin lesions, e.g., cutaneous papillomas or warts (5). In addition, there is growing evidence of an etiological role of beta HPVs in non-melanoma skin cancer (NMSC), most likely in association with ultraviolet (UV) radiation [reviewed in (19–21)].

In this review, we describe the natural history of mucosal and cutaneous HPV infections, and discuss the transforming properties of a subset of them that have been shown or are suspected to play a causative role in various human cancers. In particular, we focus on describing the state of knowledge about the role of cutaneous HPV types in NMSC, because many existing reviews already cover all the aspects of mucosal HPV type infection and its role in transformation.

GENOMIC ORGANIZATION OF HPV AND VIRAL GENE PRODUCTS

The HPV genome is organized into three major regions: (i) a long control region (LCR), also called the upstream regulatory region (URR), located between the L1 and E6 open reading frames (ORFs), which contains the early promoter and regulatory element involved in viral DNA replication and transcription; (ii) the early region, which encodes the E1, E2, E4, E5, E6, and E7 proteins involved in viral gene expression, replication, and survival; and (iii) the late region, which encodes the structural proteins (L1 and L2). This organization is shared among all alpha HPVs (**Figure 1**). However, only four ORFs (those of E1, E2, L1, and L2) are necessary to fulfill the requirements to ensure the viral replication and shedding of the virus, and are present in all known PVs (22). Certain HPV genera and types lack an ORF. For example, the E5 ORF is lacking from HPV types that belong to the genera beta, gamma, and mu, and both the E5 and E6 ORFs are lacking from three gamma HPV types (HPV101, 103, and 108) (23, 24). LR HPV types (i.e. HPV6 and 11) encode for two E5-like proteins E5γ and E5δ (25). Beta and gamma HPV types have the particularity of harboring a shorter LCR compared with members of other genera (**Figure 1**).

All PVs also have the potential to express an E8^E2C transcript (or equivalent), encoding for a protein that includes the E8 domain fused to the hinge and DNA-binding domains of E2 (26). This protein acts as a transcriptional repressor, and represses E1/E2-dependent replication of the viral origin (27–29).

NATURAL HISTORY OF MUCOSAL HPV INFECTIONS

Mucosal HPV infections occur during the first sexual exposures in early adulthood (30), although non-sexual infection may also be possible (31). Although a majority of sexually active women will acquire a genital HPV infection, most (>90%) cervical HPV infections are resolved by the host immune system within 1–2 years (32, 33) and give rise only to asymptomatic infections. However, a minority of HPV infections become persistent. The risk of developing epithelial cell abnormalities and cancer is then increased (32). Persistent infection may be explained by several factors. Alcohol consumption and the HR HPV load synergistically increased the risk of persistent HR HPV infection in women (34). Cigarette smoking alone also plays an important role in the acquisition of persistent HPV infections in women,

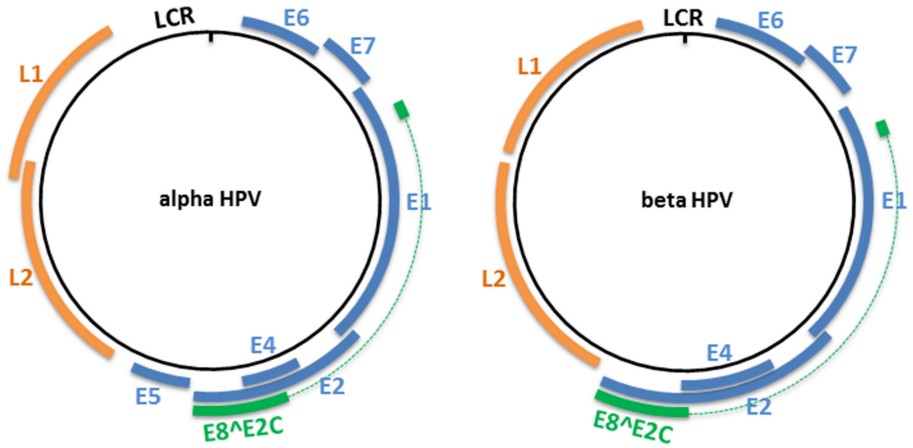

FIGURE 1 | Genome organization of alpha and beta HPV types. The circular dsDNA genome of high-risk alpha and beta HPV types is represented by a black circle. The positions of the early and late genes are represented by blue and orange, respectively. Both represented PV genera have the potential to express an E8^E2C transcript that encodes for a protein that includes an E8 domain fused to the hinge and DNA-binding domains of E2. LCR, long control region.

by decreasing the probability of clearing oncogenic infections (35). Smoking is also a risk factor for a persistent oral HPV infection (36). Host genetic risk factors may also predispose an individual to persistent HPV infection and developing cervical cancer, as supported by the high rate of heritability of cervical cancer (37–39). Defective immune response due to genetic variations (e.g., inflammasome genetics) has been associated with virus persistence and progression to cervical cancer (39–41). Human leukocyte antigen (HLA) genes also play a key role in the persistence of HPV infections and progression to cervical cancer, depending on their ability to bind to HPV antigens (39, 42).

Many natural HPV intra-type variants that differ from the prototypes have been identified, and some of them have been associated with the persistence and clinical outcome of cervical HPV infections (43–46). Several studies showed that EUR-350 T and EUR-350 G, which are HPV16 E6 variants, could influence viral persistence (47, 48). Recently, Mirabello et al. (49) evaluated a large collection of 5,570 HPV16-infected case–control samples using HPV whole-genome sequencing, and showed that E7 variation from the prototype sequence greatly decreases the risk of invasive cancer. That study also highlighted the presence of rarely detected variants of HPV16 that are consistent with the antiviral activity of the human APOBEC3 cytidine deaminase family. Moreover, APOBEC3A is stabilized in human keratinocytes by HR HPV E7, but not by E7 of LR HPV types (50).

The HR mucosal HPV genome integrates into host chromosomes in anogenital and oropharyngeal cancers, and it is considered an important driver of carcinogenesis.

There is not yet consensus on whether this is an early or a late event in carcinogenesis (51, 52). The integration occurs frequently within the E1 or E2 proteins, leading to dysregulation of expression of the oncoproteins E6 and E7 due to loss of the E2 repression function (53). The stability of E6 and E7 mRNAs is increased when HPV16 DNA is integrated into the human genome (54).

The frequency of viral integration increases with the severity of the cervical precancerous lesions. Integration of HPV into the host genome is reported in a majority of cervical cancers (83%) (55), and HPV18 has been found integrated into almost all cervical cancers (56, 57). HPV16 can be present as episomal or integrated forms, or both (58).

The DNA integration event could contribute to the HPV-driven malignant transformation of cervical cells, by affecting the expression of key host genes near the integration site (59). In addition, the existence of long-distance regulation among the integrated HPV fragment, the MYC gene, and the 8q24.22 region has been reported (60).

Although it was commonly accepted that integration occurred randomly into the host genome, several studies have shown that non-random HPV integration occurs. Integration of HPV16 sequences appears to take place preferentially in common fragile sites in human chromosomes (61, 62), in transcriptionally active regions (63), or near microRNAs (64).

Carcinogenesis can also be enhanced by epigenetic events. In cervical cancer, HPV16 E2 binding sites (E2 BS1 and E2 BS2), which play a role in transcriptional repression of E6 and E7 oncoproteins, are heavily methylated. The binding of E2 protein on these sites is then prevented, which leads to the upregulation of E6 and E7 oncoproteins (65). Host or viral epigenetic changes may be enhanced by the integration of HPV. A recent study showed that L1 gene methylation increases significantly according to the grade of the cervical lesion, and a high methylation rate of this gene correlates with the physical status of HPV integration (66, 67).

Data on HPV integration in HNC are still sparse and contradictory. Parfenov et al. (68) reported that 25 out of 35 HNCs showed integration of HPV into the human genome. Other studies reported HPV integration rates of 39% in oral

squamous cell carcinoma (SCC) (69) and 43% in HNC (70), which is lower compared with HPV-positive cervical cancer. Episomal, integrated, and mixed forms of HPV are found in HPV-positive HNC. Moreover, a recent study, based on analysis of the Cancer Genome Atlas sequencing data, reported for the first time the presence of viral–human hybrid episomes in HPV-positive HNC (71).

NATURAL HISTORY OF CUTANEOUS HPV INFECTIONS AND CLINICAL IMPLICATIONS

Cutaneous HPV types are ubiquitous and are widespread in the general population. Up to 90% of healthy individuals tested positive for beta HPV types (72–74). The infection occurs in young children through skin-to-skin contact (75, 76). The cutaneous HPVs have been proposed to infect hair follicle stem cells of healthy individuals, where they constitute a reservoir of persistent infection (77, 78). DNA from cutaneous HPVs is frequently detected in hair bulbs, independently of the anatomical region where the hairs were plucked (79). Schmitt et al. (80) showed that keratinocyte stem cells (KSCs) in rabbit hair follicles co-localize with the primary target cells of cottontail rabbit papillomavirus (CRPV). They showed presence of CRPV early transcripts in clonogenic cells of the hair follicles soon after infection, suggesting that hair follicle stem cells are the initial target cells for CRPV (80), which was shown to induce cutaneous warts and carcinomas (81).

Epidemiological studies in humans showed that the cutaneous HPV population present in hair follicles mirrors the HPV prevalence in the skin of the same individual, which makes eyebrow hair an excellent sampling method to characterize the individual cutaneous HPV population (79, 82). Studies on intra-familial transmission showed that similar spectra of beta HPV types are present within members of the same family. Babies and their parents share some of the beta HPV types (83), which can persist for many years on healthy skin (84, 85). Transmission of beta and gamma HPVs has been demonstrated in couples (86).

Exposure to cutaneous HPV is common. Serological studies measuring antibodies against the type-specific L1 major capsid protein of HPV showed that 52% of the Dutch population and 67% of the Italian population were exposed to beta HPV infections (74). A recent seroprevalence study, based on ten beta HPV types, showed that 39% of healthy men were seropositive for at least one beta HPV type (87). Seroconversion for beta and gamma HPV types appears to be slow and to increase with age (88). Low viral loads in immunocompetent individuals (89) and continuous renewal of the infected keratinocytes may explain why only half of the infected individuals develop antibodies against cutaneous HPV types (90, 91).

Cutaneous HPVs are highly prevalent in the rare hereditary disease epidermodysplasia verruciformis (EV), which tends to progress to cutaneous SCC (cSCC), frequently located at sun-exposed anatomical sites (92). HPV5 and 8, two members of species β1, were isolated from cSCC in patients with EV (93). These two types were classified as possibly carcinogenic to humans (IARC Group 2B) (10).

In organ transplant recipients, a 65–250-fold increased risk of developing SCC compared with the general population was reported (94, 95). In addition, in HIV-positive individuals, several studies reported at least a 2-fold increased risk of cSCC compared with HIV-uninfected people (96, 97). The correlation between the immunodeficiency state and an increased risk of developing NMSC suggested a possible role of infectious agents (98), such as cutaneous HPV.

This observation is corroborated by growing evidence showing an association between cutaneous infection and the risk of developing NMSC in immunocompetent individuals under certain conditions (e.g., UV radiation exposure) [reviewed in (21, 99)], in particular in cSCC (100–102).

Epidemiological and biological data suggested that beta HPV types, and species β1 and β2 in particular, may be linked to cSCC development in immunocompetent individuals (99, 100) [reviewed in (21)].

Interestingly, the cutaneous HPV viral load was higher in actinic keratosis, which is considered to be the precursor lesion of cSCC, compared with cSCC (<1 copy per cell), suggesting a possible role of cutaneous HPV types in the initiation of skin carcinogenesis but not in the maintenance of the cancer phenotype, by exacerbating the accumulation of UV radiation-induced DNA breaks and somatic mutations (the hit-and-run mechanism) (103, 104). The absence of HPV mRNA (105) and the lack of evidence for the integration event in skin tumors support this scenario.

The *Mastomys natalensis* papillomavirus (MnPV)-infected rodent model *Mastomys coucha* was previously described and used to evaluate the role of PVs in NMSC. In these mice, which can be naturally infected by MnPV, skin lesions such as papillomas or keratoacanthomas can be induced (106). Using this model, Hasche et al. (107) clearly demonstrated cooperation between UVB radiation exposure and MnPV infection in the first step of NMSC initiation. MnPV-positive animals chronically exposed to UVB radiation developed lesions significantly more frequently than MnPV-negative animals (107). Moreover, the authors showed higher MnPV viral loads in well-differentiated and keratinizing SCCs (KSCCs) compared with normal skin. Of note, these KSCC lesions share histological similarities to human SCC pre-malignant lesions, such as actinic keratosis, that normally show higher cutaneous HPV DNA loads than SCC, as discussed above. In addition, non-keratinizing SCCs (nKSCCs) occurring in mice UV-irradiated at higher doses showed low levels of (or no) viral DNA and often harbored p53 mutations, again mirroring the evolution of SCC in humans. The loss of viral DNA and viral gene expression in nKSCC lesions is most likely due to their undifferentiated state, which no longer sustains the infection. However, despite the lack of viral DNA, animals with nKSCCs developed antibody response to MnPV capsids, which clearly highlights past exposure to the virus, similar to the situation observed in SCC cases (108). Therefore, the *Mastomys* model shows parallels with a natural infection by cutaneous HPV and provides a good model to study the association between cutaneous HPV infection, UV radiation and

SCC. Most importantly, this model provides further evidence for a hit-and-run mechanism.

Many independent studies reported the presence of beta HPV types at different anatomical regions other than the skin, such as the oral mucosal epithelium, genital sites, and the anal canal (109–112), or investigated the role of cutaneous HPV in various malignancies (e.g., male external lesions, breast cancers, salivary gland tumors, esophageal cancer) other than skin cancer, without showing any association (113–116). However, a recent study reported an increased risk of HNC in individuals who tested positive for HPV5 of species β1, as well as other HPV types of species γ11 and γ12 (117). The same trend was reported in another study, in which β1 HPV5 and β2 HPV122 were significantly associated with HNC (118).

Whereas, beta HPVs are known to infect cutaneous tissues, epidemiological data showed that β3 HPV types are also present in the mucosal epithelium, suggesting a dual tropism (111, 119). Moreover, this species shares biological similarities with mucosal HR HPV types such as HPV16 in *in vitro* and *in vivo* experimental models (120, 121). A study showed that β3 HPV49 transgenic mice, after 4-nitroquinoline 1-oxide treatment, were prone to develop upper digestive tract tumors (121).

Epidemiological and biological data on the role of gamma HPV types are sparse, and do not support an etiological role in NMSC. However, this genus includes an increasing number of members that may deserve more investigation. Using deep sequencing, a member of species γ27 (HPV197) has been isolated exclusively from skin cancers. However, additional studies are required to demonstrate an etiological link between this type and skin cancer (122, 123).

ENTRY AND LIFE CYCLE OF HPV

The viral particle reaches the basal layer of the epithelium via micro-wounds or micro-fissures, or via hair follicles (80, 124, 125), to infect basal keratinocytes or stem epithelial cells. In the cervix, HR HPV may directly reach the single layer of cuboidal epithelial cells from the squamocolumnar junction located between the endocervix and the ectocervix (126); this anatomical region is therefore more sensitive to infection. In the oral cavity, HPV infection is believed to occur in tonsillar crypts, where a comparable cellular structure exists (127).

The infectious entry of the virus into the cell is intimately linked to proliferating cells of the regenerating basal epithelium. Broniarczyk et al. (128) showed that the viral particle can remain infectious after several weeks on the surface of senescent cells, which are resistant to HPV infection; however, reactivation of the cell cycle by p53 siRNA led to the entry of the virus (128).

To initiate the infection, the virus binds to the heparan sulfate chains of proteoglycans (HSPGs) located on the cell membrane or on the extracellular matrix (ECM) through the major capsid protein L1, leading to cyclophilin (CyP) B-mediated conformational changes of the capsid structure, which expose the minor capsid protein L2 on the surface of the viral particle (**Figure 2**). The cleavage of L1 in the extracellular space by a serine protease, kallikrein-8, appears to be crucial for the efficient externalization of L2 (129). On the ECM, the virus can also bind to a transient binding receptor, laminin-322 (formerly called laminin-5) (130).

Next, the N-terminus of the minor capsid protein L2 is cleaved with a CyP-independent mechanism at a conserved furin cleavage consensus site to expose the L2 amino acids 17–36 (the RG1 neutralizing epitope), which appears to be important for the interaction of the capsid with an unidentified secondary receptor (131, 132). The binding of HPV to the secondary receptor allows subsequent internalization.

Using HPV16 pseudovirion (PsV) particles covalently labeled with some fluorophores Schelhaas et al. (133) showed that HPV16 PsVs moved along the outside cell membrane in correspondence to the actin protrusions and were transported inside the cells by actin retrograde flow. Upon inhibition of the actin flow by blebbistatin, the virus particles could not be actively transported along actin-rich protrusions, which resulted in reduced efficiency but not in complete abolishment of the infection (133). In a more recent work, using different compound inhibitors and siRNAs, Schelhaas et al. (134) showed that the entry of HPV16 into HeLA and HaCaT cells was actin-dependent and clathrin-, AP2-, caveolin-, flotillin-, and dynamin-2-independent (134). Although the exact mechanism of membrane protrusion/vesicle formation during virus endocytosis remains unclear, Schelhaas's study clearly showed that actin polymerization and depolymerization are crucial for HPV16 endocytosis, in particular for scission of endocytic vesicles (134). Once inside the cell, the virus needs to reach the replication machinery within the nucleus (**Figure 2**). The virus is delivered to an early endosome or macropinosome-like endosome that matures into a late endosome [reviewed in (135)]. Inside the late endosomes, the capsid of the viral particle is disassembled in a low-pH environment, leading to the dissociation of the major capsid protein L1 from the minor capsid L2, mediated by host-cell CyPs (136). However, a recent study showed that residual conformationally intact L1 protein remains in complex with the viral genome (137). The L2–viral DNA complex traffics to the trans-Golgi network, mediated by the retromer complex (138). Retromer is a heteropentameric complex composed of dimer of sorting nexins (SNX1, SNX2, SNX5, and SNX6) and a vacuolar protein sorting (Vps) trimer containing Vps26, Vps29, and Vps35, which play a central role in the retrieval of several different cargo proteins from the endosome to the trans-Golgi network. The trafficking also involves retriever, a multi-protein complex analogous to retromer (139), and involves the cellular adaptor protein SNX17. This complex is associated with endosomes and is essential for the recycling of multiple cargoes. A proteomic approach has shown that SNX17 interacts with HPV16 L2 (140). The observation that the SNX17 binding site is conserved among different PV genera suggests that it has an important role in regulating the PV life cycle and replication. The SNX17/L2 interaction is indeed required for HPV16 infection and appears to play key roles at different stages of the infection, being important for effective capsid disassembly and L1 dissociation. The interaction of L2 with SNX17 also contributes to the maintenance of viral capsids in the late endosomal compartment, by protecting them

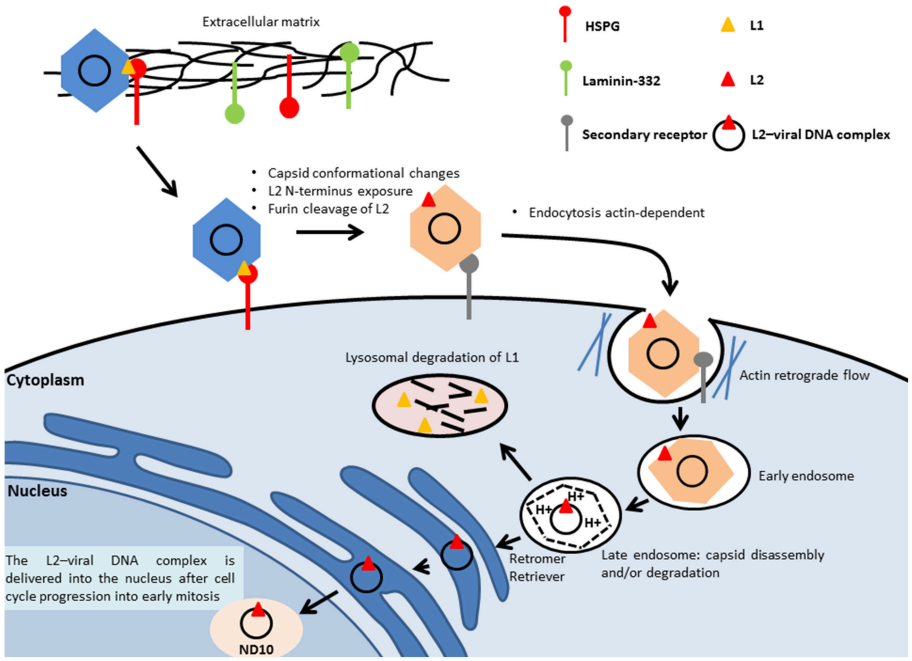

FIGURE 2 | Entry of HPV. The virus binds to the heparan sulfate chains of proteoglycans (HSPGs) located on the cell membrane or on the extracellular matrix through the major capsid protein L1, which leads to the conformational change of the capsid and the subsequent L2 externalization on the surface of the viral particle. Next, the N-terminus of the minor capsid protein L2 is cleaved at a conserved furin cleavage consensus site, which facilitates the binding of HPV to an unidentified secondary receptor, allowing subsequent internalization that is actin-dependent. The L2–viral DNA complex and a portion of L1 are delivered to the trans-Golgi network, through retromer- and retriever-mediated vesicular trafficking. Inside the late endosomes, the capsid of the viral particle is disassembled in a low-pH environment, leading to the dissociation of the major capsid protein L1 from the minor capsid L2, mediated by host-cell cyclophilins. The L2–viral DNA complex is then delivered into the nucleus to the nuclear domain (ND10), during cell mitosis.

from lysosomal degradation and allowing L2–DNA complexes to egress from the endosomes (141). A related protein, SNX27, is part of the retromer complex and also interacts with L2, but through its PDZ domain. SNX27 appears to be important for virion trafficking and to cooperate with SNX17 in this event (142).

L2 is required for efficient trafficking of the viral genome to the nucleus (138) and remains associated with the viral DNA when the transport vesicles deliver the L2–viral DNA complex inside the nucleus [reviewed in (143)], most likely together with residual L1.

Delivery of the L2–viral DNA complex into the nucleus requires cell cycle progression into early mitosis (144). Nuclear envelope disruption is also required for nuclear import of the L2–viral DNA complex (145) (**Figure 2**).

Inside the nucleus, L2 mediates the viral genome delivery to the nuclear domain (ND10) (146, 147), also known as promyelocytic leukemia (PML) bodies, where early viral transcription and replication will take place, as for many DNA viruses. ND10 structures are host restriction factors that limit viral infection as part of the intrinsic defense against viral infection (148). Stepp et al. (149) showed that Sp100 nuclear antigen, one of the components of ND10, represses transcription, replication, and establishment of incoming HPV DNA in the early stages of infection. Upon initial infection, the minor capsid protein L2 leads to the alteration of the composition of ND10 protein, leading to the release or degradation of Sp100 (150). The Daxx protein, another component of ND10, is also recruited (150). It was previously reported that this protein modulates the early gene expression and the transient replication of HPV genomes in U2OS cells (151).

The replication cycle of HPV is linked to the differentiation of the infected epithelium, and starts with a first step, called "establishment replication," which consists of maintaining a constant number of episomal copies (50–100 per cell) (**Figure 3**) [reviewed in (152)]. Viral DNA replication relies on the host DNA replication machinery and is supported by the early viral proteins E1 and E2 (153). Upon nuclear entry, viral DNA replication is initiated by the binding of E2 on specific sites located on the LCR, which is required for the recruitment and binding of E1 helicase to the viral origin of replication (154). The cellular proteins TopBP1 and Brd4 are involved in the initiation of the HPV16 E1/E2-mediated DNA replication (155). Limited viral genome amplification may be obtained by the interaction of NCOR/SMRT repressor complexes with E8^E2C proteins, which inhibits viral replication (156, 157). After this initial step, which aimed to generate a low copy number of genomes, the maintenance phase is initiated (**Figure 3**) [reviewed in (152)]. This phase consists of creating the conditions to maintain a constant number of

viral genomes in the nuclei of undifferentiated basal cells as an extrachromosomal genome to create a persistent infection. The viral genomes need to be correctly segregated during cell division through attachment/tethering of the virus genome to the host-cell chromosome. The transactivation domain at the N-terminal part of E2, with the bromodomain protein Brd4, interacts with the host mitotic chromosomes. Concomitantly, the E2 DNA-binding/dimerization domain binds to E2 binding sites in the LCR of the viral genome (158–162). Brd4 interacts with the E2 transactivation domain of most PVs (163). ChIR1, an ATP-dependent DNA helicase, seems to play a key role by regulating the chromatin association of HPV16 E2, and in maintaining the episomal form of HPV16 (164–166). The structural maintenance of chromosomes (SMC) proteins SMC5 and SMC6 may also play a role in viral genome maintenance by interacting with E2 (167). In addition to E1 and E2, the oncoproteins E6 and/or E7 are required for stable episomal maintenance of HPV (168, 169).

Upon cell differentiation in stratified epithelium, vegetative or productive viral replication is initiated, with the subsequent production of progeny virions (170, 171), [reviewed in (152)] (**Figure 3**). Uninfected cells leave the basal layer for terminal differentiation, are withdrawn from the cell cycle, and stop the replication of the DNA. Because the amplification of the viral genome requires cellular conditions that enable cell proliferation and thus DNA replication, the virus has developed strategies to prevent cell-cycle arrest and apoptosis signals. Here, the oncoproteins E6 and E7 expressed at relatively low levels in differentiated cells play a key role by inactivating tumor suppressor proteins (e.g., p53, retinoblastoma protein [pRb]) and activating signal transduction, to ensure that the infected cells remain active and progress to the S phase. The activation in differentiated epithelial cells of the late promoter (P670 for HPV16, P811 for HPV18, and P742 for HPV31), located in the E7 region, leads to the production of the high levels of E1 and E2 viral proteins required to ensure viral DNA replication. E4 and E5 contribute to efficient productive replication (172, 173). The capsid proteins L1 and L2 are expressed from the late promoter, and are involved in the encapsidation of newly replicated genomes, resulting in virion release in the superficial layers during desquamation (174) (**Figure 3**). E4 also plays a role at this step of the viral life cycle, by interacting with the keratin network (175). A more complete description of the function of each of the viral proteins is provided in **Table 1**.

Epigenetic regulation of HPV transcription plays an important role in the virus life cycle. HPV regulatory epigenetic mechanisms have recently been partly elucidated by Pentland et al. (176). The key players in this regulation are the chromatin-organizing CCCTC-binding factor (CTCF) and Yin Yang 1 (YY1), binding, respectively, to the E2 ORF and the viral LCR. CTCF and YY1 are involved in the formation of a loop allowing E2 and LCR interaction and leading to the formation of epigenetically repressed chromatin in the HPV18 genome. This event leads to attenuated expression of oncoproteins in undifferentiated cells. CTCF plays a key role in this event; in fact, the HPV18 ΔCTCF genome, carrying a mutation in the CTCF binding site, shows higher H3K4me3 and reduced H3K27me3 marks in the viral enhancer and in the downstream early promoter. This is coherent with reduced recruitment of polycomb repressor complex 2 (PCR2), enrichment of RNA pol II binding, and a consequent increase in the transcriptional activity of the viral early promoter. Pentland et al. (176) also showed that both CTCF and YY1 are necessary to repress viral transcription. During cell differentiation, a drop in YY1 expression levels leads to loss of chromatin loop formation and to increased expression of the oncoproteins consecutive to epigenetic depression of the viral genome (176).

TRANSFORMING ACTIVITIES OF PVS

Studies performed on mucosal HR HPV types, such as HPV16 and 18, showed that both oncoproteins E6 and E7 play a key role in cervical cancer by altering pathways involved in the host immune response to establish a persistent infection and promote cellular transformation. The E6 (150 amino acids) and E7 (100 amino acids) oncoproteins play a central role in carcinogenesis by interacting with a large number of cellular proteins involved in key cellular events, such as the cell cycle and apoptosis control. In addition to E6 and E7, HR HPV types encode a small hydrophobic oncoprotein of <90 amino acids, E5, which also appears to play a role in HPV-induced carcinogenesis. Continuous expression of the E6 and E7 oncoproteins is essential to initiate and to maintain the cellular transformation process. Indeed, the use of different strategies to inhibit the function of E6 and E7 in HPV-positive cancer cells resulted in cell growth arrest by apoptosis or senescence (177, 178). The main targets of the E6 and E7 oncoproteins are p53 and pRb, respectively; p53 regulates DNA damage response and apoptosis, and pRb tightly controls the cell cycle. Although E6 and E7 of mucosal HR HPV types were extensively studied, functional data on E6 and E7 of beta HPV types are sparse. Moreover, the absence of cutaneous E6 and E7 transcripts in skin tumors suggests an alternative mechanism for promoting cancer development (hit-and-run mechanism). The beta and gamma HPVs lack the E5 ORF.

Transforming Activities of E6

The E6 oncoprotein of HPV16 is a small protein of ∼150 amino acids, made up of two zinc-like fingers joined by an interdomain linker (179). E6 of HR HPV types has the ability to bind to the LXXLL peptide motif of cellular proteins. Among them, the E6-associated protein (E6AP) is an E3 ubiquitin ligase that targets proteins for ubiquitination and degradation by the proteasome (180). The best-characterized E6/E6AP interaction is with the p53 tumor suppressor (181). In normal cells, p53 plays a key role to safeguard the integrity of the cellular genome, avoiding the proliferation of cells with damaged DNA. Therefore, upon DNA damage insults, p53 will either induce cell-cycle arrest at the G1 phase to allow DNA repair or, if the DNA damage is too extensive, activate apoptotic pathways. E6/E6AP interaction leads to conformational changes of E6 that allow the association with the p53 pro-apoptotic tumor suppressor (182), leading to its ubiquitin-mediated proteasome degradation and, as a consequence, to accumulation of DNA damage and to genomic instability (183).

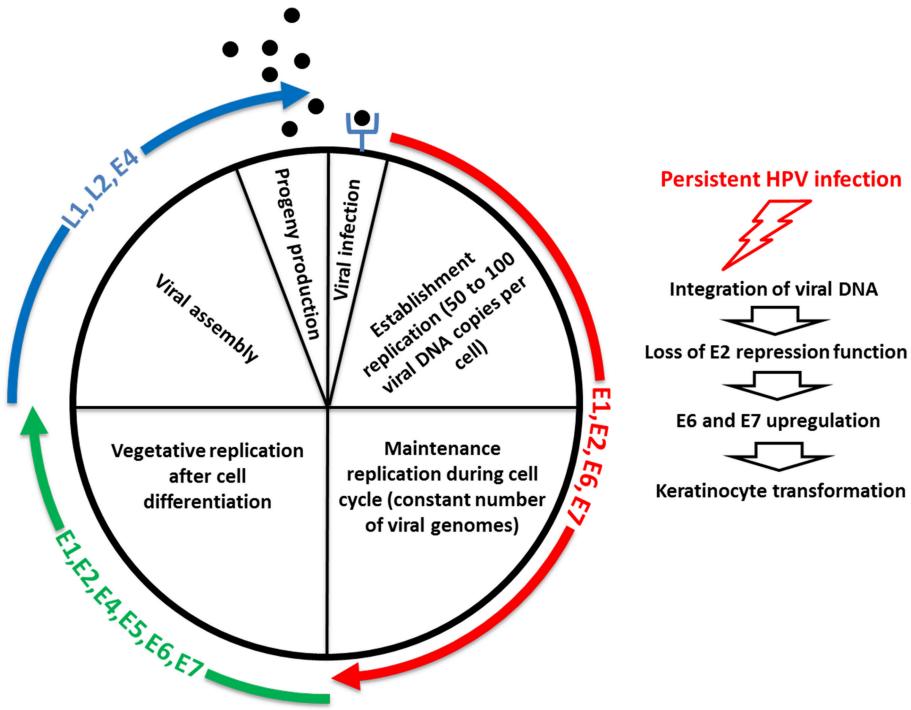

FIGURE 3 | Life cycle of HPV. The first step of the replication cycle of HPV, called "establishment replication," consists of maintaining a constant number of episomal copies. Viral DNA replication relies on the host DNA replication machinery and is supported by the early viral proteins E1 and E2. After this initial step, the maintenance phase is initiated. This phase consists of creating the conditions to maintain a constant number of viral genomes in the nuclei of undifferentiated basal cells as an extrachromosomal genome to create a persistent infection. In addition to E1 and E2, the oncoproteins E6 and/or E7 are required for stable episomal maintenance of HPV. Upon cell differentiation in stratified epithelium, vegetative or productive viral replication is initiated, with the subsequent production of progeny virions. Here, the oncoproteins E6 and E7 expressed at relatively low levels in differentiated cells play a key role by inactivating tumor suppressor proteins. The activation in differentiated epithelial cells of the late promoter, located in the E7 region, leads to the production of the high levels of E1 and E2 viral proteins required to ensure viral DNA replication. E4 and E5 contribute to efficient productive replication. The capsid proteins L1 and L2 are expressed from the late promoter, and are involved in the encapsidation of newly replicated genomes, resulting in virion release in the superficial layers during desquamation. E4 also plays a role at this step of the viral life cycle, by interacting with the keratin network. The right side of the schematic representation illustrates that a minority of HPV infections become persistent, thus increasing the risk of keratinocyte transformation, through integration of viral DNA into the human genome, which leads to the loss of E2 repression function and subsequent E6 and E7 upregulation.

Although E6 of the β3 HPV49 type also showed the ability to degrade p53 by an E6AP-dependent mechanism, E6s of HPV species β2 do not share this property and have evolved alternative mechanisms to counteract p53 functions [reviewed in (19)]. For example, E6 of HPV23 interferes with the ability of the homeodomain-interacting protein kinase 2 (HIPK2) to phosphorylate p53 on serine 46 to activate its apoptotic function upon UVB irradiation (184).

In addition to inducing its degradation, E6 of the mucosal HR HPV types can inactivate p53 and abolish its transactivation *in vivo* (185), by targeting its transcriptional coactivator CBP/p300. This event leads to the displacement of p53 from CBP (186) and to the inhibition of p300-dependent p53 acetylation (187).

As for HR HPV types, the E6 oncoproteins of β1 HPV types (HPV5, 8) also have the ability to interact with p300 with high efficiency (188). HPV5 and 8 E6s inhibit the association of AKT with the p300 C-terminus that is needed to ensure p300 stability, thus leading to its proteasomal degradation (188). The degradation of p300 results in decreased protein levels of ATR, a PI3 kinase family member, which plays a key role in UV radiation damage signaling. This event in turn reduces ATR's ability to protect cells against UVB radiation-induced damage (189). The reduced ATR level results in decreased phosphorylation and subsequent attenuated accumulation of p53 in cells in response to UVB radiation exposure. In addition, in HPV5, 8, and 38 E6-expressing cells, arrest in the G1 phase of the cell cycle is prevented, which hampers DNA repair. Both events lead to increased persistence of thymine dimers and UVB radiation-induced double-strand breaks in these cells (189). More recently, Hufbauer et al. (190) showed a lack of phosphorylation of ATM, ATR, and Chk1 in HPV8 E6 expressing-monolayer and organotypic cultures leading to the impairment of DNA damage sensing and repair. Consistent with impaired activation of the ATM/ATR pathway, immunohistochemical analysis revealed the presence of thymine dimers in UVB radiation-treated E6-expressing cells (190), further confirming a role of beta HPV E6 proteins in facilitating UV radiation-induced DNA damage.

TABLE 1 | Main features and functions of the early and late gene products from alpha and beta HPV types.

Gene products	Alpha HPV features	Beta HPV features
LCR	• Also called upstream regulatory region (URR), contains the early promoter and regulatory element involved in viral DNA replication and transcription	
E6	• Required for the maintenance of the cancer phenotype • Required for stable episomal maintenance	• Not required for the maintenance of the cancer phenotype • Inhibition of UV radiation-induced damage repair • Hampers the differentiation of HPV8-expressing keratinocytes by targeting the PDZ domain-containing protein syntenin 2 • Interacts with Notch pathway and promote the transformation process of the infected keratinocytes
	• Deregulation of cell cycle • Inhibition of apoptosis • Cell polarity, migration and attachment • The PDZ domain-binding motif of E6 proteins regulates HPV life cycle • Upregulation of the hTERT promoter activity	
E7	• Required for the maintenance of the cancer phenotype • Required for stable episomal maintenance	• Not required for the maintenance of the cancer phenotype • E7 from HPV38 shows the ability to counteract p53-mediated apoptosis by inducing accumulation of the p73 isoform, ΔNp73
	• Deregulation of cell cycle • Inhibition of apoptosis	
E5	• Not required for the maintenance of the oncogenic phenotype • Increases the immortalization effects of HPV16 E6 and E7 • Promotes tumor cell motility and cancer metastasis • Promotes cell-cycle progression • Inhibition of apoptosis	• The E5 ORF is lacking
E8^E2	• Acts as a transcriptional repressor, and represses E1/E2-dependent replication of the viral origin	
E1	• Viral DNA replication	
E2	• Ensure the segregation of the viral genome during cell division	
E4	• Contributes to efficient productive replication in differentiating cells	
L1	• Major capsid protein	
L2	• Minor capsid protein • L2 is required for an efficient trafficking of the viral genome to the nucleus • L2 mediates the viral genome delivery to the nuclear domain (ND10)	

E6 of HPV8 may also play a role in skin carcinogenesis by down-regulating CCAAT/enhancer-binding protein α (C/EBPα), a transcription factor with a role in cell differentiation. MicroRNA-203 (miR-203), previously shown to be an important regulator of epidermal proliferation and differentiation, is induced by C/EBPα. In HPV8 E6 expressing keratinocytes, downregulation of C/EBPα via E6-mediated p300 degradation led to loss of miR-203 expression and induction of the ΔNP63α protein, altering the proliferation abilities of the infected epithelial cells (191).

Similar to E6s of HPV species β1, E6 of HPV38 (species β2) was able to prevent p300-mediated acetylation of p53, an event that resulted in its inhibition and thus led to the immortalization of primary keratinocytes (192), indicating that the ability of E6 proteins of HR cutaneous HPVs to induce changes in p53 acetylation status is crucial for the transforming ability of these viruses (192).

E6 has additional cellular targets that play an important role in apoptosis (e.g., Bak, survivin, TNF R1, FADD, procaspase 8) (193–197).

The anti-apoptotic protein Bak, a member of the Bcl-2 family of proteins, plays an important role in regulating the apoptotic process by forming pores with Bax to permeabilize the outer mitochondrial membrane. Bak has been shown to be targeted for degradation by different HPVs from the alpha and beta genera, an event that contributes to preventing UV radiation-induced apoptosis. The ability of the E6 proteins of beta HPVs (e.g., HPV5, 8, or 38) to target Bak for degradation, is important for promoting the survival of DNA-damaged cells and therefore for the progression of NMSC. Bak interacts with the ubiquitin ligase E6AP; however, E6 proteins of HPV5 or 8 that do not interact with E6AP (198) can still induce Bak degradation. Moreover, HPV5 E6 mediates Bak proteolysis when expressed in E6AP-null mouse embryo fibroblast (MEFs) (199), indicating that HPV5 degrades Bak via an E6AP-independent mechanism. Holloway et al. (200) have shown that HERC1 ubiquitin ligase is required for HPV5 E6-mediated Bak degradation (200). In response to UV irradiation, Bak is dephosphorylated on K113 and interacts with HERC1. The association of HERC1 and Bak is dependent upon E6 expression. In fact, HERC1 is recruited by HPV5

E6 to degrade Bak upon UV radiation-induced damage. The degradation of Bak by E6 prevents the release of proapoptotic factors including apoptosis-inducing factor (AIF) from the mitochondria into the nucleus, an event that prevents cells from undergoing UV radiation-induced apoptosis (201).

Those experiments highlight the ability of beta HPVs to enhance UV radiation-induced DNA damage, supporting a role of beta HPVs at the early stage of skin carcinogenesis.

Beta HPVs display additional features for increasing the oncogenic potential of UV radiation exposure by attenuating DNA damage repair. For instance, the binding and destabilizing of p300 by beta HPV5 and 8 E6s also lead to lower levels of BRCA1 and BRCA2, critical to the homology-dependent repair of double-strand breaks (202). Viarisio et al. (104, 121) reported a strong reduction of IL-18 production in HPV38 transgenic mice exposed to UV radiation (104, 121). IL-18 reduces UV radiation-induced DNA damage by the induction of DNA repair (203), suggesting that by inducing a reduction of IL-18 secretion in the mouse skin, HPV38 early proteins may repress the UV radiation-induced inflammasome response, favoring DNA damage accumulation and therefore promoting the carcinogenic process (104).

All HR HPV E6 proteins contain C-terminal PDZ domain-binding motifs (PBMs) that enable interaction with several cellular PDZ domain-containing proteins involved in the regulation of different processes, such as cell polarity, migration, and attachment (204). The interaction of HR HPV E6 with the PDZ proteins leads to their proteasome-mediated degradation (205). The PBM is absent from E6s of LR HPV types, suggesting a key role for this domain in HR HPV-induced carcinogenesis.

A phosphor-acceptor site for protein kinase A (PKA) is located within the PBM. Phosphorylation at this site negatively regulates the PDZ domain-binding activity. The PKA recognition sequence is highly conserved among E6 of HR HPV types and is not present in LR HPV E6 oncoproteins, further suggesting that the PDZ domain-binding activity is crucial in HR HPV-mediated transformation (206, 207). In addition, phosphorylation at this site is required for HPV18 E6 to interact with 14-3-3ξ, contributing to the stabilization of the oncoprotein (208).

Different PDZ domain-containing proteins have been identified as targets of E6 of HR HPV types: DLG1, a human homolog of *Drosophila* discs large 1, and hScrib, a homolog of the *Drosophila* scribble protein (209–211), which play an important role in the polarity of the epithelial cells and have been identified as tumor suppressors (212); the molecular scaffolds membrane-associated guanylate kinase homology proteins, such as MAGI-1 (213), which is involved in the modulation of epithelial cell adhesion and tight junction integrity, acts as a tumor suppressor through the stabilization of PTEN (214, 215); and another HPV16 PDZ domain-containing protein, CAL, which is involved in the regulation of intracellular vesicular trafficking (216). The multi-PDZ domain protein MUPP1, which negatively regulates cellular proliferation, is targeted for degradation by HPV18 E6 and possibly by other HR HPV E6 oncoproteins, thus contributing to the transforming activities of E6 oncoproteins (217). The PDZ domain-containing proteins PSD95, TIP-2/GIPC, and NHERF1, which are involved in different signaling pathways, are also targeted by E6 (218–220).

In addition to playing a role in many cellular processes, the PBM of E6 proteins also regulates the HPV life cycle; mutations of this motif were shown to lead to lower levels of episomal HPV genome and cell growth (221). HPV8 E6, which lacks the PBM, can target the PDZ domain-containing protein syntenin 2, by reducing its expression level in a MAML1-independent manner, by an epigenetic mechanism. This event plays a key role in hampering differentiation of HPV8-expressing keratinocytes (222) and provides a further example of how skin and mucosal HPV types evolved different mechanisms to alter normal cell homeostasis and induce cellular transformation.

Using a proteomic approach, Thomas et al. (223) compared the ability of E6 PBMs of HPV types with different oncogenic potentials to interact with PDZ substrates (223). This comprehensive analysis showed that the number of PDZ substrates varies according to the HPV type. Most of the PDZ substrates were recognized by HR types (i.e., HPV16 and 18), whereas HPV66, which is a possible HR HPV type, recognizes only a limited number of substrates. Of note, whereas PDZ substrates such as DLG1 are common targets of the E6 proteins irrespective of their oncogenic potential, the ability of E6 PBMs to interact with hScrib, a component of the Scribble polarity complex, is correlated with the transforming ability of the studied HPV types (223).

Telomerase activity also plays an important role during cervical carcinogenesis, because it increases with the grade of the cervical lesions (224). The ability to activate the promoter of hTERT (human telomerase reverse transcriptase), the catalytic subunit of the telomerase, by different HPVs was assessed using a luciferase-based assay. The results of that study showed that several HR HPV types significantly upregulated hTERT promoter activity (225). The induction of hTERT requires the E6/E6AP complex to interact with the heterodimer c-Myc/Max (226–229). Moreover, E6 recruits histone acetyltransferase (HAT) at the hTERT promoter, which facilitates the access of transcriptional activators to key regulatory sites through the opening of the chromatin (230).

NFX1-91, a constitutive hTERT repressor, is targeted by E6/E6AP for ubiquitin-dependent degradation (230). The induction of hTERT expression by E6/E6AP is also dependent on the activation of NFX1-123, which constitutes a level of post-transcriptional regulation (231). Together, these events lead to extending the life span of primary human keratinocytes, and to their immortalization. Proteomics studies comparing the cellular protein-binding partners of the E6s of HPV types of different genera have shown that whereas all the studied alpha HPV E6s have a high affinity for E6AP and HERC2 (232), beta and gamma HPV E6s specifically interact with the cellular transcriptional co-activator MAML1 (233). The latter interaction is required to inhibit Notch1, a key player in skin differentiation and in the activation of transcription of the cell-cycle inhibitor p21. Interaction of beta HPV E6 with the Notch pathway appears to be important to promote the transformation process of the infected keratinocytes (234, 235). The different affinities of E6s of different

genera for specific cellular targets could be a consequence of their tropism and co-evolution with different type of tissues (236).

Transforming Activities of E7

The E7 oncoprotein is made up of ∼100 amino acids and has a C-terminal zinc-binding domain. HPV E7 presents three conserved regions, homologous to adenovirus E1A: CR1, CR2 in the N-terminal part, and CR3 in the C-terminal part. Moreover, HPV E7 comprises two nuclear localization sequences (NLSs) and one nuclear export sequence (NES), which enable E7 to be located in both the nuclear and cytoplasmic compartments, where E7 has different functions (237).

The CR1 domain has an UBR4/p600 binding site; the 600-kDa pRb-associated factor p600 is required for membrane morphogenesis, anchorage-independent growth, and cellular transformation (238, 239). Moreover, the interaction between BPV-1 E7 and p600 inhibits apoptosis, contributing to viral-induced transformation (240). UBR4/p600 ubiquitin ligase also appears to be involved in E7-mediated proteasomal degradation of the potential tumor suppressor PTPN14 (241), identified as a target of HR HPV E7 in cervical tumors (241, 242). The ability of HPV16 E7 to bind to p600 correlates with its capacity to transform cells (238). Similarly to E6, E7 binds to CBP/p300 and to its associated factor (pCAF) by interacting with the CBP TAZ2 domain (also known as CH3). The E7 binding site responsible for its interaction with the CBP TAZ2 domain overlaps with the LXCXE motif, which is crucial for E7 to bind to pRb. Formation of the ternary complex of E7/pRb and pCAF leads to the acetylation of pRb, an event that appears to play an important role in HPV-mediated transformation, probably by stabilizing the E7/pRb interaction (243, 244).

The CR2 domain comprises serine residues at positions 31 and 32 that are susceptible to phosphorylation by casein kinase II (CKII). Phosphorylation at these sites contributes to modulating some of the E7 functions. For example, CKII phosphorylation of E7 increases the binding affinity of HPV16 E7 for the TATA box-binding protein (TBP) (245), and it is required for efficient transformation by E7 (246). Interestingly, an additional phosphorylation site at serine position 29 exists in a natural E7 variant (N29S) and leads to increased levels of phosphorylation by CKII, which increases the interaction of E7 with TBP and pRb, and its transforming activity in primary rodent cells (247).

CR2 also comprises a conserved LXCXE motif located at residues 22–26 in HPV16 E7. This motif is essential for the interaction with pRb and related pocket proteins (p107 and p130) (248). An intact LXCXE motif is needed to induce cellular DNA synthesis and mediates cell transformation of immortalized NIH3T3 cells. Residues in the CR3 domain of E7 also seem to be required for its interaction with pRb (249). pRb and related pocket proteins (p107 and p130) play a key role in the regulation of the cell cycle by binding to and inhibiting E2F transcription factors (E2Fs), which regulates the expression of genes required for DNA synthesis and cell division. HR HPV E7 oncoproteins are able to target pRb and other members of the retinoblastoma family for proteasome-mediated degradation. This event requires the recruitment of the cullin-2 ubiquitin ligase complex, which binds to the CR1 region (250). Upon pRb interaction with HPV E7, E2F is released, with subsequent transcriptional induction of G1–S cell-cycle genes, such as cyclin A and cyclin E. The latter products are required for the activation of the cyclin-dependent kinase (CDK) complexes, which force the entry of HPV-infected cells into the S phase (251). This event leads to unscheduled growth and accumulation of genomic instability. LR HPV E7s bind to but are not efficient in targeting pRb for degradation (252). The ability of E7 to interact with pRb is not sufficient for HPV-induced transformation, which, as mentioned above, requires the interaction of E7 with additional cellular proteins (253).

The functionally and structurally pRb-related proteins, p107 and p130, are also targeted by the HPV E7 via the LXCXE motif, and both have the ability to bind to and inactivate E2Fs. These proteins are important negative regulators of the cell cycle, and they are believed to also act as tumor suppressors. However, unlike pRb, their role as tumor suppressors is still controversial, because related genetic alterations are not frequent in human cancers. Both HR and LR HPV E7s target p130 for proteasomal degradation, most likely by different mechanisms, thus highlighting an important role of this pocket protein in the HPV life cycle (254).

Analysis of HPV E7-interacting proteins showed that HPV16 E7 interacts with E2F6, which has the particularity of lacking the binding domain for pRb and acts as a transcriptional repressor. The association of HPV16 E7 with E2F6 abrogates its repressive activity on target genes, which results in increased DNA replication of HPV16-infected cells (255).

Using an organotypic culture of skin, Akgül et al. (256) showed that beta HPV8 E7-transduced keratinocytes displayed an invasive phenotype, in addition to losing their polarity (256). HPV8 E7-transduced keratinocytes also showed enhanced production of extracellular proteinases (MMP-1, MMP-8, and MT1-MMP) that play a role in the degradation of components of the basement membrane and the ECM during cell invasion. In another study, the same group used a transgenic mouse model expressing the E7 oncoprotein of HPV8 in the epidermis to investigate the molecular basis for HPV-induced invasion of skin keratinocytes. HPV8 E7 expression in mouse skin led to a reduction of E-cadherin and a subsequent upregulation of N-cadherin, an event that causes epithelial–mesenchymal transition. E7-positive keratinocytes also showed increased fibronectin expression and secretion. In addition, HPV8 E7-expressing cell surface showed enhanced levels of the integrin α3 chain, which plays a crucial role at the pre-invasive stage (257). E7 of beta-HPVs may play an important role in promoting an invasive phenotype in keratinocytes and may provide additional evidence to support a role of cutaneous HPVs in skin carcinogenesis.

HPV8 also has the ability to maintain cells in a "stemness-like" phenotype. Using *in vitro* colony formation and tumor sphere assays, Hufbauer et al. (258) showed that beta HPVs are able to increase the number of stem cell-like cells, as determined by measuring the number of HPV8 E2-, E6-, and E7-expressing cells harboring high levels of epithelial stemness cell surface markers, such as CD44 and epithelial cell adhesion molecule (EpCAM). EpCAM induction was also observed in organotypic skin cultures of HPV5, 8, and 16 E7-expressing cells, as well as in skin lesions of patients with EV (258). E7 expression

also led to a reduction of the epithelial differentiation marker calgranulin B. Together, these results highlight a possible role of beta HPVs in skin carcinogenesis by increasing the number of stem cell-like cells present during early carcinogenesis and delaying cell differentiation.

Lanfredini et al. (259) evaluated KSC populations located in different regions of the hair follicle (259). In that study, using HPV8 transgenic (HPV8tg) mice that express the entire HPV8 early region, the authors identified KSCs from the upper part of the junctional zone as the site from which the expansion to the interfollicular epidermis takes place during the initial step of skin carcinogenesis. This population of stem cells express leucine-rich repeats and immunoglobulin-like domains protein 1 (Lrig-1) and are characterized by nuclear staining for the stemness marker p63. Skin accumulation of the p63 isoform, ΔNp63, which is induced by the expression of HPV8 early proteins, promotes the proliferation and expansion of Lrig1-positive KSCs. In healthy skin, the ΔNP63α transcript is directly targeted by miR-203, an event that plays a major role in the regulation of epidermal proliferation and differentiation, leading to "stemness" loss (260, 261). Of note, the skin lesions of patients with EV show strong p63 immunostaining associated with very low levels of miR-203.

In addition to features cited above, E7 can also deregulate the cell cycle by binding and abrogating the inhibitory effect of CDK inhibitors like $p21^{Cip1}$ and $p27^{Kip1}$. This interaction abolishes their inhibitory effect on the cell cycle and highlights a different mechanism by which E7 favors cell proliferation (262–264).

E6 and E7 of HPV38 and 49, which can efficiently immortalize human primary keratinocytes, do not have the ability to target pRb for degradation when expressed in these cells (120). However, similarly to E7 of LR mucosal types HPV38 and 49 E7s can efficiently bind to pRb. In contrast, beta HPV8 E7 binds to pRb to a lesser extent compared with HPV16 E7, but it is still able to repress pRb levels when expressed in keratinocytes (265, 266).

Studies from our laboratory have shown that different beta HPV types (e.g., HPV38) are able to trigger pRb hyperphosphorylation, an event that leads to release of E2F and to the transcriptional activation of E2F target genes [reviewed in (19)]. In addition, E7 of HPV38 shows the ability to counteract p53-mediated apoptosis by inducing accumulation of the p73 isoform, ΔNp73, by two different mechanisms: (i) by inducing the recruitment of a post-translationally modified form of p53 to the promoter of ΔNp73, which activates its transcription (267), and (ii) by mediating ΔNp73 protein stabilization with an IκB kinase beta (IKKβ)-mediated mechanism (268). The latter studies highlight that cutaneous HPVs have evolved different mechanisms than HR mucosal HPVs, to target key cellular proteins (pRb and p53) and promote cellular transformation.

Transforming Activities of E5

Although the transforming activities of E6 and E7 oncoproteins are well characterized, the role of the E5 oncoprotein is still poorly understood (269). E5 is a hydrophobic membrane-associated protein that localizes to the endoplasmic reticulum, Golgi apparatus, and plasma membrane (270, 271). E5 forms a hexameric viroporin complex, which modulates ion homeostasis (269). E5 viroporin is required for the hyperactivation of mitotic signaling, and it plays a key role during the life cycle of HR HPV types. Recombinant HPV16 E5 membrane channel activity is sensitive to compounds such as adamantine and rimantadine. Inhibition of E5 viroporin activity in primary human keratinocytes harboring HPV18 genome leads to decreased ERK-MAPK phosphorylation and cyclin B1 levels, and to an increase in the expression of differentiation marker (272). E5 viroporin activity is therefore critical for maintaining mitogenic signaling and delaying expression of differentiation marker during the productive stages of the HPV18 life cycle; therefore, targeting viroporin could pave the way for an effective antiviral strategy.

The expression of E5 increased the immortalization effects of HPV16 E6 and E7 (273). E5 is involved in the transformation of primary human keratinocytes by potentiating epidermal growth factor receptor (EGFR) signaling (274). In 16E5 transgenic mice, EGFR is required for epithelial hyperplasia (275). The activation of EGFR leads to induction of the expression of cyclooxygenase-2 (COX-2), which plays an important role in cervical carcinogenesis, through nuclear factor-kappa B (NF-κB) and AP-1 (276). Via stimulation of EGFR, HPV16 E5 induces Met, a growth factor receptor involved in tumor cell motility and cancer metastasis; moreover, Met signaling is required for proper differentiation-dependent viral gene expression (277).

HPV16 E5 protein enhances, in cooperation with EGFR signaling, the down-regulation of tumor suppressor $p27^{Kip1}$ levels, which promotes cell-cycle progression (278). The level of another CDK inhibitor, p21, is reduced in cells that over-express E5 (279). Both events lead to an increase in cell proliferation and in the percentage of cells in the S phase.

HPV16 E5 also contributes to carcinogenesis by inhibiting apoptosis by multiple molecular strategies. The effect of HPV16 E5 on apoptosis was assessed using raft cultures in which E5 prevented FasL- or TRAIL-mediated apoptosis (280). A recent study reported that HPV16 E5 specifically down-regulates miR-196a in cervical cancer cells. The same study showed that miR-196a expression affects cell proliferation, cell growth, and apoptosis, thus confirming the role of HPV16 E5 in cervical cancer development (281). Another anti-apoptotic strategy consists of decreasing pro-apoptotic Bak and Bax levels, and increasing the expression of the anti-apoptotic Bcl-2. HPV16 E5 inhibits apoptosis of cervical cancer cells by stimulating the ubiquitin-proteasome-mediated degradation of Bax protein (282). It has also been reported that HPV16 E5 protects E5-expressing foreskin keratinocytes from UVB radiation-induced apoptosis (283).

All these data indicate that expression of E5 contributes to HPV-driven cellular transformation. However, unlike E6 and E7, E5 is not required for the maintenance of the oncogenic phenotype, because the E5 ORF is frequently disrupted during HPV integration, thus rather highlighting a role of E5 at the initial step of carcinogenesis (284).

HPV types from different genera (beta, gamma, and mu) lack the E5 ORF, suggesting that the corresponding functions are performed by other viral proteins.

E5 forms a complex with EVER1 and EVER2, proteins encoded by the TMC6 and TMC8 genes, respectively, (92). Most of the patients with EV who are susceptible to persistent beta HPV infections carry mutations in these genes. The EVER proteins interact with the zinc transporter ZnT-1 in order to regulate intracellular zinc homeostasis. Zinc sequestration by the ZnT-1/EVER complex may inhibit the replication of EV-associated HPV types. In contrast, mutations in EVER proteins lead to the disruption of the ZnT-1/EVER complex, and an increase in the level of zinc in the cytoplasm, resulting in increased replication of EV-associated HPV types (285), [reviewed in (286)]. A recent study reported that patients with EV can also display the typical EV phenotype, bearing non-synonymous calcium- and integrin-binding protein 1 (CIB1) mutations and lacking TMC6 and TMC8 mutations (287). CIB1 does not seem to interact with ZnT-1, but this protein forms a complex with EVER1 and EVER2. The CIB1-EVER1-EVER2 complex appears to act as a restriction factor for infection by cutaneous HPVs that do not express E5. In patients with EV, impairment of CIB1, EVER1, or EVER2 allows replication of these HPV types and development of EV lesions on the skin (287).

CONCLUSIVE REMARKS, CHALLENGES, AND OPEN QUESTIONS

In the past decades, epidemiological and biological studies have clearly demonstrated the role of mucosal HR HPV types in human carcinogenesis. While co-evolving with the human host, HPVs have developed strategies to evade the immune system and create the optimal conditions to persist in the host for many years. During HPV chronic infection, the E6 and E7 oncoproteins interact with different cellular proteins to prevent apoptosis, counteract the cellular senescence program, and promote unscheduled cell growth. These activities of HR HPV E6 and E7 are part of a viral strategy to complete the viral life cycle and generate progeny. The expression of the oncoproteins is kept under the tight control of E2. Loss of E2 repressive functions, by viral integration or by other mechanisms, which are discussed in this review, leads to constitutive high expression of E6 and E7, a key event in HPV-mediated transformation (**Figure 3**). Although the mechanisms leading to cervical cancer have been well-characterized, there is still a gap in the understanding of the pathogenesis of other HPV-related malignancies, e.g., oropharyngeal cancer. The proportion of HPV-driven oropharyngeal cancers has been increasing over the past decades in many parts of the world, mainly in Europe and North America (288–290). The natural history of HPV infection in the oral cavity and oropharynx is poorly studied and needs further investigation.

Of note, recent studies identified HPV16 E6 antibody as a good diagnostic and prognostic marker of oropharyngeal cancer. HPV16 E6 seropositivity was detected more than 10 years before the diagnosis (291, 292).

Moreover, although the available prophylactic vaccines have demonstrated their effectiveness in the context of prevention of anogenital cancers (293), very limited epidemiological data are available on the efficacy of the HPV vaccine on oral HPV infection (294). This deserves further investigation.

Prophylactic vaccines show a good efficiency in covering targeted HPVs, but they are inefficient in existing infections. Therefore, therapeutic vaccines are needed to fill this gap. Several studies have reported the development of promising therapeutic vaccines. Their use in the near future could be expected; however, their cost could be high. This limitation could be overcome by the use of inexpensive technologies (i.e., plant-made therapeutics) (295–299).

One of the most recurrent questions regarding the prophylactic vaccine is whether HPV vaccination will result in the occupation of a newly vacant ecological niche by non-targeted HPV types. This hypothesis has been referred as "type replacement. " To date, the available data do not support this hypothesis. However, as reported in recent studies, a few types (i.e., HPV51 and 52) may need to be monitored after vaccination (300, 301).

The bacterial microbiome composition may also play a role in the outcome of mucosal HPV infection. A chronic mycoplasma infection was shown to promote cervical dysplasia induced by HPV (302). Therefore, identifying the bacterial microbiome could allow the identification of patients at high risk for developing cervical cancer.

Our knowledge of the biology of beta and gamma HPV types is still uncomplete. For example, the expression patterns of cutaneous HPV types and associated splicing patterns are largely unknown, except for HPV5 and 8 (https://pave.niaid.nih.gov/). There are also important gaps in the understanding of the life cycle of cutaneous HPV types, which also deserve further research.

In February, 2009, an IARC Monograph Working Group met at IARC to assess the carcinogenicity of biological agents including beta and gamma HPV types. HPV5 and 8 were classified as possibly carcinogenic to humans in patients with EV (IARC Group 2B), whereas the other cutaneous HPV types were each determined to be "not classifiable as to its carcinogenicity to humans" (IARC Group 3), on the basis of insufficient epidemiological and biological data available at that time (10). In fact, although several epidemiological and biological studies support a role of β1 and β2 HPV types in NMSC, the lack of cutaneous HPV E6 and E7 expression in SCC has raised the question of whether the viral proteins are required in the carcinogenesis process.

Since that 2009 meeting, strong epidemiological and experimental data in favor of a role of cutaneous HPV types as cofactors in skin carcinogenesis have been published, as described in this review. Indeed, recent findings from our group and others have shown that unlike the expression of oncoproteins of HR HPV types, cutaneous HPV E6 and E7 expression appears to be required only at the initial step of skin carcinogenesis by exacerbating the deleterious effects of UV radiation (104). Over the past decade, studies using experimental animals (HPV38 transgenic mice and the MnPV-infected rodent model *Mastomys coucha*) have provided strong *in vivo* evidence for a synergism between UV radiation and cutaneous HPV types

in the development of cSCC (104, 107). Most importantly, both models provided further evidence for a hit-and-run mechanism. However, additional studies are still necessary to validate this hypothesis in humans.

In addition to these experimental data, a recent prospective study of two organ transplant recipient cohorts showed that having five or more different beta HPV types in eyebrow hair, and a high beta HPV viral load, was associated with cSCC carcinogenesis, providing strong evidence for a role of beta HPVs in this cancer, whereas no evidence was found for basal cell carcinoma (303). The development of vaccination strategies that target cutaneous HPV infections would be of great benefit to these patients (304, 305).

Another open question regards the tropism of certain HPVs. As previously specified in this review, a dual tropism of HPV species β3 has been proposed. Moreover, this species displays biological similarities with mucosal HR HPV types (111, 120). Our recent data highlighted that E6 and E7 of HPV49 share some features with HR mucosal types, and efficiently transform epithelial cells in *in vitro* and *in vivo* models (120, 121). In addition, in line with these biological data, epidemiological studies have shown β3 HPVs and other cutaneous types in the oral cavity mucosa. Beta and gamma HPV types have also been isolated from the nasopharynx and the nasal mucosa (119, 306). Moreover, a recent study based on a prospective design reported that beta or gamma HPV types were associated with the incidence of HNC (117). Those findings warrant further studies to better understand the role of those so-called "cutaneous HPV types" in mucosotropic cancers (111, 119).

A large number of gamma HPV types have been isolated; however, their biological activities are poorly studied and deserve more investigation. Finally, some of the known but not yet biologically characterized beta or gamma HPV types may display transforming activity and also deserve attention. In addition to these not-yet-studied types, one may also consider the large number of novel HPVs found in recent years, as well as the rapid evolution of viral detection and screening techniques and large data set analysis pipelines.

For all these reasons, we believe that in the coming years the field of HPV research will continue to be a source of surprises and excitement for the scientific community.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication. TG wrote the manuscript and designed the figures.

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REFERENCES

1. Bravo IG, de Sanjose S, Gottschling M. The clinical importance of understanding the evolution of papillomaviruses. *Trends Microbiol.* (2010) 18:432–8. doi: 10.1016/j.tim.2010.07.008
2. Lopez-Bueno A, Mavian C, Labella AM, Castro D, Borrego JJ, Alcami A, et al. Concurrence of iridovirus, polyomavirus, and a unique member of a new group of fish papillomaviruses in lymphocystis disease-affected gilthead sea bream. *J Virol.* (2016) 90:8768–79. doi: 10.1128/JVI.01369-16
3. Bravo IG, Felez-Sanchez M. Papillomaviruses: Viral evolution, cancer and evolutionary medicine. *Evol Med Public Health*. (2015) 2015:32–51. doi: 10.1093/emph/eov003
4. Gottschling M, Goker M, Stamatakis A, Bininda-Emonds OR, Nindl I, Bravo IG. Quantifying the phylodynamic forces driving papillomavirus evolution. *Mol Biol Evol*. (2011) 28:2101–13. doi: 10.1093/molbev/msr030
5. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. *Virology*. (2004) 324:17–27. doi: 10.1016/j.virol.2004.03.033
6. Muhr LSA, Eklund C, Dillner J. Towards quality and order in human papillomavirus research. *Virology*. (2018) 519:74–6. doi: 10.1016/j.virol.2018.04.003
7. Brancaccio RN, Robitaille A, Dutta S, Cuenin C, Santare D, Skenders G, et al. Generation of a novel next-generation sequencing-based method for the isolation of new human papillomavirus types. *Virology*. (2018) 520:1–10. doi: 10.1016/j.virol.2018.04.017
8. Pastrana DV, Peretti A, Welch NL, Borgogna C, Olivero C, Badolato R, et al. Metagenomic Discovery of 83 new human papillomavirus types in patients with immunodeficiency. *mSphere*. (2018) 3:e00645–18. doi: 10.1128/mSphereDirect.00645-18
9. Halec G, Schmitt M, Dondog B, Sharkhuu E, Wentzensen N, Gheit T, et al. Biological activity of probable/possible high-risk human papillomavirus types in cervical cancer. *Int J Cancer*. (2013) 132:63–71. doi: 10.1002/ijc.27605
10. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. A review of human carcinogens–Part B: biological agents. *Lancet Oncol.* (2009) 10:321–2. doi: 10.1016/S1470-2045(09)70096-8
11. Haedicke J, Iftner T. Human papillomaviruses and cancer. *Radiother Oncol.* (2013) 108:397–402. doi: 10.1016/j.radonc.2013.06.004
12. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Global Health*. (2016) 4:e609–16. doi: 10.1016/S2214-109X(16)30143-7
13. Ball SL, Winder DM, Vaughan K, Hanna N, Levy J, Sterling JC, et al. Analyses of human papillomavirus genotypes and viral loads in anogenital warts. *J Med Virol.* (2011) 83:1345–50. doi: 10.1002/jmv.22111
14. Derkay CS, Wiatrak B. Recurrent respiratory papillomatosis: a review. *Laryngoscope*. (2008) 118:1236–47. doi: 10.1097/MLG.0b013e31816a7135
15. McLaughlin-Drubin ME, Meyers J, Munger K. Cancer associated human papillomaviruses. *Curr Opin Virol.* (2012) 2:459–66. doi: 10.1016/j.coviro.2012.05.004
16. King CM, Johnston JS, Ofili K, Tam M, Palefsky J, Da Costa M, et al. Human papillomavirus types 2, 27, and 57 Identified in plantar verrucae from HIV-positive and HIV-negative individuals. *J Am Pediatric Med Assoc*. (2014) 104:141–6. doi: 10.7547/0003-0538-104.2.141
17. Hogendoorn GK, Bruggink SC, de Koning MNC, Eekhof JAH, Hermans KE, Rissmann R, et al. Morphological characteristics and human papillomavirus genotype predict the treatment response in cutaneous warts. *Br J Dermatol.* (2018) 178:253–60. doi: 10.1111/bjd.15758
18. Bruggink SC, de Koning MN, Gussekloo J, Egberts PF, Ter Schegget J, Feltkamp MC, et al. Cutaneous wart-associated HPV types: prevalence and relation with patient characteristics. *J Clin Virol*. (2012) 55:250–5. doi: 10.1016/j.jcv.2012.07.014

19. Accardi R, Gheit T. Cutaneous HPV and skin cancer. *Presse Med*. (2014) 43:e435–43. doi: 10.1016/j.lpm.2014.08.008
20. Viarisio D, Gissmann L, Tommasino M. Human papillomaviruses and carcinogenesis: well-established and novel models. *Curr Opin Virol*. (2017) 26:56–62. doi: 10.1016/j.coviro.2017.07.014
21. Tommasino M. The biology of beta human papillomaviruses. *Virus Res*. (2017) 231:128–38. doi: 10.1016/j.virusres.2016.11.013
22. Van Doorslaer K, McBride AA. Molecular archeological evidence in support of the repeated loss of a papillomavirus gene. *Sci Rep*. (2016) 6:33028. doi: 10.1038/srep33028
23. Chen Z, Schiffman M, Herrero R, Desalle R, Burk RD. Human papillomavirus (HPV) types 101 and 103 isolated from cervicovaginal cells lack an E6 open reading frame (ORF) and are related to gamma-papillomaviruses. *Virology*. (2007) 360:447–53. doi: 10.1016/j.virol.2006.10.022
24. Nobre RJ, Herraez-Hernandez E, Fei JW, Langbein L, Kaden S, Grone HJ, et al. E7 oncoprotein of novel human papillomavirus type 108 lacking the E6 gene induces dysplasia in organotypic keratinocyte cultures. *J Virol*. (2009) 83:2907–16. doi: 10.1128/JVI.02490-08
25. Bravo IG, Alonso A. Mucosal human papillomaviruses encode four different E5 proteins whose chemistry and phylogeny correlate with malignant or benign growth. *J Virol*. (2004) 78:13613–26. doi: 10.1128/JVI.78.24.13613-13626.2004
26. McBride AA. The papillomavirus E2 proteins. *Virology*. (2013) 445:57–79. doi: 10.1016/j.virol.2013.06.006
27. Zobel T, Iftner T, Stubenrauch F. The papillomavirus E8-E2C protein represses DNA replication from extrachromosomal origins. *Mol Cell Biol*. (2003) 23:8352-62. doi: 10.1128/MCB.23.22.8352-8362.2003
28. Lace MJ, Anson JR, Thomas GS, Turek LP, Haugen TH. The E8–E2 gene product of human papillomavirus type 16 represses early transcription and replication but is dispensable for viral plasmid persistence in keratinocytes. *J Virol*. (2008) 82:10841–53. doi: 10.1128/JVI.01481-08
29. Ammermann I, Bruckner M, Matthes F, Iftner T, Stubenrauch F. Inhibition of transcription and DNA replication by the papillomavirus E8-E2C protein is mediated by interaction with corepressor molecules. *J Virol*. (2008) 82:5127–36. doi: 10.1128/JVI.02647-07
30. Burchell AN, Winer RL, de Sanjose S, Franco EL. Chapter 6: Epidemiology and transmission dynamics of genital HPV infection. *Vaccine*. (2006) 24 (Suppl. 3):S3/52–61. doi: 10.1016/j.vaccine.2006.05.031
31. Ryndock EJ, Meyers C. A risk for non-sexual transmission of human papillomavirus? *Exp Rev Anti-Infect Ther*. (2014) 12:1165–70. doi: 10.1586/14787210.2014.959497
32. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. *Lancet*. (2007) 370:890–907. doi: 10.1016/S0140-6736(07)61416-0
33. Maucort-Boulch D, Plummer M, Castle PE, Demuth F, Safaeian M, Wheeler CM, et al. Predictors of human papillomavirus persistence among women with equivocal or mildly abnormal cytology. *Int J Cancer*. (2010) 126:684–91. doi: 10.1002/ijc.24752
34. Oh HY, Seo SS, Kim MK, Lee DO, Chung YK, Lim MC, et al. Synergistic effect of viral load and alcohol consumption on the risk of persistent high-risk human papillomavirus infection. *PLoS ONE*. (2014) 9:e104374. doi: 10.1371/journal.pone.0104374
35. Giuliano AR, Sedjo RL, Roe DJ, Harri R, Baldwi S, Papenfuss MR, et al. Clearance of oncogenic human papillomavirus (HPV) infection: effect of smoking (United States). *Cancer Causes Control*. (2002) 13:839–46. doi: 10.1023/A:1020668232219
36. Haukioja A, Asunta M, Soderling E, Syrjanen S. Persistent oral human papillomavirus infection is associated with smoking and elevated salivary immunoglobulin G concentration. *J Clin Virol*. (2014) 61:101–6. doi: 10.1016/j.jcv.2014.06.012
37. Magnusson PK, Sparen P, Gyllensten UB. Genetic link to cervical tumours. *Nature*. (1999) 400:29–30. doi: 10.1038/21801
38. Hemminki K, Dong C, Vaittinen P. Familial risks in cervical cancer: is there a hereditary component? *Int J Cancer*. (1999) 82:775–81. doi: 10.1002/(SICI)1097-0215(19990909)82:6<775::AID-IJC1>3.0.CO;2-V
39. Chattopadhyay K. A comprehensive review on host genetic susceptibility to human papillomavirus infection and progression to cervical cancer. *Indian J Human Genet*. (2011) 17:132–44. doi: 10.4103/0971-6866.92087
40. Wang SS, Gonzalez P, Yu K, Porras C, Li Q, Safaeian M, et al. Common genetic variants and risk for HPV persistence and progression to cervical cancer. *PLoS ONE*. (2010) 5:e8667. doi: 10.1371/journal.pone.0008667
41. Pontillo A, Bricher P, Leal VN, Lima S, Souza PR, Crovella S. Role of inflammasome genetics in susceptibility to HPV infection and cervical cancer development. *J Med Virol*. (2016) 88:1646–51. doi: 10.1002/jmv.24514
42. Bernal-Silva S, Granados J, Gorodezky C, Alaez C, Flores-Aguilar H, Cerda-Flores RM, et al. HLA-DRB1 Class II antigen level alleles are associated with persistent HPV infection in Mexican women; a pilot study. *Infect Agent Cancer*. (2013) 8:31. doi: 10.1186/1750-9378-8-31
43. Chen AA, Heideman DA, Boon D, Gheit T, Snijders PJ, Tommasino M, et al. Human papillomavirus 45 genetic variation and cervical cancer risk worldwide. *J Virol*. (2014) 88:4514–21. doi: 10.1128/JVI.03534-13
44. Chen AA, Heideman DA, Boon D, Chen Z, Burk RD, De Vuyst H, et al. Human papillomavirus 33 worldwide genetic variation and associated risk of cervical cancer. *Virology*. (2014) 448:356–62. doi: 10.1016/j.virol.2013.10.033
45. Cornet I, Gheit T, Iannacone MR, Vignat J, Sylla BS, Del Mistro A, et al. HPV16 genetic variation and the development of cervical cancer worldwide. *Br J Cancer*. (2013) 108:240–4. doi: 10.1038/bjc.2012.508
46. Chen AA, Gheit T, Franceschi S, Tommasino M, Clifford GM, Group IHVS. Human Papillomavirus 18 genetic variation and cervical cancer risk worldwide. *J Virol*. (2015) 89:10680–7. doi: 10.1128/JVI.01747-15
47. Gheit T, Cornet I, Clifford GM, Iftner T, Munk C, Tommasino M, et al. Risks for persistence and progression by human papillomavirus type 16 variant lineages among a population-based sample of Danish women. *Cancer Epidemiol Biomarkers Prev*. (2011) 20:1315–21. doi: 10.1158/1055-9965.EPI-10-1187
48. Cornet I, Gheit T, Clifford GM, Combes JD, Dalstein V, Franceschi S, et al. Human papillomavirus type 16 E6 variants in France and risk of viral persistence. *Infect Agent Cancer*. (2013) 8:4. doi: 10.1186/1750-9378-8-4
49. Mirabello L, Yeager M, Yu K, Clifford GM, Xiao Y, Zhu B, et al. HPV16 E7 genetic conservation is critical to carcinogenesis. *Cell*. (2017) 170:1164–74 e6. doi: 10.1016/j.cell.2017.08.001
50. Westrich JA, Warren CJ, Klausner MJ, Guo K, Liu CW, Santiago ML, et al. Human Papillomavirus 16 E7 Stabilizes APOBEC3A protein by inhibiting Cullin 2-dependent protein degradation. *J Virol*. (2018) 92:e01318–17. doi: 10.1128/JVI.01318-17
51. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst*. (2000) 92:690–8. doi: 10.1093/jnci/92.9.690
52. Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol*. (2002) 40:886–91. doi: 10.1128/JCM.40.3.886-891.2002
53. Collins SI, Constandinou-Williams C, Wen K, Young LS, Roberts S, Murray PG, et al. Disruption of the E2 gene is a common and early event in the natural history of cervical human papillomavirus infection: a longitudinal cohort study. *Cancer Res*. (2009) 69:3828–32. doi: 10.1158/0008-5472.CAN-08-3099
54. Jeon S, Lambert PF. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. *Proc Natl Acad Sci USA*. (1995) 92:1654–8. doi: 10.1073/pnas.92.5.1654
55. Cancer Genome Atlas Research N, Albert Einstein College of M, Analytical Biological S, Barretos Cancer H, Baylor College of M, Beckman Research Institute of City of H, et al. Integrated genomic and molecular characterization of cervical cancer. *Nature*. (2017) 543:378–84. doi: 10.1038/nature21386
56. Xu B, Chotewutmontri S, Wolf S, Klos U, Schmitz M, Durst M, et al. Multiplex Identification of human papillomavirus 16 DNA integration sites in cervical carcinomas. *PLoS ONE*. (2013) 8:e66693. doi: 10.1371/journal.pone.0066693
57. Gray E, Pett MR, Ward D, Winder DM, Stanley MA, Roberts I, et al. *In vitro* progression of human papillomavirus 16 episome-associated cervical neoplasia displays fundamental similarities to

integrant-associated carcinogenesis. *Cancer Res.* (2010) 70:4081–91. doi: 10.1158/0008-5472.CAN-09-3335
58. Hong D, Liu J, Hu Y, Lu X, Li B, Li Y, et al. Viral E6 is overexpressed via high viral load in invasive cervical cancer with episomal HPV16. *BMC Cancer*. (2017) 17:136. doi: 10.1186/s12885-017-3124-9
59. Zhang R, Shen C, Zhao L, Wang J, McCrae M, Chen X, et al. Dysregulation of host cellular genes targeted by human papillomavirus (HPV) integration contributes to HPV-related cervical carcinogenesis. *Int J Cancer*. (2016) 138:1163–74. doi: 10.1002/ijc.29872
60. Shen C, Liu Y, Shi S, Zhang R, Zhang T, Xu Q, et al. Long-distance interaction of the integrated HPV fragment with MYC gene and 8q24.22 region upregulating the allele-specific MYC expression in HeLa cells. *Int J Cancer*. (2017) 141:540–8. doi: 10.1002/ijc.30763
61. Glover TW, Berger C, Coyle J, Echo B. DNA polymerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. *Hum Genet*. (1984) 67:136–42. doi: 10.1007/BF00272988
62. Thorland EC, Myers SL, Gostout BS, Smith DI. Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. *Oncogene*. (2003) 22:1225–37. doi: 10.1038/sj.onc.1206170
63. Christiansen IK, Sandve GK, Schmitz M, Durst M, Hovig E. Transcriptionally active regions are the preferred targets for chromosomal HPV integration in cervical carcinogenesis. *PLoS ONE*. (2015) 10:e0119566. doi: 10.1371/journal.pone.0119566
64. Schmitz M, Driesch C, Jansen L, Runnebaum IB, Durst M. Non-random integration of the HPV genome in cervical cancer. *PLoS ONE*. (2012) 7:e39632. doi: 10.1371/journal.pone.0039632
65. Leung TW, Liu SS, Leung RC, Chu MM, Cheung AN, Ngan HY. HPV 16 E2 binding sites 1 and 2 become more methylated than E2 binding site 4 during cervical carcinogenesis. *J Med Virol*. (2015) 87:1022–33. doi: 10.1002/jmv.24129
66. Oka N, Kajita M, Nishimura R, Ohbayashi C, Sudo T. L1 gene methylation in high-risk human papillomaviruses for the prognosis of cervical intraepithelial neoplasia. *Int J Gynecol Cancer*. (2013) 23:235–43. doi: 10.1097/IGC.0b013e31827da1f6
67. Torres-Rojas FI, Alarcon-Romero LDC, Leyva-Vazquez MA, Ortiz-Ortiz J, Mendoza-Catalan MA, Hernandez-Sotelo D, et al. Methylation of the L1 gene and integration of human papillomavirus 16 and 18 in cervical carcinoma and premalignant lesions. *Oncol Lett*. (2018) 15:2278–86. doi: 10.3892/ol.2017.7596
68. Parfenov M, Pedamallu CS, Gehlenborg N, Freeman SS, Danilova L, Bristow CA, et al. Characterization of HPV and host genome interactions in primary head and neck cancers. *Proc Natl Acad Sci USA*. (2014) 111:15544–9. doi: 10.1073/pnas.1416074111
69. Olthof NC, Speel EJ, Kolligs J, Haesevoets A, Henfling M, Ramaekers FC, et al. Comprehensive analysis of HPV16 integration in OSCC reveals no significant impact of physical status on viral oncogene and virally disrupted human gene expression. *PLoS ONE*. (2014) 9:e88718. doi: 10.1371/journal.pone.0088718
70. Vojtechova Z, Sabol I, Salakova M, Turek L, Grega M, Smahelova J, et al. Analysis of the integration of human papillomaviruses in head and neck tumours in relation to patients' prognosis. *Int J Cancer*. (2016) 138:386–95. doi: 10.1002/ijc.29712
71. Nulton TJ, Olex AL, Dozmorov M, Morgan IM, Windle B. Analysis of the cancer genome Atlas sequencing data reveals novel properties of the human papillomavirus 16 genome in head and neck squamous cell carcinoma. *Oncotarget*. (2017) 8:17684–99. doi: 10.18632/oncotarget.15179
72. Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson BG. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. *J Virol*. (2000) 74:11636–41. doi: 10.1128/JVI.74.24.11636-11641.2000
73. de Koning MN, Weissenborn SJ, Abeni D, Bouwes Bavinck JN, Euvrard S, Green AC, et al. Prevalence and associated factors of betapapillomavirus infections in individuals without cutaneous squamous cell carcinoma. *J Gen Virol*. (2009) 90:1611–21. doi: 10.1099/vir.0.010017-0
74. Bouwes Bavinck JN, Neale RE, Abeni D, Euvrard S, Green AC, Harwood CA, et al. Multicenter study of the association between betapapillomavirus infection and cutaneous squamous cell carcinoma. *Cancer Res.* (2010) 70:9777–86. doi: 10.1158/0008-5472.CAN-10-0352
75. Hsu JY, Chen AC, Keleher A, McMillan NA, Antonsson A. Shared and persistent asymptomatic cutaneous human papillomavirus infections in healthy skin. *J Med Virol*. (2009) 81:1444–9. doi: 10.1002/jmv.21529
76. Antonsson A, Karanfilovska S, Lindqvist PG, Hansson BG. General acquisition of human papillomavirus infections of skin occurs in early infancy. *J Clin Microbiol*. (2003) 41:2509–14. doi: 10.1128/JCM.41.6.2509-2514.2003
77. Plasmeijer EI, Struijk L, Bouwes Bavinck JN, Feltkamp MC. Epidemiology of cutaneous human papillomavirus infections. *Cancer Treat Res.* (2009) 146:143–57. doi: 10.1007/978-0-387-78574-5_13
78. Egawa N, Egawa K, Griffin H, Doorbar J. Human papillomaviruses; epithelial tropisms, and the development of neoplasia. *Viruses*. (2015) 7:3863–90. doi: 10.3390/v7072802
79. Kohler A, Forschner T, Meyer T, Ulrich C, Gottschling M, Stockfleth E, et al. Multifocal distribution of cutaneous human papillomavirus types in hairs from different skin areas. *Br J Dermatol*. (2007) 156:1078–80. doi: 10.1111/j.1365-2133.2007.07809.x
80. Schmitt A, Rochat A, Zeltner R, Borenstein L, Barrandon Y, Wettstein FO, et al. The primary target cells of the high-risk cottontail rabbit papillomavirus colocalize with hair follicle stem cells. *J Virol*. (1996) 70:1912–22.
81. Rous P, Beard JW. The progression to carcinoma of virus-induced rabbit papillomas (Shope). *J Exp Med*. (1935) 62:523–48. doi: 10.1084/jem.62.4.523
82. Plasmeijer EI, Neale RE, Buettner PG, de Koning MN, Ter Schegget J, Quint WG, et al. Betapapillomavirus infection profiles in tissue sets from cutaneous squamous cell-carcinoma patients. *Int J Cancer*. (2010) 126:2614–21. doi: 10.1002/ijc.24991
83. Weissenborn SJ, De Koning MN, Wieland U, Quint WG, Pfister HJ. Intrafamilial transmission and family-specific spectra of cutaneous betapapillomaviruses. *J Virol*. (2009) 83:811–6. doi: 10.1128/JVI.01338-08
84. Hazard K, Karlsson A, Andersson K, Ekberg H, Dillner J, Forslund O. Cutaneous human papillomaviruses persist on healthy skin. *J Invest Dermatol*. (2007) 127:116–9. doi: 10.1038/sj.jid.5700570
85. de Koning MN, Struijk L, Bavinck JN, Kleter B, ter Schegget J, Quint WG, et al. Betapapillomaviruses frequently persist in the skin of healthy individuals. *J Gen Virol*. (2007) 88:1489–95. doi: 10.1099/vir.0.82732-0
86. Moscicki AB, Ma Y, Gheit T, McKay-Chopin S, Farhat S, Widdice LE, et al. Prevalence and transmission of beta and gamma human papillomavirus in heterosexual couples. *Open Forum Infect Dis*. (2017) 4:ofw216. doi: 10.1093/ofid/ofw216
87. Rahman S, Pierce Campbell CM, Waterboer T, Rollison DE, Ingles DJ, Torres BN, et al. Seroprevalence of cutaneous human papillomaviruses (HPVs) among men in the multinational HPV Infection in Men study. *J Gen Virol*. (2016) 97:3291–301. doi: 10.1099/jgv.0.000620
88. Michael KM, Waterboer T, Pfister H, Gariglio M, Majewski S, Favre M, et al. Seroreactivity of 38 human papillomavirus types in epidermodysplasia verruciformis patients, relatives, and controls. *J Invest Dermatol*. (2010) 130:841–8. doi: 10.1038/jid.2009.356
89. Weissenborn S, Neale RE, Waterboer T, Abeni D, Bavinck JN, Green AC, et al. Beta-papillomavirus DNA loads in hair follicles of immunocompetent people and organ transplant recipients. *Med Microbiol Immunol*. (2012) 201:117–25. doi: 10.1007/s00430-011-0212-3
90. Dillner J. The serological response to papillomaviruses. *Semin Cancer Biol*. (1999) 9:423–30. doi: 10.1006/scbi.1999.0146
91. Michael KM, Waterboer T, Sehr P, Rother A, Reidel U, Boeing H, et al. Seroprevalence of 34 human papillomavirus types in the German general population. *PLoS Pathog*. (2008) 4:e1000091. doi: 10.1371/journal.ppat.1000091
92. Orth G. Host defenses against human papillomaviruses: lessons from epidermodysplasia verruciformis. *Curr Topics Microbiol Immunol*. (2008) 321:59–83. doi: 10.1007/978-3-540-75203-5_3
93. Orth G, Jablonska S, Favre M, Croissant O, Jarzabek-Chorzelska M, Rzesa G. Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis. *Proc Natl Acad Sci USA*. (1978) 75:1537–41. doi: 10.1073/pnas.75.3.1537
94. O'Reilly Zwald F, Brown M. Skin cancer in solid organ transplant recipients: advances in therapy and management: part I. epidemiology of skin cancer in solid organ transplant recipients. *J Am Acad Dermatol*. (2011) 65:253–61. doi: 10.1016/j.jaad.2010.11.062

95. Nindl I, Rosl F. Molecular concepts of virus infections causing skin cancer in organ transplant recipients. *Am J Transl.* (2008) 8:2199–204. doi: 10.1111/j.1600-6143.2008.02392.x
96. Silverberg MJ, Leyden W, Warton EM, Quesenberry CP Jr., Engels EA, Asgari MM. HIV infection status, immunodeficiency, and the incidence of non-melanoma skin cancer. *J Natl Cancer Inst.* (2013) 105:350–60. doi: 10.1093/jnci/djs529
97. Asgari MM, Ray GT, Quesenberry CP, Jr., Katz KA, Silverberg MJ. Association of multiple primary skin cancers with human immunodeficiency virus infection, CD4 count, and viral load. *JAMA Dermatol.* (2017) 153:892–6. doi: 10.1001/jamadermatol.2017.1716
98. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet.* (2007) 370:59–67. doi: 10.1016/S0140-6736(07)61050-2
99. Howley PM, Pfister HJ. Beta genus papillomaviruses and skin cancer. *Virology.* (2015) 479–480:290–6. doi: 10.1016/j.virol.2015.02.004
100. Iannacone MR, Gheit T, Pfister H, Giuliano AR, Messina JL, Fenske NA, et al. Case-control study of genus-beta human papillomaviruses in plucked eyebrow hairs and cutaneous squamous cell carcinoma. *Int J Cancer.* (2014) 134:2231–44. doi: 10.1002/ijc.28552
101. Iannacone MR, Gheit T, Waterboer T, Giuliano AR, Messina JL, Fenske NA, et al. Case-control study of cutaneous human papillomaviruses in squamous cell carcinoma of the skin. *Cancer Epidemiol Biomarkers Prev.* (2012) 21:1303–13. doi: 10.1158/1055-9965.EPI-12-0032
102. Faust H, Andersson K, Luostarinen T, Gislefoss RE, Dillner J. Cutaneous human papillomaviruses and squamous cell carcinoma of the skin: nested case-control study. *Cancer Epidemiol Biomarkers Prev.* (2016) 25:721–4. doi: 10.1158/1055-9965.EPI-15-1290
103. Weissenborn SJ, Nindl I, Purdie K, Harwood C, Proby C, Breuer J, et al. Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J Invest Dermatol.* (2005) 125:93–7. doi: 10.1111/j.0022-202X.2005.23733.x
104. Viarisio D, Muller-Decker K, Accardi R, Robitaille A, Durst M, Beer K, et al. Beta HPV38 oncoproteins act with a hit-and-run mechanism in ultraviolet radiation-induced skin carcinogenesis in mice. *PLoS Pathog.* (2018) 14:e1006783. doi: 10.1371/journal.ppat.1006783
105. Arron ST, Ruby JG, Dybbro E, Ganem D, Derisi JL. Transcriptome sequencing demonstrates that human papillomavirus is not active in cutaneous squamous cell carcinoma. *J Invest Dermatol.* (2011) 131:1745–53. doi: 10.1038/jid.2011.91
106. Nafz J, Kohler A, Ohnesorge M, Nindl I, Stockfleth E, Rosl F. Persistence of *Mastomys natalensis* papillomavirus in multiple organs identifies novel targets for infection. *J Gen Virol.* (2007) 88:2670–8. doi: 10.1099/vir.0.82955-0
107. Hasche D, Stephan S, Braspenning-Wesch I, Mikulec J, Niebler M, Grone HJ, et al. The interplay of UV and cutaneous papillomavirus infection in skin cancer development. *PLoS Pathog.* (2017) 13:e1006723. doi: 10.1371/journal.ppat.1006723
108. Andersson K, Waterboer T, Kirnbauer R, Slupetzky K, Iftner T, de Villiers EM, et al. Seroreactivity to cutaneous human papillomaviruses among patients with nonmelanoma skin cancer or benign skin lesions. *Cancer Epidemiol Biomarkers Prev.* (2008) 17:189–95. doi: 10.1158/1055-9965.EPI-07-0405
109. Wong MCS, Vlantis AC, Liang M, Wong PY, Ho WCS, Boon SS, et al. Prevalence and epidemiologic profile of oral infection with alpha, beta, and gamma papillomaviruses in an asian Chinese population. *J Infect Dis.* (2018) 218:388–97. doi: 10.1093/infdis/jiy160
110. Nunes EM, Lopez RVM, Sudenga SL, Gheit T, Tommasino M, Baggio ML, et al. Concordance of Beta-papillomavirus across anogenital and oral anatomic sites of men: the HIM Study. *Virology.* (2017) 510:55–9. doi: 10.1016/j.virol.2017.07.006
111. Hampras SS, Rollison DE, Giuliano AR, McKay-Chopin S, Minoni L, Sereday K, et al. Prevalence and concordance of cutaneous beta human papillomavirus infection at mucosal and cutaneous sites. *J Infect Dis.* (2017) 216:92–6. doi: 10.1093/infdis/jix245
112. Smelov V, Muwonge R, Sokolova O, McKay-Chopin S, Eklund C, Komyakov B, et al. Beta and gamma human papillomaviruses in anal and genital sites among men: prevalence and determinants. *Sci Rep.* (2018) 8:8241. doi: 10.1038/s41598-018-26589-w
113. Pierce Campbell CM, Messina JL, Stoler MH, Jukic DM, Tommasino M, Gheit T, et al. Cutaneous human papillomavirus types detected on the surface of male external genital lesions: a case series within the HPV Infection in Men Study. *J Clin Virol.* (2013) 58:652–9. doi: 10.1016/j.jcv.2013.10.011
114. ElAmrani A, Gheit T, Benhessou M, McKay-Chopin S, Attaleb M, Sahraoui S, et al. Prevalence of mucosal and cutaneous human papillomavirus in Moroccan breast cancer. *Papillomavirus Res.* (2018) 5:150–5. doi: 10.1016/j.pvr.2018.04.003
115. Chen AA, Gheit T, Stellin M, Lupato V, Spinato G, Fuson R, et al. Oncogenic DNA viruses found in salivary gland tumors. *Oral Oncol.* (2017) 75:106–10. doi: 10.1016/j.oraloncology.2017.11.005
116. Agalliu I, Chen Z, Wang T, Hayes RB, Freedman ND, Gapstur SM, et al. Oral alpha, beta, and gamma HPV types and risk of incident esophageal cancer. *Cancer Epidemiol Biomarkers Prev.* (2018) 27:1168–75. doi: 10.1158/1055-9965.EPI-18-0287
117. Agalliu I, Gapstur S, Chen Z, Wang T, Anderson RL, Teras L, et al. Associations of oral alpha-, beta-, and gamma-human papillomavirus types with risk of incident head and neck cancer. *JAMA Oncol.* (2016) 2:599–606. doi: 10.1001/jamaoncol.2015.5504
118. Sabol I, Smahelova J, Klozar J, Mravak-Stipetic M, Gheit T, Tommasino M, et al. Beta-HPV types in patients with head and neck pathology and in healthy subjects. *J Clin Virol.* (2016) 82:159–65. doi: 10.1016/j.jcv.2016.07.019
119. Forslund O, Johansson H, Madsen KG, Kofoed K. The nasal mucosa contains a large spectrum of human papillomavirus types from the Betapapillomavirus and Gammapapillomavirus genera. *J Infect Dis.* (2013) 208:1335–41. doi: 10.1093/infdis/jit326
120. Cornet I, Bouvard V, Campo MS, Thomas M, Banks L, Gissmann L, et al. Comparative analysis of transforming properties of E6 and E7 from different beta human papillomavirus types. *J Virol.* (2012) 86:2366–70. doi: 10.1128/JVI.06579-11
121. Viarisio D, Muller-Decker K, Zanna P, Kloz U, Aengeneyndt B, Accardi R, et al. Novel ss-HPV49 transgenic mouse model of upper digestive tract cancer. *Cancer Res.* (2016) 76:4216–25. doi: 10.1158/0008-5472.CAN-16-0370
122. Grace M, Munger K. Proteomic analysis of the gamma human papillomavirus type 197 E6 and E7 associated cellular proteins. *Virology.* (2017) 500:71–81. doi: 10.1016/j.virol.2016.10.010
123. Arroyo Muhr LS, Hultin E, Bzhalava D, Eklund C, Lagheden C, Ekstrom J, et al. Human papillomavirus type 197 is commonly present in skin tumors. *Int J Cancer.* (2015) 136:2546–55. doi: 10.1002/ijc.29325
124. Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. *Proc Natl Acad Sci USA.* (2009) 106:20458–63. doi: 10.1073/pnas.0908502106
125. Schiller JT, Day PM, Kines RC. Current understanding of the mechanism of HPV infection. *Gynecol Oncol.* (2010) 118(Suppl. 1):S12–7. doi: 10.1016/j.ygyno.2010.04.004
126. Herfs M, Yamamoto Y, Laury A, Wang X, Nucci MR, McLaughlin-Drubin ME, et al. A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. *Proc Natl Acad Sci USA.* (2012) 109:10516–21. doi: 10.1073/pnas.1202684109
127. Rautava J, Syrjanen S. Biology of human papillomavirus infections in head and neck carcinogenesis. *Head Neck Pathol.* (2012) 6 (Suppl. 1):S3–15. doi: 10.1007/s12105-012-0367-2
128. Broniarczyk J, Ring N, Massimi P, Giacca M, Banks L. HPV-16 virions can remain infectious for 2 weeks on senescent cells but require cell cycle re-activation to allow virus entry. *Sci Rep.* (2018) 8:811. doi: 10.1038/s41598-017-18809-6
129. Cerqueira C, Samperio Ventayol P, Vogeley C, Schelhaas M. Kallikrein-8 Proteolytically processes human papillomaviruses in the extracellular space to facilitate entry into host cells. *J Virol.* (2015) 89:7038–52. doi: 10.1128/JVI.00234-15
130. Culp TD, Budgeon LR, Marinkovich MP, Meneguzzi G, Christensen ND. Keratinocyte-secreted laminin 5 can function as a transient receptor for

human papillomaviruses by binding virions and transferring them to adjacent cells. *J Virol.* (2006) 80:8940–50. doi: 10.1128/JVI.00724-06
131. Bronnimann MP, Calton CM, Chiquette SF, Li S, Lu M, Chapman JA, et al. Furin cleavage of l2 during papillomavirus infection: minimal dependence on cyclophilins. *J Virol.* (2016) 90:6224–34. doi: 10.1128/JVI.00038-16
132. Rubio I, Seitz H, Canali E, Sehr P, Bolchi A, Tommasino M, et al. The N-terminal region of the human papillomavirus L2 protein contains overlapping binding sites for neutralizing, cross-neutralizing and non-neutralizing antibodies. *Virology.* (2011) 409:348–59. doi: 10.1016/j.virol.2010.10.017
133. Schelhaas M, Ewers H, Rajamaki ML, Day PM, Schiller JT, Helenius A. Human papillomavirus type 16 entry: retrograde cell surface transport along actin-rich protrusions. *PLoS Pathog.* (2008) 4:e1000148. doi: 10.1371/journal.ppat.1000148
134. Schelhaas M, Shah B, Holzer M, Blattmann P, Kuhling L, Day PM, et al. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. *PLoS Pathog.* (2012) 8:e1002657. doi: 10.1371/journal.ppat.1002657
135. Siddiqa A, Broniarczyk J, Banks L. Papillomaviruses and endocytic trafficking. *Int J Mol Sci*. (2018) 19:E2619 . doi: 10.3390/ijms19092619
136. Bienkowska-Haba M, Williams C, Kim SM, Garcea RL, Sapp M. Cyclophilins facilitate dissociation of the human papillomavirus type 16 capsid protein L1 from the L2/DNA complex following virus entry. *J Virol.* (2012) 86:9875–87. doi: 10.1128/JVI.00980-12
137. DiGiuseppe S, Bienkowska-Haba M, Guion LGM, Keiffer TR, Sapp M. Human papillomavirus major capsid protein L1 remains associated with the incoming viral genome throughout the entry process. *J Virol.* (2017) 91:e00537–17. doi: 10.1128/JVI.00537-17
138. Popa A, Zhang W, Harrison MS, Goodner K, Kazakov T, Goodwin EC, et al. Direct binding of retromer to human papillomavirus type 16 minor capsid protein L2 mediates endosome exit during viral infection. *PLoS Pathog.* (2015) 11:e1004699. doi: 10.1371/journal.ppat.1004699
139. McNally KE, Faulkner R, Steinberg F, Gallon M, Ghai R, Pim D, et al. Retriever is a multiprotein complex for retromer-independent endosomal cargo recycling. *Nat Cell Biol.* (2017) 19:1214–25. doi: 10.1038/ncb3610
140. Bergant Marusic M, Ozbun MA, Campos SK, Myers MP, Banks L. Human papillomavirus L2 facilitates viral escape from late endosomes via sorting nexin 17. *Traffic*. (2012) 13:455–67. doi: 10.1111/j.1600-0854.2011.01320.x
141. Bergant M, Peternel S, Pim D, Broniarczyk J, Banks L. Characterizing the spatio-temporal role of sorting nexin 17 in human papillomavirus trafficking. *J Gen Virol*. (2017) 98:715–25. doi: 10.1099/jgv.0.000734
142. Pim D, Broniarczyk J, Bergant M, Playford MP, Banks L. A Novel PDZ domain interaction mediates the binding between human papillomavirus 16 L2 and sorting nexin 27 and modulates virion trafficking. *J Virol.* (2015) 89:10145–55. doi: 10.1128/JVI.01499-15
143. DiGiuseppe S, Bienkowska-Haba M, Sapp M. Human papillomavirus entry: hiding in a bubble. *J Virol.* (2016) 90:8032–5. doi: 10.1128/JVI.01065-16
144. Pyeon D, Pearce SM, Lank SM, Ahlquist P, Lambert PF. Establishment of human papillomavirus infection requires cell cycle progression. *PLoS Pathog.* (2009) 5:e1000318. doi: 10.1371/journal.ppat.1000318
145. Aydin I, Weber S, Snijder B, Samperio Ventayol P, Kuhbacher A, Becker M, et al. Large scale RNAi reveals the requirement of nuclear envelope breakdown for nuclear import of human papillomaviruses. *PLoS Pathog.* (2014) 10:e1004162. doi: 10.1371/journal.ppat.1004162
146. Ascoli CA, Maul GG. Identification of a novel nuclear domain. *J Cell Biol.* (1991) 112:785–95. doi: 10.1083/jcb.112.5.785
147. Day PM, Baker CC, Lowy DR, Schiller JT. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proc Natl Acad Sci USA.* (2004) 101:14252–7. doi: 10.1073/pnas.0404229101
148. Tavalai N, Adler M, Scherer M, Riedl Y, Stamminger T. Evidence for a dual antiviral role of the major nuclear domain 10 component Sp100 during the immediate-early and late phases of the human cytomegalovirus replication cycle. *J Virol.* (2011) 85:9447–58. doi: 10.1128/JVI.00870-11
149. Stepp WH, Meyers JM, McBride AA. Sp100 provides intrinsic immunity against human papillomavirus infection. *mBio.* (2013) 4:e00845–13. doi: 10.1128/mBio.00845-13
150. Florin L, Schafer F, Sotlar K, Streeck RE, Sapp M. Reorganization of nuclear domain 10 induced by papillomavirus capsid protein l2. *Virology*. (2002) 295:97–107. doi: 10.1006/viro.2002.1360
151. Kivipold P, Vosa L, Ustav M, Kurg R. DAXX modulates human papillomavirus early gene expression and genome replication in U2OS cells. *Virol J.* (2015) 12:104. doi: 10.1186/s12985-015-0335-z
152. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, et al. The biology and life-cycle of human papillomaviruses. *Vaccine.* (2012) 30 (Suppl. 5):F55–70. doi: 10.1016/j.vaccine.2012.06.083
153. McKinney CC, Kim MJ, Chen D, McBride AA. Brd4 activates early viral transcription upon human papillomavirus 18 infection of primary keratinocytes. *mBio*. (2016) 7:e01644–16. doi: 10.1128/mBio.01644-16
154. Sanders CM, Stenlund A. Recruitment and loading of the E1 initiator protein: an ATP-dependent process catalysed by a transcription factor. *EMBO J*. (1998) 17:7044–55. doi: 10.1093/emboj/17.23.7044
155. Gauson EJ, Donaldson MM, Dornan ES, Wang X, Bristol M, Bodily JM, et al. Evidence supporting a role for TopBP1 and Brd4 in the initiation but not continuation of human papillomavirus 16 E1/E2-mediated DNA replication. *J Virol.* (2015) 89:4980–91. doi: 10.1128/JVI.00335-15
156. Dreer M, Fertey J, van de Poel S, Straub E, Madlung J, Macek B, et al. Interaction of NCOR/SMRT repressor complexes with papillomavirus E8^E2C proteins inhibits viral replication. *PLoS Pathog*. (2016) 12:e1005556. doi: 10.1371/journal.ppat.1005556
157. Dreer M, van de Poel S, Stubenrauch F. Control of viral replication and transcription by the papillomavirus E8^E2 protein. *Virus Res.* (2017) 231:96–102. doi: 10.1016/j.virusres.2016.11.005
158. Bastien N, McBride AA. Interaction of the papillomavirus E2 protein with mitotic chromosomes. *Virology*. (2000) 270:124–34. doi: 10.1006/viro.2000.0265
159. Jang MK, Shen K, McBride AA. Papillomavirus genomes associate with BRD4 to replicate at fragile sites in the host genome. *PLoS Pathog.* (2014) 10:e1004117. doi: 10.1371/journal.ppat.1004117
160. McBride AA, Jang MK. Current understanding of the role of the Brd4 protein in the papillomavirus lifecycle. *Viruses.* (2013) 5:1374–94. doi: 10.3390/v5061374
161. Abroi A, Ilves I, Kivi S, Ustav M. Analysis of chromatin attachment and partitioning functions of bovine papillomavirus type 1 E2 protein. *J Virol.* (2004) 78:2100–13. doi: 10.1128/JVI.78.4.2100-2113.2004
162. Rogers A, Waltke M, Angeletti PC. Evolutionary variation of papillomavirus E2 protein and E2 binding sites. *Virol J.* (2011) 8:379. doi: 10.1186/1743-422X-8-379
163. McPhillips MG, Oliveira JG, Spindler JE, Mitra R, McBride AA. Brd4 is required for e2-mediated transcriptional activation but not genome partitioning of all papillomaviruses. *J Virol.* (2006) 80:9530–43. doi: 10.1128/JVI.01105-06
164. Parish JL, Bean AM, Park RB, Androphy EJ. ChlR1 is required for loading papillomavirus E2 onto mitotic chromosomes and viral genome maintenance. *Mol Cell*. (2006) 24:867–76. doi: 10.1016/j.molcel.2006.11.005
165. Parish JL, Rosa J, Wang X, Lahti JM, Doxsey SJ, Androphy EJ. The DNA helicase ChlR1 is required for sister chromatid cohesion in mammalian cells. *J Cell Sci.* (2006) 119:4857–65. doi: 10.1242/jcs.03262
166. Harris L, McFarlane-Majeed L, Campos-Leon K, Roberts S, Parish JL. The cellular DNA helicase ChlR1 regulates chromatin and nuclear matrix attachment of the human papillomavirus 16 e2 protein and high-copy-number viral genome establishment. *J Virol.* (2017) 91:16. doi: 10.1128/JVI.01853-16
167. Bentley P, Tan MJA, McBride AA, White EA, Howley PM. The SMC5/6 complex interacts with the papillomavirus E2 protein and influences maintenance of viral episomal DNA. *J Virol.* (2018) 92:e00356–18. doi: 10.1128/JVI.00356-18
168. Thomas JT, Hubert WG, Ruesch MN, Laimins LA. Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc Natl Acad Sci USA.* (1999) 96:8449–54. doi: 10.1073/pnas.96.15.8449
169. Lorenz LD, Rivera Cardona J, Lambert PF. Inactivation of p53 rescues the maintenance of high risk HPV DNA genomes deficient in expression of E6. *PLoS Pathog.* (2013) 9:e1003717. doi: 10.1371/journal.ppat.1003717

170. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. *Nat Rev Cancer.* (2010) 10:550–60. doi: 10.1038/nrc2886
171. Maglennon GA, McIntosh P, Doorbar J. Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. *Virology*. (2011) 414:153–63. doi: 10.1016/j.virol.2011.03.019
172. Hummel M, Hudson JB, Laimins LA. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *J Virol.* (1992) 66:6070–80.
173. Fehrmann F, Klumpp DJ, Laimins LA. Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. *J Virol.* (2003) 77:2819–31. doi: 10.1128/JVI.77.5.2819-2831.2003
174. Longworth MS, Laimins LA. Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol Mol Biol Rev*. (2004) 68:362–72. doi: 10.1128/MMBR.68.2.362-372.2004
175. Khan J, Davy CE, McIntosh PB, Jackson DJ, Hinz S, Wang Q, et al. Role of calpain in the formation of human papillomavirus type 16 E1^E4 amyloid fibers and reorganization of the keratin network. *J Virol.* (2011) 85:9984–97. doi: 10.1128/JVI.02158-10
176. Pentland I, Campos-Leon K, Cotic M, Davies KJ, Wood CD, Groves IJ, et al. Disruption of CTCF-YY1-dependent looping of the human papillomavirus genome activates differentiation-induced viral oncogene transcription. *PLoS Biol*. (2018) 16:e2005752. doi: 10.1371/journal.pbio.2005752
177. Butz K, Ristriani T, Hengstermann A, Denk C, Scheffner M, Hoppe-Seyler F. siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells. *Oncogene*. (2003) 22:5938–45. doi: 10.1038/sj.onc.1206894
178. Fujii T, Saito M, Iwasaki E, Ochiya T, Takei Y, Hayashi S, et al. Intratumor injection of small interfering RNA-targeting human papillomavirus 18 E6 and E7 successfully inhibits the growth of cervical cancer. *Int J Oncol*. (2006) 29:541–8. doi: 10.3892/ijo.29.3.541
179. Howie HL, Katzenellenbogen RA, Galloway DA. Papillomavirus E6 proteins. *Virology*. (2009) 384:324–34. doi: 10.1016/j.virol.2008.11.017
180. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell.* (1993) 75:495–505. doi: 10.1016/0092-8674(93)90384-3
181. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell.* (1990) 63:1129–36. doi: 10.1016/0092-8674(90)90409-8
182. Huibregtse JM, Scheffner M, Howley PM. Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Mol Cell Biol.* (1993) 13:4918–27. doi: 10.1128/MCB.13.8.4918
183. Martinez-Zapien D, Ruiz FX, Poirson J, Mitschler A, Ramirez J, Forster A, et al. Structure of the E6/E6AP/p53 complex required for HPV-mediated degradation of p53. *Nature*. (2016) 529:541–5. doi: 10.1038/nature16481
184. Muschik D, Braspenning-Wesch I, Stockfleth E, Rosl F, Hofmann TG, Nindl I. Cutaneous HPV23 E6 prevents p53 phosphorylation through interaction with HIPK2. *PLoS ONE*. (2011) 6:e27655. doi: 10.1371/journal.pone.0027655
185. Pim D, Storey A, Thomas M, Massimi P, Banks L. Mutational analysis of HPV-18 E6 identifies domains required for p53 degradation *in vitro*, abolition of p53 transactivation *in vivo* and immortalisation of primary BMK cells. *Oncogene*. (1994) 9:1869–76.
186. Zimmermann H, Degenkolbe R, Bernard HU, O'Connor MJ. The human papillomavirus type 16 E6 oncoprotein can down-regulate p53 activity by targeting the transcriptional coactivator CBP/p300. *J Virol.* (1999) 73:6209–19.
187. Thomas MC, Chiang CM. E6 oncoprotein represses p53-dependent gene activation via inhibition of protein acetylation independently of inducing p53 degradation. *Mol Cell.* (2005) 17:251–64. doi: 10.1016/j.molcel.2004.12.016
188. Howie HL, Koop JI, Weese J, Robinson K, Wipf G, Kim L, et al. Beta-HPV 5 and 8 E6 promote p300 degradation by blocking AKT/p300 association. *PLoS Pathog.* (2011) 7:e1002211. doi: 10.1371/journal.ppat.1002211
189. Wallace NA, Robinson K, Howie HL, Galloway DA. HPV 5 and 8 E6 abrogate ATR activity resulting in increased persistence of UVB induced DNA damage. *PLoS Pathog.* (2012) 8:e1002807. doi: 10.1371/journal.ppat.1002807
190. Hufbauer M, Cooke J, van der Horst GT, Pfister H, Storey A, Akgul B. Human papillomavirus mediated inhibition of DNA damage sensing and repair drives skin carcinogenesis. *Mol Cancer.* (2015) 14:183. doi: 10.1186/s12943-015-0453-7
191. Marthaler AM, Podgorska M, Feld P, Fingerle A, Knerr-Rupp K, Grasser F, et al. Identification of C/EBPalpha as a novel target of the HPV8 E6 protein regulating miR-203 in human keratinocytes. *PLoS Pathog.* (2017) 13:e1006406. doi: 10.1371/journal.ppat.1006406
192. Muench P, Probst S, Schuetz J, Leiprecht N, Busch M, Wesselborg S, et al. Cutaneous papillomavirus E6 proteins must interact with p300 and block p53-mediated apoptosis for cellular immortalization and tumorigenesis. *Cancer Res.* (2010) 70:6913–24. doi: 10.1158/0008-5472.CAN-10-1307
193. Thomas M, Banks L. Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. *J Gen Virol.* (1999) 80:1513–7. doi: 10.1099/0022-1317-80-6-1513
194. Borbely AA, Murvai M, Konya J, Beck Z, Gergely L, Li F, et al. Effects of human papillomavirus type 16 oncoproteins on survivin gene expression. *J Gen Virol.* (2006) 87:287–94. doi: 10.1099/vir.0.81067-0
195. Filippova M, Song H, Connolly JL, Dermody TS, Duerksen-Hughes PJ. The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. *J Biol Chem.* (2002) 277:21730–9. doi: 10.1074/jbc.M200113200
196. Filippova M, Parkhurst L, Duerksen-Hughes PJ. The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. *J Biol Chem.* (2004) 279:25729–44. doi: 10.1074/jbc.M401172200
197. Filippova M, Johnson MM, Bautista M, Filippov V, Fodor N, Tungteakkhun SS, et al. The large and small isoforms of human papillomavirus type 16 E6 bind to and differentially affect procaspase 8 stability and activity. *J Virol.* (2007) 81:4116–29. doi: 10.1128/JVI.01924-06
198. Bedard KM, Underbrink MP, Howie HL, Galloway DA. The E6 oncoproteins from human betapapillomaviruses differentially activate telomerase through an E6AP-dependent mechanism and prolong the lifespan of primary keratinocytes. *J Virol.* (2008) 82:3894–902. doi: 10.1128/JVI.01818-07
199. Simmonds M, Storey A. Identification of the regions of the HPV 5 E6 protein involved in Bak degradation and inhibition of apoptosis. *Int J Cancer.* (2008) 123:2260–6. doi: 10.1002/ijc.23815
200. Holloway A, Simmonds M, Azad A, Fox JL, Storey A. Resistance to UV-induced apoptosis by beta-HPV5 E6 involves targeting of activated BAK for proteolysis by recruitment of the HERC1 ubiquitin ligase. *Int J Cancer*. (2015) 136:2831–43. doi: 10.1002/ijc.29350
201. Leverrier S, Bergamaschi D, Ghali L, Ola A, Warnes G, Akgul B, et al. Role of HPV E6 proteins in preventing UVB-induced release of pro-apoptotic factors from the mitochondria. *Apoptosis*. (2007) 12:549–60. doi: 10.1007/s10495-006-0004-1
202. Wallace NA, Robinson K, Howie HL, Galloway DA. beta-HPV 5 and 8 E6 disrupt homology dependent double strand break repair by attenuating BRCA1 and BRCA2 expression and foci formation. *PLoS Pathog.* (2015) 11:e1004687. doi: 10.1371/journal.ppat.1004687
203. Schwarz T, Schwarz A. DNA repair and cytokine responses. *J Invest Dermatol Symp Proc*. (2009) 14:63–6. doi: 10.1038/jidsymp.2009.3
204. Javier RT. Cell polarity proteins: common targets for tumorigenic human viruses. *Oncogene.* (2008) 27:7031–46. doi: 10.1038/onc.2008.352
205. Songyang Z, Fanning AS, Fu C, Xu J, Marfatia SM, Chishti AH, et al. Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science.* (1997) 275:73–7. doi: 10.1126/science.275.5296.73
206. Kuhne C, Gardiol D, Guarnaccia C, Amenitsch H, Banks L. Differential regulation of human papillomavirus E6 by protein kinase A: conditional degradation of human discs large protein by oncogenic E6. *Oncogene*. (2000) 19:5884–91. doi: 10.1038/sj.onc.1203988
207. Zhang Y, Dasgupta J, Ma RZ, Banks L, Thomas M, Chen XS. Structures of a human papillomavirus (HPV) E6 polypeptide bound to MAGUK proteins: mechanisms of targeting tumor suppressors by a high-risk HPV oncoprotein. *J Virol.* (2007) 81:3618–26. doi: 10.1128/JVI.02044-06
208. Boon SS, Banks L. High-risk human papillomavirus E6 oncoproteins interact with 14-3-3zeta in a PDZ binding motif-dependent manner. *J Virol.* (2013) 87:1586–95. doi: 10.1128/JVI.02074-12

209. Nakagawa S, Huibregtse JM. Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol Cell Biol.* (2000) 20:8244–53. doi: 10.1128/MCB.20.21.8244-8253.2000
210. Gardiol D, Kuhne C, Glaunsinger B, Lee SS, Javier R, Banks L. Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. *Oncogene.* (1999) 18:5487–96. doi: 10.1038/sj.onc.1202920
211. Kranjec C, Tomaic V, Massimi P, Nicolaides L, Doorbar J, Banks L. The high-risk HPV E6 target scribble (hScrib) is required for HPV E6 expression in cervical tumour-derived cell lines. *Papillomavirus Res.* (2016) 2:70–7. doi: 10.1016/j.pvr.2016.04.001
212. Kapil S, Sharma BK, Patil M, Elattar S, Yuan J, Hou SX, et al. The cell polarity protein Scrib functions as a tumor suppressor in liver cancer. *Oncotarget.* (2017) 8:26515–31. doi: 10.18632/oncotarget.15713
213. Kranjec C, Banks L. A systematic analysis of human papillomavirus (HPV) E6 PDZ substrates identifies MAGI-1 as a major target of HPV type 16 (HPV-16) and HPV-18 whose loss accompanies disruption of tight junctions. *J Virol.* (2011) 85:1757–64. doi: 10.1128/JVI.01756-10
214. Dobrosotskaya IY, James GL. MAGI-1 interacts with beta-catenin and is associated with cell-cell adhesion structures. *Biochem Biophys Res Commun.* (2000) 270:903–9. doi: 10.1006/bbrc.2000.2471
215. Kotelevets L, van Hengel J, Bruyneel E, Mareel M, van Roy F, Chastre E. Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. *FASEB J.* (2005) 19:115–7. doi: 10.1096/fj.04-1942fje
216. Jeong KW, Kim HZ, Kim S, Kim YS, Choe J. Human papillomavirus type 16 E6 protein interacts with cystic fibrosis transmembrane regulator-associated ligand and promotes E6-associated protein-mediated ubiquitination and proteasomal degradation. *Oncogene.* (2007) 26:487–99. doi: 10.1038/sj.onc.1209837
217. Lee SS, Glaunsinger B, Mantovani F, Banks L, Javier RT. Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. *J Virol.* (2000) 74:9680–93. doi: 10.1128/JVI.74.20.9680-9693.2000
218. Handa K, Yugawa T, Narisawa-Saito M, Ohno S, Fujita M, Kiyono T. E6AP-dependent degradation of DLG4/PSD95 by high-risk human papillomavirus type 18 E6 protein. *J Virol.* (2007) 81:1379–89. doi: 10.1128/JVI.01712-06
219. Favre-Bonvin A, Reynaud C, Kretz-Remy C, Jalinot P. Human papillomavirus type 18 E6 protein binds the cellular PDZ protein TIP-2/GIPC, which is involved in transforming growth factor beta signaling and triggers its degradation by the proteasome. *J Virol.* (2005) 79:4229–37. doi: 10.1128/JVI.79.7.4229-4237.2005
220. Accardi R, Rubino R, Scalise M, Gheit T, Shahzad N, Thomas M, et al. E6 and E7 from human papillomavirus type 16 cooperate to target the PDZ protein Na/H exchange regulatory factor 1. *J Virol.* (2011) 85:8208–16. doi: 10.1128/JVI.00114-11
221. Lee C, Laimins LA. Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. *J Virol.* (2004) 78:12366–77. doi: 10.1128/JVI.78.22.12366-12377.2004
222. Marx B, Miller-Lazic D, Doorbar J, Majewski S, Hofmann K, Hufbauer M, et al. HPV8-E6 Interferes with Syntenin-2 expression through deregulation of differentiation, methylation and phosphatidylinositide-kinase dependent mechanisms. *Front Microbiol.* (2017) 8:1724. doi: 10.3389/fmicb.2017.01724
223. Thomas M, Myers MP, Massimi P, Guarnaccia C, Banks L. Analysis of multiple HPV E6 PDZ interactions defines type-specific PDZ fingerprints that predict oncogenic potential. *PLoS Pathog.* (2016) 12:e1005766. doi: 10.1371/journal.ppat.1005766
224. Branca M, Giorgi C, Ciotti M, Santini D, Di Bonito L, Costa S, et al. Upregulation of telomerase (hTERT) is related to the grade of cervical intraepithelial neoplasia, but is not an independent predictor of high-risk human papillomavirus, virus persistence, or disease outcome in cervical cancer. *Diag Cytopathol.* (2006) 34:739–48. doi: 10.1002/dc.20554
225. Van Doorslaer K, Burk RD. Association between hTERT activation by HPV E6 proteins and oncogenic risk. *Virology.* (2012) 433:216–9. doi: 10.1016/j.virol.2012.08.006
226. Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein composition of catalytically active human telomerase from immortal cells. *Science.* (2007) 315:1850–3. doi: 10.1126/science.1138596
227. Wolyniec K, Shortt J, de Stanchina E, Levav-Cohen Y, Alsheich-Bartok O, Louria-Hayon I, et al. E6AP ubiquitin ligase regulates PML-induced senescence in Myc-driven lymphomagenesis. *Blood.* (2012) 120:822–32. doi: 10.1182/blood-2011-10-387647
228. James MA, Lee JH, Klingelhutz AJ. HPV16-E6 associated hTERT promoter acetylation is E6AP dependent, increased in later passage cells and enhanced by loss of p300. *Int J Cancer.* (2006) 119:1878–85. doi: 10.1002/ijc.22064
229. Lebel R, McDuff FO, Lavigne P, Grandbois M. Direct visualization of the binding of c-Myc/Max heterodimeric b-HLH-LZ to E-box sequences on the hTERT promoter. *Biochemistry.* (2007) 46:10279–86. doi: 10.1021/bi700076m
230. Xu M, Luo W, Elzi DJ, Grandori C, Galloway DA. NFX1 interacts with mSin3A/histone deacetylase to repress hTERT transcription in keratinocytes. *Mol Cell Biol.* (2008) 28:4819–28. doi: 10.1128/MCB.01969-07
231. Katzenellenbogen RA, Egelkrout EM, Vliet-Gregg P, Gewin LC, Gafken PR, Galloway DA. NFX1-123 and poly(A) binding proteins synergistically augment activation of telomerase in human papillomavirus type 16 E6-expressing cells. *J Virol.* (2007) 81:3786–96. doi: 10.1128/JVI.02007-06
232. Mortensen F, Schneider D, Barbic T, Sladewska-Marquardt A, Kuhnle S, Marx A, et al. Role of ubiquitin and the HPV E6 oncoprotein in E6AP-mediated ubiquitination. *Proc Natl Acad Sci USA.* (2015) 112:9872–7. doi: 10.1073/pnas.1505923112
233. Brimer N, Drews CM, Vande Pol SB. Association of papillomavirus E6 proteins with either MAML1 or E6AP clusters E6 proteins by structure, function, and evolutionary relatedness. *PLoS Pathog.* (2017) 13:e1006781. doi: 10.1371/journal.ppat.1006781
234. Brimer N, Lyons C, Wallberg AE, Vande Pol SB. Cutaneous papillomavirus E6 oncoproteins associate with MAML1 to repress transactivation and NOTCH signaling. *Oncogene.* (2012) 31:4639–46. doi: 10.1038/onc.2011.589
235. Meyers JM, Spangle JM, Munger K. The human papillomavirus type 8 E6 protein interferes with NOTCH activation during keratinocyte differentiation. *J Virol.* (2013) 87:4762–7. doi: 10.1128/JVI.02527-12
236. White EA, Howley PM. Proteomic approaches to the study of papillomavirus-host interactions. *Virology.* (2013) 435:57–69. doi: 10.1016/j.virol.2012.09.046
237. Knapp AA, McManus PM, Bockstall K, Moroianu J. Identification of the nuclear localization and export signals of high risk HPV16 E7 oncoprotein. *Virology.* (2009) 383:60–8. doi: 10.1016/j.virol.2008.09.037
238. Huh KW, DeMasi J, Ogawa H, Nakatani Y, Howley PM, Munger K. Association of the human papillomavirus type 16 E7 oncoprotein with the 600-kDa retinoblastoma protein-associated factor, p600. *Proc Natl Acad Sci USA.* (2005) 102:11492–7. doi: 10.1073/pnas.0505337102
239. Nakatani Y, Konishi H, Vassilev A, Kurooka H, Ishiguro K, Sawada J, et al. p600, a unique protein required for membrane morphogenesis and cell survival. *Proc Natl Acad Sci USA.* (2005) 102:15093–8. doi: 10.1073/pnas.0507458102
240. DeMasi J, Chao MC, Kumar AS, Howley PM. Bovine papillomavirus E7 oncoprotein inhibits anoikis. *J Virol.* (2007) 81:9419–25. doi: 10.1128/JVI.00422-07
241. Szalmas A, Tomaic V, Basukala O, Massimi P, Mittal S, Konya J, et al. The PTPN14 tumor suppressor is a degradation target of human papillomavirus E7. *J Virol.* (2017) 91:e00057–17. doi: 10.1128/JVI.00057-17
242. White EA, Munger K, Howley PM. High-risk human papillomavirus E7 proteins target PTPN14 for degradation. *mBio.* (2016) 7:16. doi: 10.1128/mBio.01530-16
243. Avvakumov N, Torchia J, Mymryk JS. Interaction of the HPV E7 proteins with the pCAF acetyltransferase. *Oncogene.* (2003) 22:3833–41. doi: 10.1038/sj.onc.1206562
244. Jansma AL, Martinez-Yamout MA, Liao R, Sun P, Dyson HJ, Wright PE. The high-risk HPV16 E7 oncoprotein mediates interaction between the transcriptional coactivator CBP and the retinoblastoma protein pRb. *J Mol Biol.* (2014) 426:4030–48. doi: 10.1016/j.jmb.2014.10.021
245. Massimi P, Pim D, Storey A, Banks L. HPV-16 E7 and adenovirus E1a complex formation with TATA box binding protein is enhanced by casein kinase II phosphorylation. *Oncogene.* (1996) 12:2325–30.

246. Barbosa MS, Edmonds C, Fisher C, Schiller JT, Lowy DR, Vousden KH. The region of the HPV E7 oncoprotein homologous to adenovirus E1a and Sv40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *EMBO J*. (1990) 9:153–60. doi: 10.1002/j.1460-2075.1990.tb08091.x
247. Zine El Abidine A, Tomaic V, Bel Haj Rhouma R, Massimi P, Guizani I, Boubaker S, et al. A naturally occurring variant of HPV-16 E7 exerts increased transforming activity through acquisition of an additional phospho-acceptor site. *Virology*. (2017) 500:218–25. doi: 10.1016/j.virol.2016.10.023
248. Munger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J*. (1989) 8:4099–105. doi: 10.1002/j.1460-2075.1989.tb08594.x
249. Todorovic B, Hung K, Massimi P, Avvakumov N, Dick FA, Shaw GS, et al. Conserved region 3 of human papillomavirus 16 E7 contributes to deregulation of the retinoblastoma tumor suppressor. *J Virol*. (2012) 86:13313–23. doi: 10.1128/JVI.01637-12
250. Huh K, Zhou X, Hayakawa H, Cho JY, Libermann TA, Jin J, et al. Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor. *J Virol*. (2007) 81:9737–47. doi: 10.1128/JVI.00881-07
251. Chellappan S, Kraus VB, Kroger B, Munger K, Howley PM, Phelps WC, et al. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. *Proc Natl Acad Sci USA*. (1992) 89:4549–53. doi: 10.1073/pnas.89.10.4549
252. Roman A, Munger K. The papillomavirus E7 proteins. *Virology*. (2013) 445:138–68. doi: 10.1016/j.virol.2013.04.013
253. Banks L, Edmonds C, Vousden KH. Ability of the HPV16 E7 protein to bind RB and induce DNA synthesis is not sufficient for efficient transforming activity in NIH3T3 cells. *Oncogene*. (1990) 5:1383–9.
254. Barrow-Laing L, Chen W, Roman A. Low- and high-risk human papillomavirus E7 proteins regulate p130 differently. *Virology*. (2010) 400:233–9. doi: 10.1016/j.virol.2010.01.034
255. McLaughlin-Drubin ME, Huh KW, Munger K. Human papillomavirus type 16 E7 oncoprotein associates with E2F6. *J Virol*. (2008) 82:8695–705. doi: 10.1128/JVI.00579-08
256. Akgul B, Garcia-Escudero R, Ghali L, Pfister HJ, Fuchs PG, Navsaria H, et al. The E7 protein of cutaneous human papillomavirus type 8 causes invasion of human keratinocytes into the dermis in organotypic cultures of skin. *Cancer Res*. (2005) 65:2216–23. doi: 10.1158/0008-5472.CAN-04-1952
257. Heuser S, Hufbauer M, Steiger J, Marshall J, Sterner-Kock A, Mauch C, et al. The fibronectin/alpha3beta1 integrin axis serves as molecular basis for keratinocyte invasion induced by betaHPV. *Oncogene*. (2016) 35:4529–39. doi: 10.1038/onc.2015.512
258. Hufbauer M, Biddle A, Borgogna C, Gariglio M, Doorbar J, Storey A, et al. Expression of betapapillomavirus oncogenes increases the number of keratinocytes with stem cell-like properties. *J Virol*. (2013) 87:12158–65. doi: 10.1128/JVI.01510-13
259. Lanfredini S, Olivero C, Borgogna C, Calati F, Powell K, Davies KJ, et al. HPV8 field cancerization in a transgenic mouse model is due to Lrig1+ keratinocyte stem cell expansion. *J Invest Dermatol*. (2017) 137:2208–16. doi: 10.1016/j.jid.2017.04.039
260. Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G, et al. miR-203 represses 'stemness' by repressing DeltaNp63. *Cell Death Different*. (2008) 15:1187–95. doi: 10.1038/cdd.2008.69
261. Yi R, Poy MN, Stoffel M, Fuchs E. A skin microRNA promotes differentiation by repressing 'stemness'. *Nature*. (2008) 452:225–9. doi: 10.1038/nature06642
262. Shin MK, Balsitis S, Brake T, Lambert PF. Human papillomavirus E7 oncoprotein overrides the tumor suppressor activity of p21Cip1 in cervical carcinogenesis. *Cancer Res*. (2009) 69:5656–63. doi: 10.1158/0008-5472.CAN-08-3711
263. Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev*. (1997) 11:2090–100. doi: 10.1101/gad.11.16.2090
264. Jones DL, Alani RM, Munger K. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev*. (1997) 11:2101–11. doi: 10.1101/gad.11.16.2101
265. Akgul B, Ghali L, Davies D, Pfister H, Leigh IM, Storey A. HPV8 early genes modulate differentiation and cell cycle of primary human adult keratinocytes. *Exp Dermatol*. (2007) 16:590–9. doi: 10.1111/j.1600-0625.2007.00569.x
266. Schmitt A, Harry JB, Rapp B, Wettstein FO, Iftner T. Comparison of the properties of the E6 and E7 genes of low- and high-risk cutaneous papillomaviruses reveals strongly transforming and high Rb-binding activity for the E7 protein of the low-risk human papillomavirus type 1. *J Virol*. (1994) 68:7051–9.
267. Accardi R, Dong W, Smet A, Cui R, Hautefeuille A, Gabet AS, et al. Skin human papillomavirus type 38 alters p53 functions by accumulation of deltaNp73. *EMBO Rep*. (2006) 7:334–40. doi: 10.1038/sj.embor.7400615
268. Accardi R, Scalise M, Gheit T, Hussain I, Yue J, Carreira C, et al. IkappaB kinase beta promotes cell survival by antagonizing p53 functions through DeltaNp73alpha phosphorylation and stabilization. *Mol Cell Biol*. (2011) 31:2210–26. doi: 10.1128/MCB.00964-10
269. Wetherill LF, Holmes KK, Verow M, Muller M, Howell G, Harris M, et al. High-risk human papillomavirus E5 oncoprotein displays channel-forming activity sensitive to small-molecule inhibitors. *J Virol*. (2012) 86:5341–51. doi: 10.1128/JVI.06243-11
270. Oetke C, Auvinen E, Pawlita M, Alonso A. Human papillomavirus type 16 E5 protein localizes to the Golgi apparatus but does not grossly affect cellular glycosylation. *Arch Virol*. (2000) 145:2183–91. doi: 10.1007/s007050070048
271. Hu L, Ceresa BP. Characterization of the plasma membrane localization and orientation of HPV16 E5 for cell-cell fusion. *Virology*. (2009) 393:135–43. doi: 10.1016/j.virol.2009.07.034
272. Wetherill LF, Wasson CW, Swinscoe G, Kealy D, Foster R, Griffin S, et al. Alkyl-imino sugars inhibit the pro-oncogenic ion channel function of human papillomavirus (HPV) E5. *Antiviral Res*. (2018) 158:113–21. doi: 10.1016/j.antiviral.2018.08.005
273. Stoppler MC, Straight SW, Tsao G, Schlegel R, McCance DJ. The E5 gene of HPV-16 enhances keratinocyte immortalization by full-length DNA. *Virology*. (1996) 223:251–4. doi: 10.1006/viro.1996.0475
274. Barbaresi S, Cortese MS, Quinn J, Ashrafi GH, Graham SV, Campo MS. Effects of human papillomavirus type 16 E5 deletion mutants on epithelial morphology: functional characterization of each transmembrane domain. *J Gen Virol*. (2010) 91:521–30. doi: 10.1099/vir.0.016295-0
275. Genther Williams SM, Disbrow GL, Schlegel R, Lee D, Threadgill DW, Lambert PF. Requirement of epidermal growth factor receptor for hyperplasia induced by E5, a high-risk human papillomavirus oncogene. *Cancer Res*. (2005) 65:6534–42. doi: 10.1158/0008-5472.CAN-05-0083
276. Kim SH, Oh JM, No JH, Bang YJ, Juhnn YS, Song YS. Involvement of NF-kappaB and AP-1 in COX-2 upregulation by human papillomavirus 16 E5 oncoprotein. *Carcinogenesis*. (2009) 30:753–7. doi: 10.1093/carcin/bgp066
277. Scott ML, Coleman DT, Kelly KC, Carroll JL, Woodby B, Songock WK, et al. Human papillomavirus type 16 E5-mediated upregulation of Met in human keratinocytes. *Virology*. (2018) 519:1–11. doi: 10.1016/j.virol.2018.03.021
278. Pedroza-Saavedra A, Lam EW, Esquivel-Guadarrama F, Gutierrez-Xicotencatl L. The human papillomavirus type 16 E5 oncoprotein synergizes with EGF-receptor signaling to enhance cell cycle progression and the down-regulation of p27(Kip1). *Virology*. (2010) 400:44–52. doi: 10.1016/j.virol.2010.01.009
279. Liao S, Deng D, Hu X, Wang W, Li L, Li W, et al. HPV16/18 E5, a promising candidate for cervical cancer vaccines, affects SCPs, cell proliferation and cell cycle, and forms a potential network with E6 and E7. *Int J Mol Med*. (2013) 31:120–8. doi: 10.3892/ijmm.2012.1168
280. Kabsch K, Mossadegh N, Kohl A, Komposch G, Schenkel J, Alonso A, et al. The HPV-16 E5 protein inhibits TRAIL- and FasL-mediated apoptosis in human keratinocyte raft cultures. *Intervirology*. (2004) 47:48–56. doi: 10.1159/000076642
281. Liu C, Lin J, Li L, Zhang Y, Chen W, Cao Z, et al. HPV16 early gene E5 specifically reduces miRNA-196a in cervical cancer cells. *Sci Rep*. (2015) 5:7653. doi: 10.1038/srep07653

282. Oh JM, Kim SH, Cho EA, Song YS, Kim WH, Juhnn YS. Human papillomavirus type 16 E5 protein inhibits hydrogen-peroxide-induced apoptosis by stimulating ubiquitin-proteasome-mediated degradation of Bax in human cervical cancer cells. *Carcinogenesis.* (2010) 31:402–10. doi: 10.1093/carcin/bgp318
283. Zhang B, Spandau DF, Roman A. E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis. *J Virol.* (2002) 76:220–31. doi: 10.1128/JVI.76.1.220-231.2002
284. Valle GF, Banks L. The human papillomavirus (HPV)-6 and HPV-16 E5 proteins co-operate with HPV-16 E7 in the transformation of primary rodent cells. *J Gen Virol.* (1995) 76:1239–45. doi: 10.1099/0022-1317-76-5-1239
285. Lazarczyk M, Pons C, Mendoza JA, Cassonnet P, Jacob Y, Favre M. Regulation of cellular zinc balance as a potential mechanism of EVER-mediated protection against pathogenesis by cutaneous oncogenic human papillomaviruses. *J Exp Med.* (2008) 205:35–42. doi: 10.1084/jem.20071311
286. Kalinska-Bienias A, Kowalewski C, Majewski S. The EVER genes - the genetic etiology of carcinogenesis in epidermodysplasia verruciformis and a possible role in non-epidermodysplasia verruciformis patients. *Postepy Dermatol Alergol.* (2016) 33:75–80. doi: 10.5114/ada.2016.59145
287. de Jong SJ, Crequer A, Matos I, Hum D, Gunasekharan V, Lorenzo L, et al. The human CIB1-EVER1-EVER2 complex governs keratinocyte-intrinsic immunity to beta-papillomaviruses. *J Exp Med.* (2018) 215:2289–310. doi: 10.1084/jem.20170308
288. Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. *J Clin Oncol.* (2008) 26:612–9. doi: 10.1200/JCO.2007.14.1713
289. Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer.* (2009) 125:362–6. doi: 10.1002/ijc.24339
290. Hong AM, Grulich AE, Jones D, Lee CS, Garland SM, Dobbins TA, et al. Squamous cell carcinoma of the oropharynx in Australian males induced by human papillomavirus vaccine targets. *Vaccine.* (2010) 28:3269–72. doi: 10.1016/j.vaccine.2010.02.098
291. Kreimer AR, Johansson M, Waterboer T, Kaaks R, Chang-Claude J, Drogen D, et al. Evaluation of human papillomavirus antibodies and risk of subsequent head and neck cancer. *J Clin Oncol.* (2013) 31:2708–15. doi: 10.1200/JCO.2012.47.2738
292. Kreimer AR, Johansson M, Yanik EL, Katki HA, Check DP, Lang Kuhs KA, et al. Kinetics of the human papillomavirus type 16 E6 antibody response prior to oropharyngeal cancer. *J Natl Cancer Inst.* (2017) 109:djx005 . doi: 10.1093/jnci/djx005
293. Harper DM, DeMars LR. HPV vaccines - a review of the first decade. *Gynecol Oncol.* (2017) 146:196–204. doi: 10.1016/j.ygyno.2017.04.004
294. Guo T, Eisele DW, Fakhry C. The potential impact of prophylactic human papillomavirus vaccination on oropharyngeal cancer. *Cancer.* (2016) 122:2313–23. doi: 10.1002/cncr.29992
295. Vici P, Pizzuti L, Mariani L, Zampa G, Santini D, Di Lauro L, et al. Targeting immune response with therapeutic vaccines in premalignant lesions and cervical cancer: hope or reality from clinical studies. *Exp Rev Vaccines.* (2016) 15:1327–36. doi: 10.1080/14760584.2016.1176533
296. Yang A, Farmer E, Wu TC, Hung CF. Perspectives for therapeutic HPV vaccine development. *J Biomed Sci.* (2016) 23:75. doi: 10.1186/s12929-016-0293-9
297. Kim HJ, Kim HJ. Current status and future prospects for human papillomavirus vaccines. *Arch Pharmacal Res.* (2017) 40:1050–63. doi: 10.1007/s12272-017-0952-8
298. Hefferon K. Plant virus expression vectors: a powerhouse for global health. *Biomedicines.* (2017) 5:E44 . doi: 10.3390/biomedicines5030044
299. Chabeda A, Yanez RJR, Lamprecht R, Meyers AE, Rybicki EP, Hitzeroth, II. Therapeutic vaccines for high-risk HPV-associated diseases. *Papillomavirus Res.* (2018) 5:46–58. doi: 10.1016/j.pvr.2017.12.006
300. Gray P, Luostarinen T, Vanska S, Eriksson T, Lagheden C, Man I, et al. Occurrence of human papillomavirus (HPV) type replacement by sexual risk-taking behaviour group: *Post-hoc* analysis of a community randomized clinical trial up to 9 years after vaccination (IV). *Int J Cancer.* (2019). doi: 10.1002/ijc.32189. [Epub ahead of print].
301. Gray P, Palmroth J, Luostarinen T, Apter D, Dubin G, Garnett G, et al. Evaluation of HPV type-replacement in unvaccinated and vaccinated adolescent females-*Post-hoc* analysis of a community-randomized clinical trial (II). *Int J Cancer.* (2018) 142:2491–500. doi: 10.1002/ijc.31281
302. Klein C, Gonzalez D, Samwel K, Kahesa C, Mwaiselage J, Aluthge N, et al. Relationship between the cervical microbiome, HIV status, and precancerous lesions. *mBio.* (2019) 10 10:e02785–18. doi: 10.1128/mBio.02785-18
303. Bouwes Bavinck JN, Feltkamp MCW, Green AC, Fiocco M, Euvrard S, Harwood CA, et al. Human papillomavirus and posttransplantation cutaneous squamous cell carcinoma: a multicenter, prospective cohort study. *Am J Transl.* (2018) 18:1220–30. doi: 10.1111/ajt.14537
304. Huber B, Schellenbacher C, Shafti-Keramat S, Jindra C, Christensen N, Kirnbauer R. Chimeric L2-based virus-like particle (VLP) vaccines targeting cutaneous human papillomaviruses (HPV). *PLoS ONE.* (2017) 12:e0169533. doi: 10.1371/journal.pone.0169533
305. Vinzon SE, Braspenning-Wesch I, Muller M, Geissler EK, Nindl I, Grone HJ, et al. Protective vaccination against papillomavirus-induced skin tumors under immunocompetent and immunosuppressive conditions: a preclinical study using a natural outbred animal model. *PLoS Pathog.* (2014) 10:e1003924. doi: 10.1371/journal.ppat.1003924
306. Ostrbenk A, Kocjan BJ, Hosnjak L, Li J, Deng Q, Sterbenc A, et al. Identification of a Novel human papillomavirus, type HPV199, isolated from a nasopharynx and anal canal, and complete genomic characterization of papillomavirus species gamma-12. *PLoS ONE.* (2015) 10:e0138628. doi: 10.1371/journal.pone.0138628

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(-)-Epigallocatechin-3-gallate inhibits human papillomavirus (HPV)-16 oncoprotein-induced angiogenesis in non-small cell lung cancer cells by targeting HIF-1α

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Abstract

PURPOSE: To investigate the effects of (-)-epigallocatechin-3-gallate (EGCG) on human papillomavirus (HPV)-16 oncoprotein-induced angiogenesis in non-small cell lung **cancer** (NSCLC) cells and the underlying mechanisms.

METHODS: NSCLC cells (A549 and NCI-H460) transfected with EGFP plasmids containing HPV-16 E6 or E7 oncogene were treated with different concentrations of EGCG for 16 h. The effects of EGCG on angiogenesis in vitro and in vivo were observed. The expression of HIF-1α, p-Akt, and p-ERK1/2 proteins in NSCLC cells was analyzed by Western blot. The levels of HIF-1α mRNA in NSCLC cells were detected by real-time RT-PCR. The concentration of VEGF and **IL-8** in the conditioned media was determined by ELISA. HIF-1α, VEGF, and CD31 expression in A549 xenografted tumors of nude mice was analyzed by immunohistochemistry.

RESULTS: HPV-16 E6 and E7 oncoproteins HIF-1α-dependently promoted angiogenesis in vitro and in vivo, which was inhibited by EGCG. Mechanistically, EGCG inhibited HPV-16 oncoprotein-induced HIF-1α protein expression but had no effect on HIF-1α mRNA expression in NSCLC cells. Additionally, 50 and 100 μmol/L of EGCG significantly reduced the secretion of VEGF and **IL-8** proteins induced by HPV-16 E7 oncoprotein in NSCLC A549 cells. Meanwhile, HPV-16 E6 and E7 oncoproteins HIF-1α-dependently enhanced Akt activation in A549 cells, which was suppressed by EGCG. Furthermore, EGCG inhibited HPV-16 oncoprotein-induced HIF-1α and HIF-1α-dependent VEGF and CD31 expression in A549 xenografted tumors.

CONCLUSIONS: EGCG inhibited HPV-16 oncoprotein-induced angiogenesis conferred by NSCLC through the **inhibition** of HIF-1α protein expression and HIF-1α-dependent expression of VEGF, **IL-8**, and CD31 as well as activation of Akt, suggesting that HIF-1α may be a potential target of EGCG against HPV-related NSCLC angiogenesis.

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Meta-Analysis Shows Importance of HPV Vaccines

By **Leah Lawrence**

July 2, 2019

Cervical Cancer, Gynecologic Cancers, News

Significant reductions in several strains of human papillomavirus (HPV) and HPV-associated diseases have occurred in the up to 8 years of follow-up since implementation of the girls-only HPV vaccination, according to results of a meta-analysis published in *Lancet Oncology.*

The review and meta-analysis included data from 65 articles from 14 high-income countries (most of which had girls/women-only programs), encompassing follow-up data from more than 60 million individuals over 8 years.

During that time, the prevalence of HPV 16 and 18 decreased by 83% among girls age 13 to 19 years old, and by 66% among women age 20 to 24 years. In addition, cross protection occurred, with the prevalence of HPV 31, 33, and 45 decreasing by 54% among girls age 13 to 19 years.

There was also evidence of herd protection from the vaccine. Specifically, anogenital warts decreased by 67% among girls age 15 to 19 and by 54% among women age 20 to 24, but also by 31% among women age 25 to 29, 48% among boys age 15 to 19, and 32% in men age 20 to 24.

“Our results provide strong evidence of HPV vaccination working to prevent cervical cancer in real-world settings, as both the cause (high-risk HPV infection) and proximal disease endpoint (CIN2+) are significantly declining,” wrote Mélanie Drolet, PhD, of Laval University in Quebec, and colleagues from the HPV Vaccination Impact Study Group. “In terms of global policy implications, these results reinforce the recently revised position of WHO recommending HPV vaccination of multiple age cohorts of girls, and provide promising early signs that the WHO call for action on cervical cancer elimination might be possible if sufficient population-level vaccination coverage can be reached.”

After a period of 5 to 9 years of vaccination, the rate of CIN2+ decreased by more than half (51%) among screened girls age 15 to 19 years and by about one-third (31%) among women age 20 to 24.

In an editorial that accompanied the article, Silvia de Sanjosé, PhD, of PATH in Seattle, and Sinead Delany-Moretlwe, MBBCh, PhD, of Wits Reproductive Health and HIV Institute in South Africa, pointed out that one limitation of the study is its lack of inclusion of data from low- and middle-income countries, “where the burden of disease accounts for over 80% of deaths attributable to HPV-related cancer.”

However, collection of these data in low- and middle-income countries would be challenging due to a lack of surveillance system to link vaccinations with outcome data. In addition, vaccine cost and inadequate supply may slow the process of vaccine introduction to these countries.

“For the next 5 years, vaccine supplies are projected to be constrained and unable to accommodate demand because of fewer requirements to introduce HPV vaccines in countries supported by Gavi, the Vaccine Alliance, increased interest in multiple age-cohort vaccination, and introduction of a gender-neutral approach,” they wrote. “The scale-up of HPV vaccine introduction in low- and middle-income countries will be particularly affected by these constraints.”

Data from studies like this meta-analysis can help implementers to concentrate on high-priority targets.

“The data presented by Drolet and colleagues emphasize the importance of redoubling our efforts to tackle the fiscal, supply, and programmatic barriers that currently limit HPV vaccine programs; with these efforts, HPV vaccination could become a hallmark investment of cancer prevention in the 21st century,” they concluded.

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ORIGINAL ARTICLE

TriCurin, a synergistic formulation of curcumin, resveratrol, and epicatechin gallate, repolarizes tumor-associated macrophages and triggers an immune response to cause suppression of HPV+ tumors

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Abstract

Our earlier studies reported a unique potentiated combination (TriCurin) of curcumin (C) with two other polyphenols. The TriCurin-associated C displays an IC50 in the low micromolar range for cultured HPV+ TC-1 cells. In contrast, because of rapid degradation in vivo, the TriCurin-associated C reaches only low nano-molar concentrations in the plasma, which are sub-lethal to tumor cells. Yet, injected TriCurin causes a dramatic suppression of tumors in TC-1 cell-implanted mice (TC-1 mice) and xenografts of Head and Neck Squamous Cell Carcinoma (HNSCC) cells in *nude/nude* mice. Here, we use the TC-1 mice to test our hypothesis that a major part of the anti-tumor activity of TriCurin is evoked by innate and adaptive immune responses. TriCurin injection repolarized arginase1^{high} (ARG1^{high}), IL10^{high}, inducible nitric oxide synthase^{low} (iNOS^{low}), IL12^{low} M2-type tumor-associated macrophages (TAM) into ARG1^{low}, IL10^{low}, iNOS^{high}, and IL12^{high} M1-type TAM in HPV+ tumors. The M1 TAM displayed sharply suppressed STAT3 and induced STAT1 and NF-kB(p65). STAT1 and NF-kB(p65) function synergistically to induce *iNOS* and *IL12* transcription. Neutralizing IL12 signaling with an IL12 antibody abrogated TriCurin-induced intra-tumor entry of activated natural killer (NK) cells and Cytotoxic T lymphocytes (CTL), thereby confirming that IL12 triggers recruitment of NK cells and CTL. These activated NK cells and CTL join the M1 TAM to elicit apoptosis of the E6+ tumor cells. Corroboratively, neutralizing IL12 signaling partially reversed this TriCurin-mediated apoptosis. Thus, injected TriCurin elicits an M2→M1 switch in TAM, accompanied by IL12-dependent intra-tumor recruitment of NK cells and CTL and elimination of cancer cells.

Keywords Curcumin · TriCurin · Papillomavirus · Tumor-associated macrophages · NK cells · CTL

Abbreviations

ARG1	Arginase-1
C	Curcumin
CD8	Cluster of differentiation 8 protein
CD8α	Cluster of differentiation 8 alpha

Rahman Hussaini and Richard White contributed equally.

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CTL	Cytotoxic T lymphocytes
CUNY	City University of New York
E	Epicatechin gallate
E6 16/18	Early 6 protein 16/18
E7	Early 7 protein
HaRas	Harvey ras protein
HCl	Hydrochloric acid
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
IACUC	Institutional Animal Care and Use Committee
Iba1	Ionized calcium-binding adapter molecule 1
IF	Integrated fluorescence
IFNγ	Interferon gamma
IgG	Immunoglobin G
IL10	Interleukin-10
IL12	Interleukin-12
iNOS	Inducible nitric oxide synthase
LEEP	Loop electrosurgical excision procedure
LR	Lower right
NCr	Athymic nude mice
NKp46	Natural killer cell p-46-related protein
NOS2	Nitric oxide synthase 2
PFA	Paraformaldehyde
P-NF-kB	Phospho-NF-kB
P-p65	Phospho-p65
P-ser	Phosphor-serine
P-STAT1/3	Phospho-STAT1/3
P-Tyr	Phospho-tyrosine
R	Resveratrol
S.E.	Standard error
SEM	Standard error of the mean
SSC	Saline-sodium citrate
TAM	Tumor-associated macrophage
TriCur + IL12Ab	'IL12 antibody infusion followed by TriCurin treatment' group
UL	Upper left
UMSCC47	University of Michigan-squamous cell carcinoma-47
UR	Upper right
w/v	Weight/volume

Introduction

The human papillomavirus (HPV) is the major risk factor for cervical cancer that claims numerous lives globally and is a major threat to women especially in the developing countries where screening and vaccinations are not affordable or realistic due to the lack of infrastructure. The incidence of cervical cancer is rather low in the developed countries because of extensive screening programs [1–3]. Though the availability of two vaccines, Cervarix® and Gardasil®, offers preventive measures against the usual tumorigenic HPV16/18+ cervical lesions, effective therapy for post-infection lesions is currently unavailable [4]. High-risk HPV16+ infection has also emerged as an etiologic factor for the development of head and neck squamous cell carcinoma (HNSCC), with new cases escalating worldwide. The standard of care for HNSCC consists of surgery, radiation therapy, chemotherapy, or a combination of treatments, which have significant side effects and/or are associated with high morbidity [2, 5, 6]. This emphasizes the need for safe therapeutic approaches to treat HPV+ cervical cancer and HNSCC.

In earlier studies, we developed a formulation, termed TriCurin, containing curcumin (C) with two other food-derived natural polyphenols, resveratrol (R) and epicatechin gallate (E) at a unique and synergistic molar ratio. TriCurin is non-toxic to normal cells and selectively eliminates cancer cells in vitro and also in HaRas+, HPV16 E6+ and E7+ TC-1 cell-evoked mouse tumors and xenografts of HNSCC tumors in athymic *nude/nude* mice (NCr) [7, 8]. Poor bioavailability of C seriously limits its anti-tumor efficacy and the increased potency of TriCurin against cancer cells has been attributed to the stabilization of C in the presence of R and E and increased uptake of C from TriCurin into tumor cells [8]. Thus, such improved delivery of C has enabled us to use TriCurin to efficiently eliminate a wide array of tumor cells [7–15]. Although enhanced delivery increases the plasma concentration of C, it never reaches beyond the low nanomolar range, which is not high enough to cause direct elimination of cancer cells, which requires micromolar curcumin (Sumit Mukherjee et al., unpublished). We have postulated that the nano-molar level of C in the plasma is sufficient to activate the innate and adaptive immune system against tumor [12, 13, 16–21].

We demonstrate here for the first time that TriCurin treatment of tumor-bearing mice leads to an overall repolarization of the milieu of HPV+ tumor-associated macrophages from an M2 state to M1 phenotype. In addition, we also demonstrate that TriCurin-evoked repolarization of TAM is associated with intra-tumor recruitment of activated NK cells and CTL which are known to have tumoricidal activity [16, 17, 19, 20]. These changes in the tumor microenvironment play a major role in TriCurin-evoked elimination of HPV+ tumors [1, 7, 8, 12, 14].

Promising clinical trials using chimeric antigen receptor (CAR) T-cell treatment and regulators of T-cell activation (checkpoint inhibitors) have recently yielded immunotherapy drugs for cancer. Our therapeutic strategy using TriCurin also depends on the activation of the immune system against tumor, but unlike other approaches, it involves

a highly potent agent (TriCurin), which does not elicit the side effects commonly experienced in other therapies [22, 23]. Thus, TriCurin is a promising immunotherapeutic agent for various types of cancer.

Materials and methods

Animals

Please see the 'Animal Source' subsection under 'Compliance With Ethical Standards'.

Preparation of TriCurin

1.28 mM + TriCurin solution in PBS was prepared according to our earlier reports [7, 8].

Cell culture

HPV+ mouse TC-1 cells and UMSCC47 human cells were procured, authenticated, and cultured as reported earlier. HPV16+ UMSCC47 HNSCC cells were obtained from Dr. Thomas Carey (University of Michigan) [7, 8].

Implantation of cancer cells in mice

To generate the TC-1 mouse model, experiments were performed according to our previous report [8]. The tumors were allowed to grow to a diameter of 0.5 cm before intra-tumor infusion of TriCurin.

To generate the pre-clinical UMSCC47-derived HNSCC (UMSCC47) xenograft tumors in athymic *nude/nude* mice (NCr), experiments were performed according to our earlier report. The tumors were allowed to be palpable before intra-tumor infusion of TriCurin [7].

Treatment of pre-clinical HPV+ tumor-bearing mice with TriCurin

When the TC-1 tumors assumed the approximate length of 0.5 cm, eight mice were randomly divided into two groups: "Vehicle" (PBS plus 5% DMSO) ($n = 6$), "TriCurin" ($n = 6$), and "TriCurin + IL12Ab" ($n = 3$). Each tumor was marked into four quadrants and 2.5 µl of the 1.28 mM + TriCurin solution (or Vehicle) was infused into each of the four quadrants every 24 h for 5 days (final estimated intra-tumor concentration for each injection: 64 µM+). On the sixth day from the first treatment (the day of first treatment was marked as day 1) (TriCurin or Vehicle or TriCurin + IL12Ab), the mice were sacrificed, tumors extricated, and the final tumor volumes were measured by displacement of water. The Tumors were divided into two halves: one-half for preparing single-cell suspensions for post-immunostaining flow cytometry and the other half for immunohistochemistry and confocal microscopy.

Athymic mice (*nu/nu*) bearing human UMSCC47 cell-derived HNSCC xenograft tumors were treated with 1.28 mM + TriCurin (or Vehicle) and tumors were subsequently processed according to our earlier report [7].

Neutralization of IL12 signaling on NK cells and CTL by intra-peritoneal infusion of IL12Ab

On day 0 (24 h before the first TriCurin treatment) and day 3, each TC-1 implanted mouse in the TriCurin + IL12Ab group received intra-peritoneal injection of IL12-neutralizing Anti-IL12 (p40/p70) antibody (BD, 554,475) (60 µg, each dose) [24]. Mice in the other two groups (Vehicle and TriCurin) received rat serum (60 µg, per mouse, per dose) and subsequent drugs (Vehicle or TriCurin) as stated in the previous section.

Preparation of single-cell suspensions from TC-1 tumors and immunostaining for flow cytometry

One-half of the extricated TC-1 tumor was placed in RPMI-1640 medium containing 100 U/ml penicillin and 100 µg/ml streptomycin. Next, the tumors were rinsed with PBS and subsequently minced into 1–2-mm pieces and immersed in serum-free RPMI-1640 medium containing 0.05 mg/ml collagenase I (Fisher #NC0847850), 0.05 mg/ml collagenase IV (Fisher #ICN19511080), 0.025 mg/ml hyaluronidase IV (Fisher #NC9725814), 0.25 mg/ml DNase I, 100 U/ml penicillin, and 100 µg/ml streptomycin. The resultant cocktail was incubated at 37 °C for 15 min with periodic agitation in a mechanical shaker. The tumor digest was strained through a 70-µm nylon filter to remove undigested tissue fragments. The resultant single tumor cells were pelleted, washed with PBS, and fixed in 4% PFA [25].

For flow cytometry, 2 million fixed cells from each animal were immunostained as described earlier [12]. After each antibody treatment, the cells were pelleted and resuspended in wash buffers. Antibodies against Iba1 (C20) (1:50), iNOS (1:100), Arginase1 (1:100), NKp46 (1:100), active-caspase 3 (Asp175) (1:100), and E6 (1:75) were used for staining. Cells treated with the secondary antibody alone were used to set the threshold. The double-stained fluorescent events from ARG1+/Iba1+ and iNOS+/Iba1+ cells appeared as sub-populations in the upper right (UR) quadrant within the coordinates 520 nm (green for ARG1 or iNOS) (FL1-A) and 580 nm (red for Iba1) (FL2-A). Single-stained fluorescent events from the scatter plots and from Nkp46+ (580 nm, red) and E6+ (520 nm, green) cells appeared as sub-populations in the upper left (UL) and the lower right (LR) quadrants, respectively. Integrated fluorescence intensity was measured

for comparison between groups by multiplying the number of positive events (single-stained or double-stained cells) by the mean fluorescence intensity per event (cell).

Immunohistochemistry

Randomly chosen sections were subjected to pre-immunostaining antigen-retrieval using formamide: 2×SSC as reported earlier [7, 8, 12] only for IL12 and IL10 by treating with 0.1% (w/v) pepsin (Fisher, AC417071000) dissolved in 0.01 N HCl for 20 min at room temperature, followed by two PBS washes. For immunostaining, the sections were blocked overnight at 4 °C in 0.1% Triton X-100, 3% goat serum and 10% rabbit serum in 100 mM PBS, and then treated overnight with primary antibodies: anti-Iba1 (goat IgG) (C20) (sc28530) (1:50), iNOS (rabbit IgG) (NOS2 sc-651) (1:100), anti-Arginase1 (rabbit IgG) (sc-20150) (1:100), anti-STAT3 (rabbit IgG) (sc-7179) (1:100), anti-P-Tyr^{705}-STAT3 (goat IgG) (sc-7993) (1:100), anti-STAT1 (rabbit IgG) (sc-592) (1:100), anti-P-Tyr^{701}-STAT1 (mouse IgG) (sc-8394) (1:100), anti-p65 (NF-kB) (mouse IgG) (sc-8008), anti-P-Ser^{276}-p65 (NF-kB) (rabbit IgG) (sc-101749) (1:100), IL12p40 (rabbit IgG) (sc-7926) (1:100), IL10 (goat IgG) (sc-1783) (1:100), anti-NKp46 (rabbit IgG) (sc-292796) (1:100), anti-CD8-α (D-9) (mouse IgG) (sc-7970) (1:100), anti-E6 antibody (mouse IgG)(sc-460) (1:75) (Santa Cruz Biotechnology), and anti-active-caspase 3 (Asp175) (Rabbit IgG) (CST#9661, Cell Signaling Technology) (1:200). All antibodies were diluted in 2% goat serum plus 2% rabbit serum and 0.1% Triton X-100 in PBS (GRT-PBS). After washing three times with PBS, the respective secondary antibodies (Alexa Fluor 568 rabbit anti-goat, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-rabbit, Alexa Fluor 633 rabbit anti-goat, Alexa Fluor 633 goat anti-mouse, Alexa Fluor 488 rabbit anti-goat, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 633 goat anti-rabbit, and Alexa Fluor goat 488 anti-mouse) (Invitrogen) (1:1000 dilutions in GRT-PBS) were added to wells treated with the respective primary antibodies as well as those not treated with primary antibodies (2° Ab controls). Following overnight incubation at 4 °C and three washes with PBS, the sections were treated with HOECHST33342 (10 µg/ml) for 30 min at room temperature, followed by three washes with PBS and mounting on microscope slides with Gold anti-fade mounting fluid. Confocal Imaging was conducted using a Leica SP2 microscope from multiple randomly chosen fields.

ImageJ was used to quantify the fluorescence intensity for iNOS, ARG1, p65 (NF-kB), P-Ser^{276}-p65, STAT-1, P-Tyr^{701}-STAT1, NKp46, E6, anti-active-caspase 3 (Asp175), and HOECHST33342. The fluorescence intensity for each marker was normalized to HOECHST33342 intensity (blue). Since STAT3, STAT1, and p65 NF-kB displayed both induction as well as phosphorylation-mediated deactivation/activation, the HOECHST-normalized staining intensities were expressed both as P-STAT3/STAT3, P-STAT1/STAT1, and P-p65 NF-kB/p65 NF-kB, as well as P-STAT3/HOECHST, P-STAT1/HOECHST, and P-p65 NF-kB/HOECHST.

Statistical analysis

We used two-tailed *t* tests with unequal variance while comparing between two groups and one-way ANOVA with Tukey for post-hoc analysis to compare among three groups ($p \leq 0.05$ was considered as significant).

Results

Five days of TriCurin treatment is adequate to cause repolarization of tumor-associated macrophages from M2 to M1 phenotype in TC-1 tumor. Immunostaining of the tumors from the Vehicle and TriCurin groups revealed that the 'tumor-core' harbors mostly E6+ tumor cells, while the 'tumor-periphery' harbors mostly Iba1+ TAM (Supplementary Fig. 1) [8, 12]. TriCurin-evoked repolarization of ARG1^{high} M2 TAM into iNOS^{high} M1 TAM was studied using flow cytometry analysis of immunostained cells dissociated from Vehicle-treated and TriCurin-treated TC-1 tumors [8, 12]. The integrated fluorescence (IF) from ARG1+/Iba1+ (M2 TAM) (Fig. 1a, b) and iNOS/Iba1+(M1 TAM) (Fig. 1e, f) double-stained events occurring in the upper right (UR) quadrant (red ellipse) was used to quantitatively compare between the two groups. Whereas the Iba1+ cells in the tumor were ARG1^{high}/iNOS^{low} (Fig. 1a, e) in the Vehicle-treated mice, in the TriCurin-treated tumors, the Iba1+ cells were ARG1^{low}/iNOS^{high} (Fig. 1b, f). We observed a 55.5% decrease in ARG1 IF (Fig. 1c, d) and a 305% increase in iNOS IF (Fig. 1e, h). The intra-tumor iNOS ^{high} M1 TAM liberate NO, which is expected to be cytotoxic toward the surrounding tumor cells [12, 13, 26].

The Iba1 IF of these double-stained events (ARG1+ or iNOS+) was also sharply dissimilar between the two groups. TriCurin treatment elicited an 80% decrease in Iba1 IF in the ARG1+ cells in the tumor (relative to Vehicle-treated) (Fig. 1i), but the Iba1 IF in the iNOS+ cells in the TriCurin-treated tumors was 989% higher than in the Vehicle-treated tumors (Fig. 1j). In contrast, the total Iba1 IF (total number of Iba1+ cells in the iNOS+/Iba1+ and ARG1+/Iba1+ populations) did not significantly differ between the TriCurin and Vehicle-treated groups (Fig. 1k). This supported the possibility that TriCurin treatment causes suppression of M2 TAM and simultaneous activation/recruitment of a discrete population of M1 TAM [12].

TriCurin treatment causes suppression of P-STAT3 in the tumor-associated macrophages. C has been known to inhibit the ARG1-inducing transcription factor STAT3, which is a

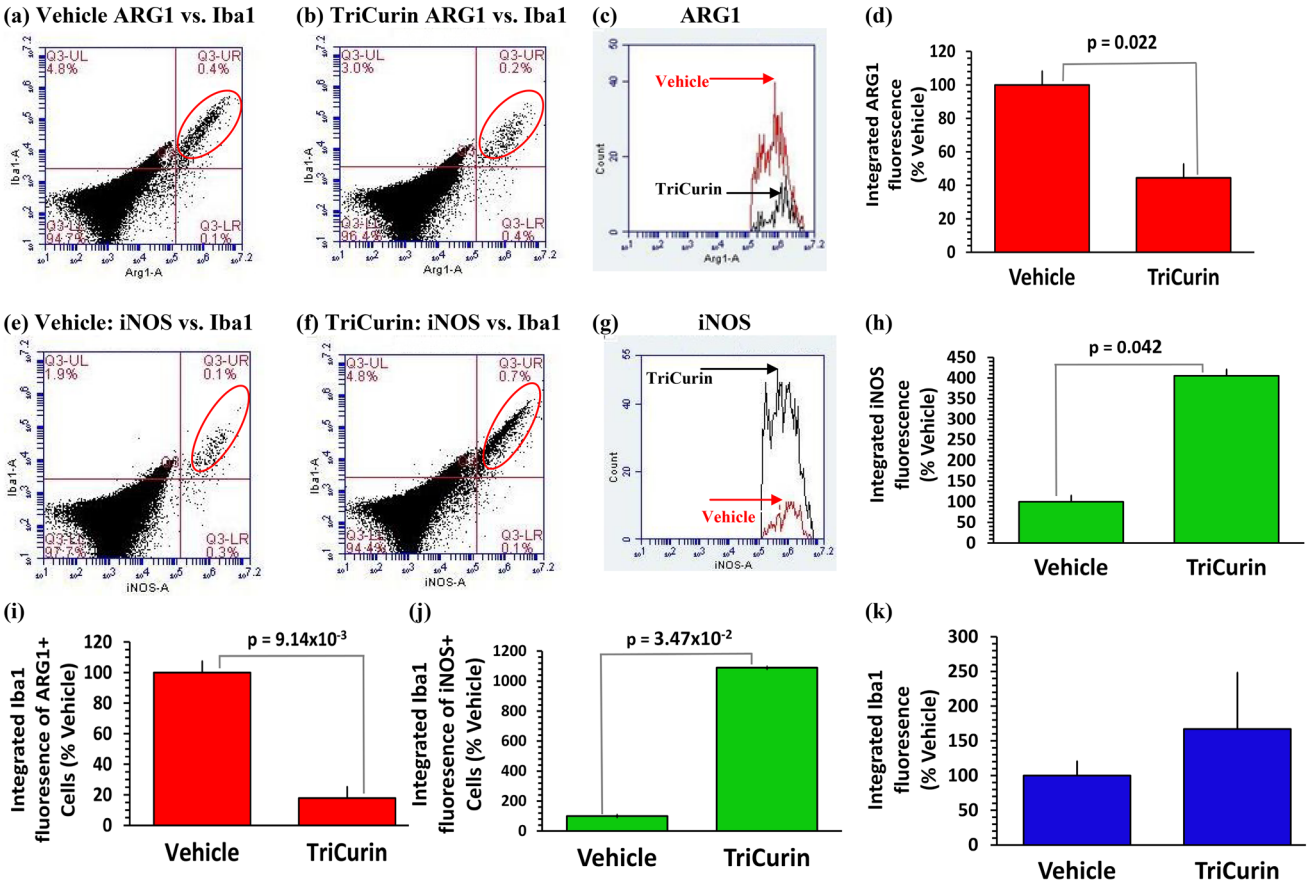

Fig. 1 Five days of TriCurin treatment causes repolarization of tumor-associated macrophages (TAM) from M2 to M1-type in TC-1 tumor. Cells from TC-1-implanted mice with 5 days of TriCurin or Vehicle (PBS) treatment (daily, see "Materials and methods") were dispersed, fixed, permeabilized, and immunostained using iNOS, ARG1, and Iba1 (marks activated macrophages) antibodies, and the stained cells analyzed by flow cytometry. **a–c** Integrated fluorescence intensity (IF) (fluorescence per event x total number of events in a segregated population) obtained from double-stained events (red ellipse, upper right (UR) quadrant) for ARG1 (green) and Iba1 (red) was considered and the IF profiles of ARG1+cells were obtained for both the groups. **e–g** Similarly, IF for iNOS+cells were obtained from both groups (Vehicle: red, TriCurin: black) values obtained from double-stained events [red ellipse, UR quadrant for iNOS (green) and Iba1 (red)]. **d, h** Graphs showing a 56% decrease in ARG1 IF ($p=0.022$) and a 305% increase in iNOS IF ($p=0.042$) (with respect to Vehicle-treated) using Vehicle-treated and TriCurin-treated mice (mean ± S.E.) ($n=4$ for each group). **i, j** Similarly, Iba1 IF quantified from **a, b, e**, and **f** shows a concomitant decrease in Iba1 integrated fluorescence in the ARG1+cells (80%) ($p=9.14\times10^{-3}$) and an increase in Iba1 integrated fluorescence (989%) in iNOS+cells ($p=0.035$) with respect to the Vehicle-treated for each group (mean ± SEM) ($n=4$ for each group). **k** No significant difference in Iba1 IF between double-positive cells from the Vehicle-treated and the TriCurin-treated groups

marker for the immunosuppressive M2-type TAM [13, 20, 27]. To understand if STAT3 was a major player in the TriCurin-mediated repolarization of Iba1+TAM (Supplementary Fig. 1) from M2 to M1-type, we performed immunohistochemistry of tumor sections from Vehicle and TriCurin groups for STAT3 and activated STAT3 (P-Y^{705}-STAT3, or P-STAT3). The M2 TAM in the Vehicle-treated TC-1 tumor harbored elevated levels of P-STAT3. In contrast, the M1 TAM in the TriCurin-treated tumors exhibited 80% suppression of P-STAT3 (Fig. 2a, b). A comparison with the corresponding total STAT3 levels revealed that the overall suppression of P-STAT3 was due to a combination of suppressed STAT3 expression (56% decrease) (Fig. 2a, c) and inhibited STAT3 phosphorylation (51% decrease) (Fig. 2a, d).

TriCurin treatment causes an induction in P-STAT1 and P-NF-KB (P-p65) in the TAM. STAT3 activation causes IL10-mediated suppression of the transcription factor STAT1 in the TAM [28]. Based on our previous results (Fig. 2), inhibition of STAT3 by TriCurin is expected to result in the activation of STAT1 [12], a transcription factor that triggers iNOS and IL12 synthesis in the TAM [29]. To investigate the activation status of STAT1 in both groups (Vehicle and TriCurin), we immunostained the TAM using antibodies against STAT1 and activated STAT1 (P-Tyr^{701}-STAT1). The Iba1+ cells in the Vehicle-treated mice showed

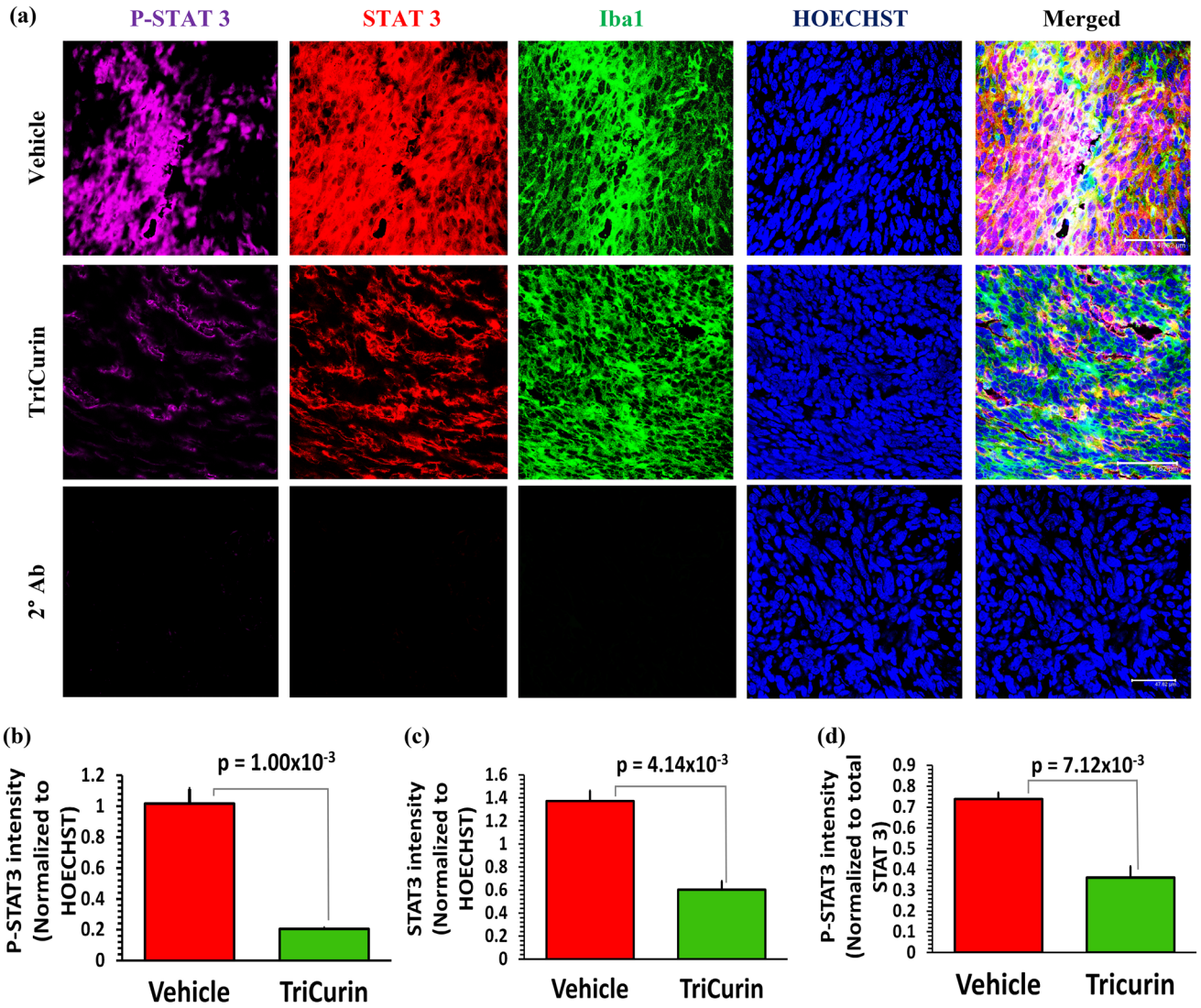

Fig. 2 Five days of TriCurin treatment causes a dramatic suppression of P-STAT3 in the tumor-associated macrophages (TAM). TC-1 tumor sections parallel to those used in Supplementary Fig. 1 were used to assess the levels of STAT3 and P-Tyr^{705}-STAT3 (activated) in the Iba1+ macrophages. **a**, upper and middle rows, and **b** the Vehicle-treated mice displayed a high level of activated STAT3 (P-Y^{705}-STAT3) in the TAM, which was suppressed by 80% ($p = 1.0 \times 10^{-3}$) in the TriCurin-treated group. This overall suppression of P-STAT3 was due to a combination of **c** suppression of STAT3 expression (STAT3 fluorescence normalized to HOECHST fluorescence) and **d** STAT3 activation (P-STAT3 normalized to STAT3). Four randomly chosen sections per mouse were used for imaging and the data (mean ± SEM) obtained from Vehicle-treated ($n = 4$) and TriCurin-treated ($n = 4$) groups. HOECHST = HOECHST33342. (Scale bar: 47.62 μm). **a**, lower row, absence of non-specific staining in sections treated with three 2° antibodies consecutive to one another (see "Materials and methods")

basal levels of activated STAT1, whereas the M1 TAM in the TriCurin-treated tumors exhibited a 532% increase in P-STAT1 (Fig. 3a, b). A comparison with the corresponding total STAT1 levels revealed that the overall increase in P-STAT1 was due to a combination of induced STAT1 expression (219% increase) (Fig. 3a, c) and increased STAT1 phosphorylation (activation) (97% increase) (Fig. 3a, d).

Activated NF-kB (p65) has been known to be overexpressed in M1 TAM [30]. In addition, earlier work in macrophages shows that co-activated NF-kB (p65) and STAT1 cooperate by binding to enhancer elements on the *iNOS* gene [12, 31, 32]. To effect such alterations in the tumor-associated macrophages, TriCurin treatment should cause simultaneous activation these two transcription factors. As expected, immunostaining of the TAM using antibodies against NF-kB (p65) and activated NF-kB p65 (P-Ser^{276}-p65) revealed a 2071% increase in P-p65 (P-NF-kB) in the TAM (Fig. 4a, b). A comparison with the corresponding total NF-kB (p65) levels revealed that the overall increase in P-p65 was due to a combination of induced NF-kB (p65)

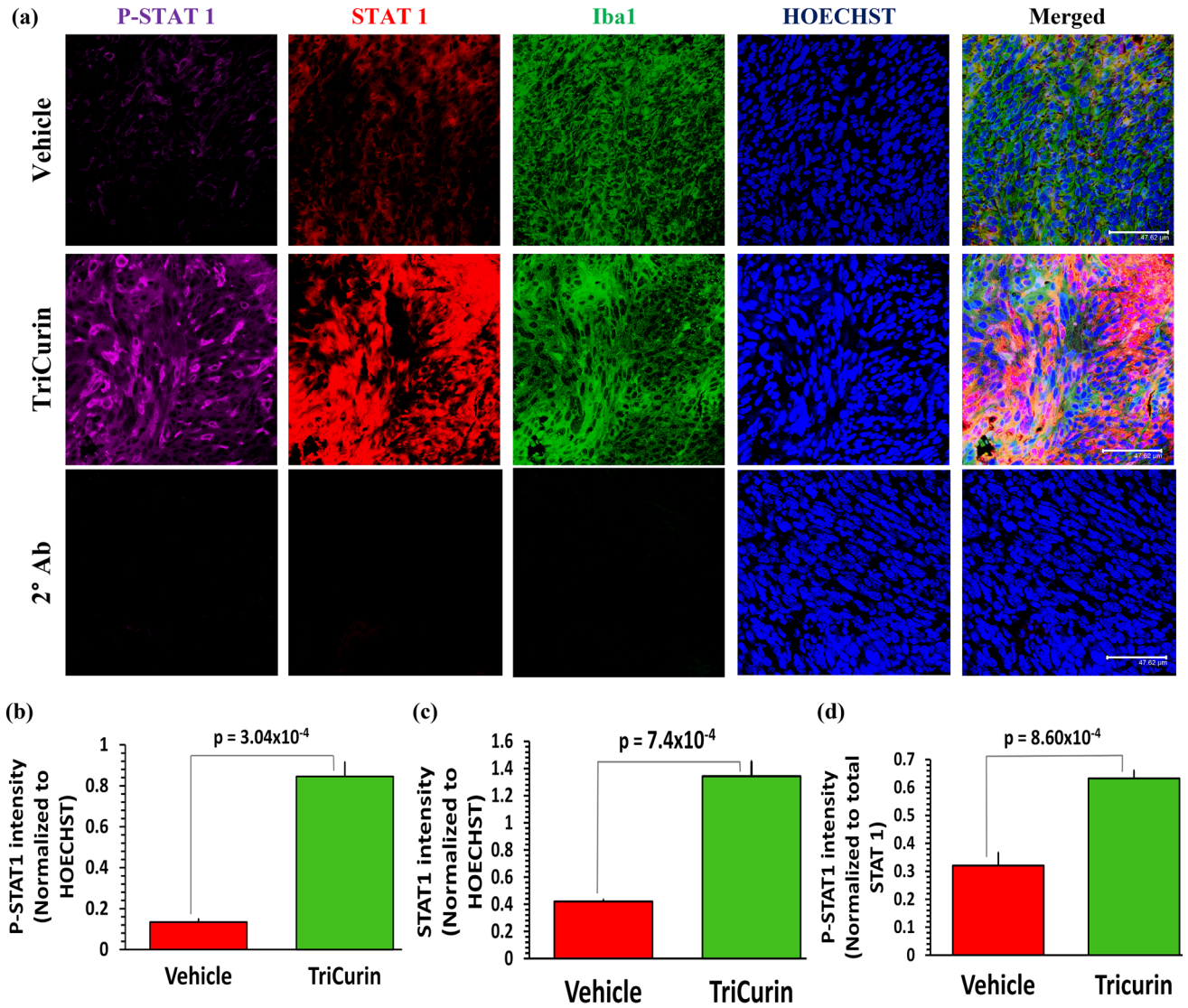

Fig. 3 TriCurin treatment causes an induction in P-STAT1 in the TAM. TC-1 tumor sections parallel to those used in Supplementary Fig. 1 were stained to determine the levels of STAT1 and P-Tyr^{701}-STAT1 (activated) in the tumors from Vehicle-treated and TriCurin-treated mice. **a** Upper and middle rows, and **b** the Vehicle-treated mice displayed basal levels of activated STAT1 (P-Tyr^{701}-STAT1) in the Iba1+TAM, which was increased by 532% ($p = 3.04 \times 10^{-4}$) in the TC-1 tumor sections from the TriCurin-treated group. This overall increase in P-STAT1 was due to a combination of (**c**) induction of STAT1 expression (STAT1 normalized to HOECHST) and **d** STAT1 activation (P-STAT1 normalized to STAT1). Four randomly chosen sections per mouse were used for imaging and the data (mean ± SEM) were compared between Vehicle-treated and TriCurin-treated groups ($n = 4$ per group). (Scale bar: 47.62 µm). (**a**, lower row) Absence of non-specific staining from the 2° antibodies (see "Materials and methods")

expression (226% increase) (Figs. 4a, 2c) and increased NF-kB (p65) phosphorylation (activation) (536% increase) (Figs. 4a, 2d).

Five days of TriCurin treatment is adquate to cause repolarization of IL12^{low}, IL10^{high} tumor-associated M2 macrophages to IL12^{high}, IL10^{low} M1-type in TC-1 tumor. Suppression of P-STAT3 is known to cause reduced IL10 (M2 TAM marker) expression along with increased IL12 (M1 TAM marker) expression [33]. Based on this understanding, we wanted to verify if the observed TriCurin-mediated M2→M1 polarization (Fig. 1) was associated with a switch in the Iba1+ TAM to IL12^{high} and IL10^{low} (M1) phenotype. As expected, tumors from the Vehicle-treated group had high IL10 and very low IL12 in the Iba1(+) TAM, whereas the TAM in tumor sections from the TriCurin-treated mice showed a 70% decrease in IL10 and a 244% increase in IL12 expression (Supplementary Fig. 2). This further demonstrated the TriCurin-evoked M2→M1 switch using a second set of markers for M2 and M1, IL10, and IL12, respectively.

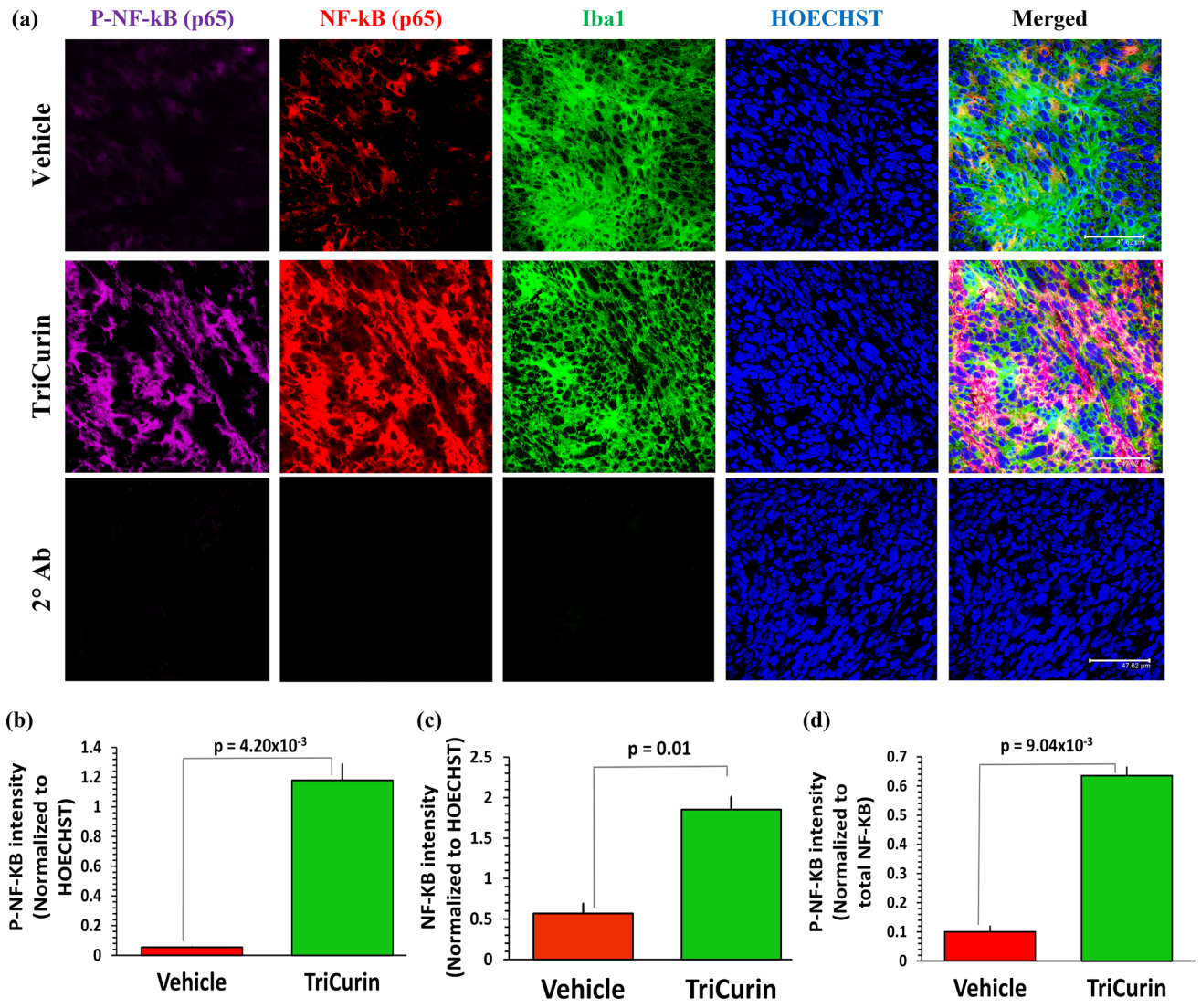

Fig. 4 TriCurin treatment causes an induction in P-NF-KB (P-p65) in the TAM. TC-1 tumor sections parallel to those used in Supplementary Fig. 1 were stained to determine the levels of NF-kB (p65) and activated NF-kB p65 (P-Ser^{276}-p65) in the tumors from Vehicle-treated and TriCurin-treated mice. **a** Upper and middle rows and **b** the Vehicle-treated mice displayed basal levels of activated NF-kB p65 (P-Ser^{276}-p65) in the iba1+TAM, which was increased by 2071% in the TriCurin-treated TC-1 tumor sections ($p = 4.20 \times 10^{-3}$). This overall increase of P-NF-kB (P-p65) was a result of **c** induction of NF-kB (p65) expression (NF-kB (p65) normalized to HOECHST) and **d** NF-kB (p65) activation (P-NF-kB (P-p65) normalized to NF-kB (p65)). Four randomly chosen sections per mouse were used for imaging and the data (mean ± SEM) compared between Vehicle-treated and TriCurin-treated groups ($n = 4$ per group). (Scale bar: 47.62 µm). **a** lower row, showed the lack of non-specific staining from the secondary antibodies (see "Materials and methods")

IL12 is known to trigger intra-tumor recruitment of activated NK cells and cytotoxic T lymphocytes [21, 29, 34–37]. To verify the role of IL12 in causing recruitment of NK cells and CTL that would cause enhanced elimination of tumors, we neutralized the IL12 receptor-mediated signaling in these immune cells by peripheral treatment with IL12p40 antibody [24, 29].

IL12Ab treatment abrogates TriCurin-evoked infiltration of natural killer (NK) cells into TC-1 tumors. C has also been reported to activate NK cells in tumors [16]. Cognizant of these studies, we investigated the possibility of TriCurin-evoked, intra-tumor recruitment of activated NK cells (in addition to M1 TAM polarization) as a mechanism for its anti-tumor activity [21]. Based on flow cytometry data from Vehicle, TriCurin, and TriCurin + IL12Ab groups, TriCurin evoked a 376% increase in infiltrating activated (NKp46+) NK cells, which was eliminated in the TriCurin + IL12Ab group NKp46+ fluorescence (UL quadrant. red ellipse) within the tumor, thus confirming infiltration of NK cells into the TC-1 tumors in response to TriCurin treatment (Fig. 5a–e). Furthermore, based on immunohistochemical data, the 5-day treatment with TriCurin caused

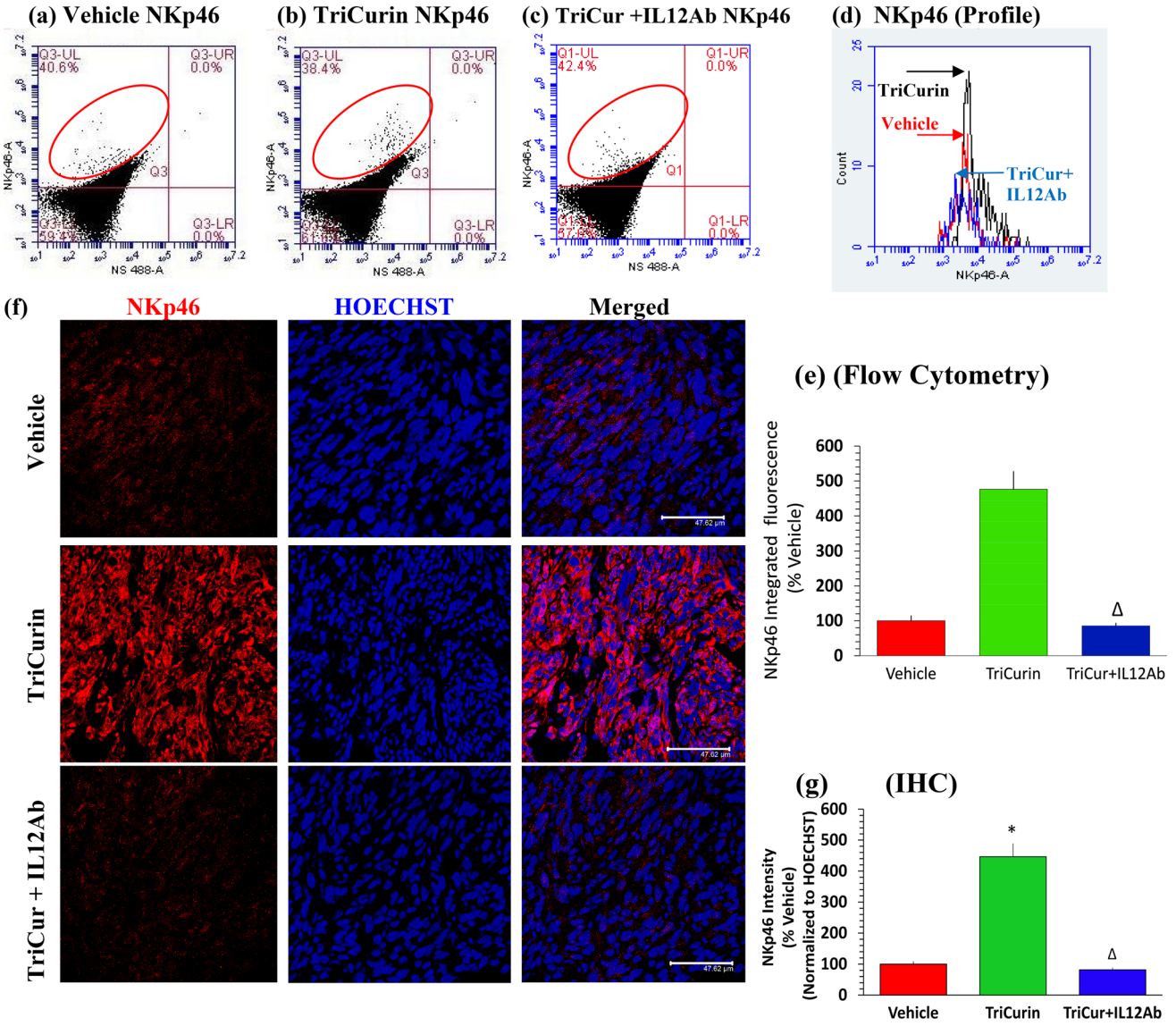

Fig. 5 IL12 Ab treatment abrogate infiltration of natural killer (NK) cells into TC-1 tumors. Cells from TC-1 tumor implanted mice with 5-day TriCurin (plus normal rat serum, see "Materials and methods"), TriCurin + IL12Ab and Vehicle (PBS) treatments were dispersed, fixed, and permeabilized, the cells immunostained using the activated NK cell-specific NKp46 antibody, and then analyzed by flow cytometry. **a–d** IF from single-stained NKp46+ events (red ellipse, UL quadrant) was considered from the three groups, expressed as mean ± S.E. (Vehicle: red, TriCurin: black, TriCurin + IL12Ab: blue) (Vehicle, $n=4$; TriCurin, $n=4$; TriCurin + IL12Ab, $n=3$). **a, b, d, e** TriCurin treatment affords a 376% increase in NKp46 IF (with respect to Vehicle-treated, $^*p=0.019$). **a, c, d, e** TriCurin + IL12Ab displayed an NKp46 IF, which was comparable to that of the Vehicle group (with respect to TriCurin-treated, $\Delta p=0.02$). **f** upper, middle, and lower rows, and **g** TC-1 tumor sections from Vehicle-, TriCurin-, and TriCurin + IL12Ab-treated mice were single-stained with the NKp46 antibody. The Vehicle-treated tumor tissue displayed minimal NKp46 fluorescence, but the TriCurin-treated mice showed a 346% increase in NKp46-staining (with respect to the Vehicle), confirming the recruitment of activated NK cells ($^*p=1.25\times10^{-3}$).TriCurin + IL12Ab treatment reversed this recruitment of NKp46+ NK cells to the level in the Vehicle group (with respect to TriCurin-treated, $\Delta p=1.0\times10^{-3}$). Four randomly chosen sections per mouse were used for imaging and the data obtained were expressed as (mean ± S.E.M.), from Vehicle-treated ($n=4$), TriCurin-treated ($n=4$), and TriCurin + IL12Ab ($n=3$) groups. (Scale bar: 47.62 µm)

a 346% increase in activated (NKp46+) NK cells in the TC-1 tumors (Fig. 5f, g) [38]. In contrast, infiltration of activated (NKp46+) NK cells was virtually eliminated in the TriCurin + IL12Ab group, thus confirming the role of TriCurin-induced IL12 (Supplementary Fig. 2) in causing this intra-tumor recruitment of activated NK cells (Fig. 5f, g). Interestingly, C is also known to cause enhanced expression of IFNγ from tumor-associated immune cells (lymphocytes like NK cells and CTL), which, in the case of TriCurin, could prolong the M1 TAM phenotype [17, 19, 29, 34, 38].

IL12 Ab treatment eliminates TriCurin-evoked infiltration of Cytotoxic T lymphocytes into TC-1 tumors. CTL are known to be highly tumoricidal and C has been known to promote intra-tumor infiltration of activated CD8+ CTL to cause tumor elimination [17, 19, 20]. In addition, M1-macrophage-derived IL12 has been known to promote the activation and infiltration of CTL [35–37]. Therefore, we next investigated if TriCurin treatment was capable of causing intra-tumor infiltration of activated CD8+ CTL and if IL12 regulated this process (Supplementary Fig. 2). As revealed by immunohistochemistry for CD8+ CTL, the 5-day treatment with TriCurin triggered a 633% increase in activated (CD8+) CTL in the TC-1 tumors (Supplementary Fig. 3a, b). In addition, data from the TriCurin + IL12Ab group revealed the lack of tumor-infiltrating activated (CD8+) CTL, despite TriCurin treatment, thereby indicating that the IL12 plays a major role in causing the intra-tumor recruitment of activated CTL (Supplementary Fig. 3a, b).

IL12 Ab treatment partially reverses TriCurin-induced apoptosis of E6+ tumor cells and tumor size reduction. In our prior studies, intra-tumor injection of TriCurin into HaRas+, HPV16 E6+, and E7+ TC-1 cell-implanted mice, every 72 h for 10 days caused an 80–90% decrease in tumor growth [8]. Here, we examined if the onco-immunotherapeutic effect of TriCurin in recruiting/activating M1 TAM, NK cells, and CTL in 5 days was also associated with a concomitant reduction in tumor load. Furthermore, we asked if neutralization of NK cells and CTL by IL12 Ab infusion influences the effect of TriCurin [21, 29, 34–37]. Five days of intra-tumor TriCurin infusion (every 24 h) caused a 61% decrease in tumor growth, whereas application of IL12 antibody (i.p.) along with TriCurin (intra-tumor) caused a partial reversal of the TriCurin-triggered inhibition of tumor growth (41% decrease) (tumor volumes were determined using water displacement) (Fig. 6a, b). Based on flow cytometry, the reduction in tumor load was associated with a flow cytometry analysis which showed a 68% inhibition of E6 expression in these tumor cells (LR quadrant: red ellipse) (Fig. 6c, d, f, g). The TriCurin + IL12Ab group showed only a 38% inhibition of E6 in these tumor cells, verifying that the neutralization of NK cells and CTL causes 30% less elimination of these tumor cells by (Fig. 6c, e–g). Based on immunohistochemistry, the tumor load reduction was concomitant with an inhibition of the oncoprotein E6 (54% decrease) (Fig. 6h, i) and activation of caspase 3 (383% increase) (Fig. 6h, j) in the tumor cells. In contrast, the TriCurin + IL12Ab group revealed a 31% inhibition of the oncoprotein E6 (Fig. 6h, i) and 67% increase in active-caspase 3 (Fig. 6h, j). Thus, neutralization of NK cells and CTL with the IL12Ab impacted tumor elimination by causing 23% less inhibition of E6 and 316% less activation of caspase 3. This is consistent with our previous in vitro observation that TriCurin treatment for 6 h causes suppression of E6, increased acetylation-mediated activation of p53, and elevation of active-caspase 3 in cultured TC-1 cells [8].

Prolonged TriCurin treatment triggers P-NF-kB and P-STAT1-mediated M2 to M1 polarization of TAM, recruitment of activated NK cells, and suppression of E6+ cells in HNSCC xenograft tumors. To translate the novel findings from our TC-1 model into a more clinically relevant human tumor model, HPV16+ HNSCC cells UMSCC47 were implanted into the flanks of athymic *nude/nude* (NCr) mice (xenograft model); and after the tumors were palpable, the immuno-modulatory effect of intra-tumor TriCurin injection for 5 weeks (3 doses per week) was assessed [7].

Immunohistochemical analysis revealed that the Vehicle-treated TAM were $iNOS^{low}$, $ARG1^{high}$, and Iba1+ M2 macrophages. In contrast, similar to the 5-day treatment, prolonged TriCurin treatment also caused a major shift in polarity of the Iba1+ TAM to the $iNOS^{high}$, $ARG1^{low}$ M1 form (Supplementary Fig. 4a). We observed an 89% decrease in ARG1 (Supplementary Fig. 4a, b) and a 684% increase in iNOS (integrated fluorescence) (Supplementary Fig. 4a, c).

Our previous results (Fig. 2) and other reports have shown that both TriCurin as well as C are a potent suppressors of activated STAT3 in M2 TAM and that STAT3 suppression causes activation of STAT1, leading to the formation of the M1 phenotype [13, 28]. Also the findings from our TriCurin-treated TC-1 tumors (Figs. 3, 4) and other reports have revealed that the co-activated transcription factors STAT1 and NF-kB (p65) are likely to trigger the expression of the M1-linked enzyme iNOS, which produces NO that is known to cause tumor elimination [12, 13, 26, 30–32]. Immunohistochemical analysis of the Iba1+ TAM in the TriCurin-treated HNSCC-implanted xenograft tumors revealed elevated levels of co-localized, P-STAT1 (993% increase), and activated P-p65 (93% increase) in the TriCurin-treated Iba1+ TAM (Supplementary Fig. 5a–c).

Given the evidence of interaction between M1 TAM and activated NK cells in the tumor microenvironment (Supplementary Fig. 2, Figs. 5, 6), we also probed for tumor-infiltrating activated NK cells in these xenograft tumors [29, 34, 39]. Immunostaining revealed elevated levels of intra-tumor NKp46+ NK cells (441% increase) in the TriCurin-treated tumors (Supplementary Fig. 6a–b). This recruitment of activated NK cells is expected to result in stabilization of the M1 TAM phenotype, thereby causing elimination of tumors by the coordinated action of TAM and NK cells [19, 21, 29, 38, 40]. As expected, the intra-tumor activation/recruitment of M1 TAM and NK cells was associated with the suppression in E6+ tumor cells (Supplementary Fig. 6a) and an 86% reduction in tumor size, as reported earlier [7]. These results were consistent with our previously reported

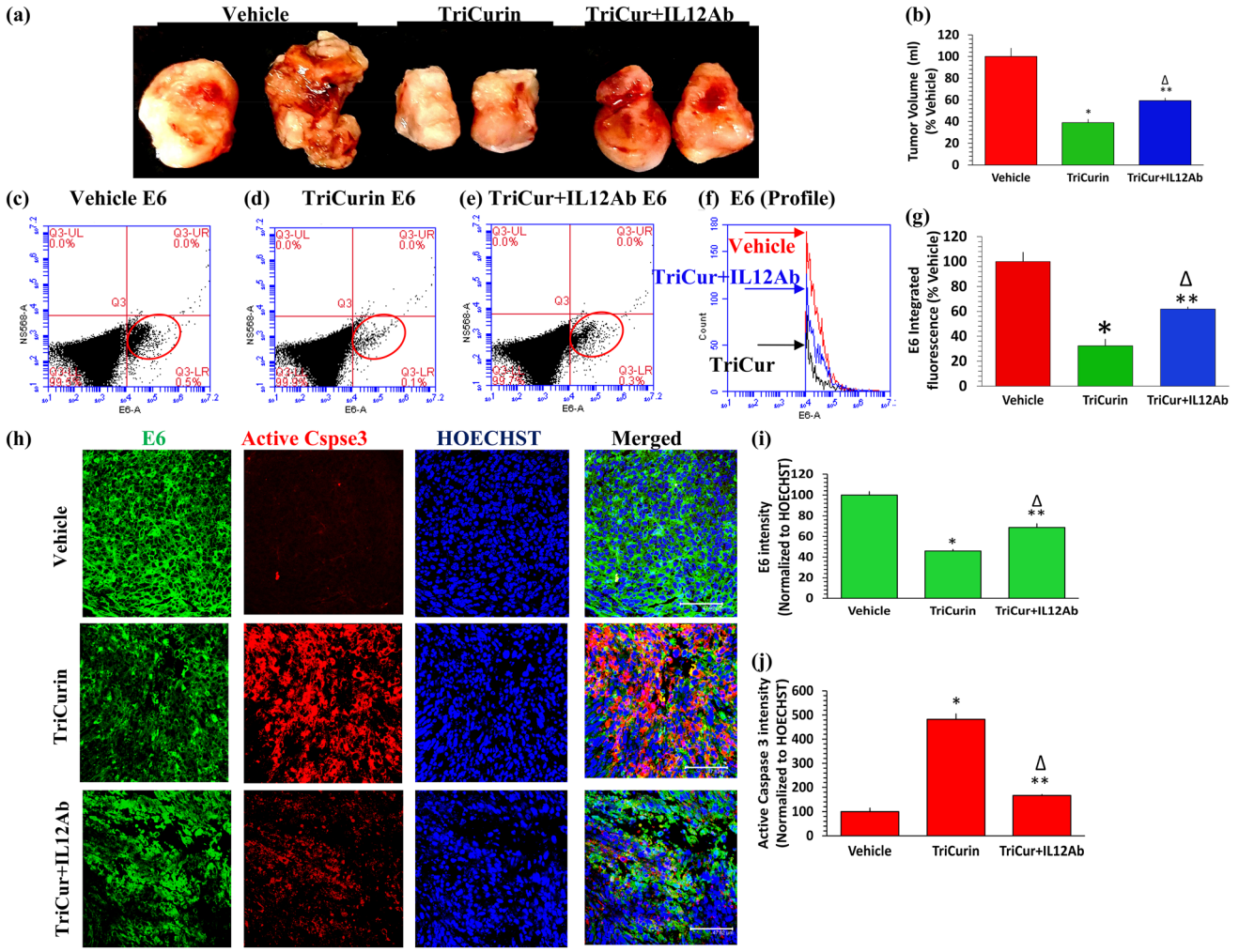

Fig. 6 IL12Ab treatment partially reverses TriCurin-induced apoptosis of E6+tumor cells and reduction in tumor load. **a** Volumes of extricated tumors from Vehicle (PBS)-treated, TriCurin-treated, and TriCurin+IL12Ab-treated mice were measured by the water displacement on the day of sacrifice (day 6). Two representative tumors from each group are shown here. **b** Even after the 5-day treatment, the TriCurin group (six mice per group) showed a dramatic decrease in tumor size (61% relative to Vehicle-treated) ($n=6$) ($^{*}p=2.54\times10^{-5}$). The TriCurin+IL12Ab group ($n=3$) exhibited a smaller decrease in tumor size (41% relative to Vehicle-treated, $^{**}p=8.4\times10^{-3}$) ($\Delta p=5.8\times10^{-3}$ relative to TriCurin-treated) (mean ± S.E.M). **c–f** Tumor cells from 5 days of TriCurin, TriCurin+IL12Ab, and Vehicle treatment were dispersed, fixed, permeabilized, immunostained using the HPV+tumor cell-specific E6 antibody, and analyzed by flow cytometry. IF from single-stained E6+events (red ellipse, LR quadrant) was obtained from the three groups (Vehicle: red, TriCurin: black, TriCurin+IL12Ab: Blue). **g** Relative to the Vehicle group ($n=5$), the TriCurin group ($n=5$) showed a 68% decrease in E6 IF ($^{*}p=0.01$) and the TriCurin+IL12Ab-treated mice ($n=3$) showed a 38% decrease in E6 IF ($^{**}p=0.04$) ($\Delta p=0.02$ relative to TriCurin-treated), **h** upper, middle, and lower rows, **i** and **j** tumor sections from these three groups also were subjected to immunohistochemistry to assess caspase 3 (Cspse3) activation and E6 expression. The Vehicle group showed high E6 expression (green) and negligible active-caspase 3 (red). Relative to the Vehicle group, the TriCurin group showed a 54% suppression of E6 ($^{*}p=1.2\times10^{-3}$) (**i**) and a sharp increase in active-caspase 3 (383% increase) ($^{*}p=4.0\times10^{-4}$) (**j**). Relative Vehicle-treated, the TriCurin+IL12Ab group showed only 31% suppression of E6 ($^{**}p=8.8\times10^{-3}$) ($\Delta p=0.03$ relative to TriCurin-treated) (**i**). Relative to Vehicle-treated, the TriCurin+IL12Ab group showed 67% upregulation of active-caspase 3 upregulation ($^{**}p=0.04$) ($\Delta p=2\times10^{-3}$ relative to TriCurin-treated) (**j**). Four randomly chosen sections per mouse were used for imaging and data quantified from Vehicle-treated ($n=4$), TriCurin-treated ($n=4$) and, TriCurin+IL12Ab-treated ($n=3$) groups. (Scale bar: 47.62 µm)

in vitro data showing suppression of E6, increase in p53 levels, and induction of apoptosis in HNSCC cells following TriCurin treatment [7].

Discussion

This project involved intra-tumor injection of TriCurin, which is expected to cause direct apoptosis of a number of tumor cells. However, due to rapid degradation

of C in vivo, the concentration of TriCurin-derived C in the body is expected to drop quickly. Interestingly, our in vivo pharmacokinetic studies [Mukherjee et al., unpublished] using a lipid-encapsulated formulation of TriCurin yielded plasma concentrations of C in the nano-molar range (610–210 nM), which is not high enough to directly kill TC-1 and UMSCC47 cells, as the IC50 values of TriCurin for these cells are much higher (13 μM+ and 3.22 μM+, respectively) [7, 8]. Based on our observation that injected TriCurin causes a dramatic reduction in tumor load, we expect that in addition to eliciting direct tumor cell death immediately after intra-tumor injection, TriCurin would elicit its anti-tumor activity also by stimulating the immune cells, which are known to eliminate tumors [12, 13, 16, 19, 20, 41]. Our current study tests this postulate by demonstrating that TriCurin, indeed, causes intra-tumor recruitment of M1 TAM, NK cells, and CTL which are known to eliminate tumor cells [21, 26, 29, 30, 38].

Our data show that TriCurin treatment causes activation/recruitment of M1 TAM, NK cells, and CTL into pre-clinical HPV+ cervical (TC-1 implanted mice) and activation/recruitment of M1 TAM and NK cells in HNSCC (xenograft) tumors (Fig. 6) [7]. Analysis of these TriCurin-treated tumors (Fig. 1, Supplementary Fig. 2 and Supplementary Fig. 4) reveals a major shift in polarity of $ARG1^{high}$, $iNOS^{low}$, $IL10^{high}$, $IL12^{low}$ M2 TAM into the $ARG1^{low}$. $iNOS^{high}$, $IL10^{low}$, and $IL12l^{high}$ M1-type [12]. This repolarization is concomitant with repression of activated STAT3 and induction of activated STAT1 and NF-kB (p65) in the TAM (Figs. 2, 3, 4 and Supplementary Fig. 5). Activated STAT1 is known to cooperate with activated NF-kB (p65) to trigger the M1 phenotype, which is marked by the induction of IL12 and iNOS which should trigger toxic NO production by the TAM (Fig. 1, Supplementary Fig. 2, and Supplementary Fig. 4) [12, 13, 28, 30–32]. We also demonstrate TriCurin-mediated, intra-tumor recruitment of activated NK cells and CTL (Fig. 5, Supplementary Fig. 3, and Supplementary Fig. 6), which are expected to contribute to the observed tumor load reduction (Fig. 6 and Supplementary Fig. 6) [7, 16, 21]. The IL12-mediated NK cell and CTL recruitment and activation are attenuated in mice pretreated with an IL12-neutralizing antibody (Supplementary Fig. 2, Supplementary Fig. 3, and Fig. 5). This recruitment of CTL and NK cells may simultaneously cause IFNγ-mediated observed stimulation of STAT1 in macrophages and M1-type polarization of TAM, thereby enabling the activated NK cells, CTL, and the M1 TAM to act as a lethal triad against cancer cells. Corroboratively, IL12Ab-mediated abrogation of intra-tumor recruitment of activated NK cells and CTL partially eliminated the tumoricidal activity of TriCurin (Fig. 6) [13, 16, 19, 21, 29, 33, 34, 36, 38, 40].

Our data also indicate that TriCurin causes this shift in polarity by silencing the M2 TAM and activating/recruiting a discrete population of M1 TAM while maintaining a constant number of overall intra-tumor Iba1+ TAM (Fig. 1i, j, k) [12]. Interestingly, NKp46+ NK cells have been reported to eliminate M2 macrophages while causing IFNγ-mediated activation and amplification of MHC class I^{high} M1 macrophages [42]. Based on the previous findings of other groups and the data presented in this manuscript, we propose a working model in which injected TriCurin elicits a switch in TAM phenotype from a tumor-promoting M2-type into a tumoricidal M1-type. Furthermore, founded on our IL12 blocking data, we postulate that TriCurin-induced M1 TAM-derived IL12 is responsible for triggering intra-tumor recruitment of NK cells and CTL and elimination of the HPV+ cervical cancer cells (Fig. 6) [7, 8].

Treatments for HNSCC include chemotherapy and/or radiation, which are fairly toxic to the body and they deplete the tumoricidal immune cells [5, 6, 43]. Cancer immunotherapy using chimeric antigen receptor (CAR) T-cell treatment and regulators of T-cell activation (checkpoint inhibitors) has recently come into prominence but with limitations and side effects [22, 23]. Thus, there is an acute need for novel immunotherapeutic strategies to better manage the growing number of HPV+ tumors. TriCurin is highly efficient in stimulating the immune system against cancer cells and can be used as a safe immunotherapeutic agent to turn the immune system against HPV+ tumors.

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Author contributions PB, SM, QP, and LP conceived and planned the experiments. SM, RH, RW, DA, AF, SS, and LP carried out the experiments. All the authors contributed to the interpretation of results. SM and PB took the lead in writing the manuscript. All authors provided critical feedback and helped in shaping the final manuscript.

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Compliance with ethical standards

Conflict of interest Probal Banerjee has a pending patent application "Activity Enhancing Curcumin Compositions and Methods of Use", PCT/US14/67819 pending. The remaining authors declare no conflict of interest.

Ethical approval and ethical standards All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care Committees (IACUC) at the College of Staten Island (approval # 11 – 008) and The Ohio State University Medical Center (approval # 2009A0172).

Animal source Adult C57BL/6 female mice (2–6 months old) used for the TC-1 tumor experiments were bred and handled at the College of Staten Island (CUNY) [8]. Athymic nude (NCr) mice were purchased from Charles River Laboratories (Wilmington, MA) for the Head and Neck Squamous Cell Carcinoma (HNSCC) UMSCC47 tumor xenograft model and housed at The Ohio State University animal facility

[7]. Both the strains mentioned above were bred and handled in accordance to the consent of respective Institutional Animal Care Committees (IACUC) at the College of Staten Island and The Ohio State University Medical Center. All animal protocols were approved by the respective Institutional Animal Care Committees (IACUC) at the College of Staten Island and The Ohio State University Medical Center.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics CA. Cancer J Clin 61:69–90
2. Kamangar F, Dores GM, Anderson WF (2006) Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 24:2137–2150. https://doi.org/10.1200/jco.2005.05.2308
3. Fowler RS (2000) Vulvar vestibulitis: response to hypocontactant vulvar therapy. J Low Genit Tract Dis 4:200–203
4. Stanley M (2010) Potential mechanisms for HPV vaccine-induced long-term protection. Gynecol Oncol 118:2–7. https://doi.org/10.1016/j.ygyno.2010.04.002
5. Leemans CR, Braakhuis BJM, Brakenhoff RH (2011) The molecular biology of head and neck cancer. Nat Rev Cancer 11:9–22. https://doi.org/10.1038/nrc2982
6. Mirghani H, Amen F, Blanchard P, Moreau F, Guigay J, Hartl DM, Lacau St Guily J (2015) Treatment de-escalation in HPV-positive oropharyngeal carcinoma: ongoing trials, critical issues and perspectives. Int J Cancer 136:1494–1503. https://doi.org/10.1002/ijc.28847
7. Piao L, Mukherjee S, Chang Q et al (2017) TriCurin, a novel formulation of curcumin, epicatechin gallate, and resveratrol, inhibits the tumorigenicity of human papillomavirus-positive head and neck squamous cell carcinoma. Oncotarget 8:60025–60035. https://doi.org/10.18632/oncotarget.10620
8. Mukherjee S, Debata PR, Hussaini R et al (2017) Unique synergistic formulation of curcumin, epicatechin gallate and resveratrol, tricurin, suppresses HPV E6, eliminates HPV+ cancer cells, and inhibits tumor progression. Oncotarget 8:60904–60916. https://doi.org/10.18632/oncotarget.16648
9. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4:807–818. https://doi.org/10.1021/mp700113r
10. Langone P, Debata PR, Dolai S, Curcio GM, Inigo JD, Raja K, Banerjee P (2012) Coupling to a cancer cell-specific antibody potentiates tumoricidal properties of curcumin. Int J Cancer 131:E569–E578. https://doi.org/10.1002/ijc.26479
11. Langone P, Debata PR, Inigo JDR, Dolai S, Mukherjee S, Halat P, Mastroianni K, Curcio GM, Castellanos MR, Raja K, Banerjee P (2014) Coupling to a glioblastoma-directed antibody potentiates anti-tumor activity of curcumin. Int J Cancer 135:710–719
12. Mukherjee S, Baidoo J, Fried A, Atwi D, Dolai S, Boockvar J, Symons M, Ruggieri R, Raja K, Banerjee P (2016) Curcumin changes the polarity of tumor-associated microglia and eliminates glioblastoma. Int J Cancer 139:2838–2849. https://doi.org/10.1002/ijc.30398
13. Zhang X, Tian W, Cai X, Wang X, Dang X, Dang W, Tang H, Cao H, Wang L, Chen T (2013) Hydrazinocurcumin encapsuled nanoparticles "Re-Educate" tumor-associated macrophages and exhibit anti-tumor effects on breast cancer following STAT3 suppression. PLoS One 8:e65896
14. Debata PR, Castellanos MR, Fata JE, Baggett S, Rajupet S, Szerszen A, Begum S, Mata A, Murty VV, Opitz LM, Banerjee P (2013) A novel curcumin-based vaginal cream Vacurin selectively eliminates apposed himan cervical cancer cells. Gynecol Oncol. 129:145–153
15. Purkayastha S, Berliner A, Fernando SS, Ranasinghe B, Ray I, Tariq H, Banerjee P (2009) Curcumin blocks brain tumor formation. Brain Res 1266:130–138. https://doi.org/10.1016/j.brainres.2009.01.066
16. Zhang HG, Kim H, Liu C, Yu S, Wang J, Grizzle WE, Kimberly RP, Barnes S (2007) Curcumin reverses breast tumor exosomes mediated immune suppression of NK cell tumor cytotoxicity. Biochim Biophys Acta 1773:1116–1123. https://doi.org/10.1016/j.bbamcr.2007.04.015
17. Bhattacharyya S, Md Sakib Hossain D, Mohanty S et al. (2010) Curcumin reverses T cell-mediated adaptive immune dysfunctions in tumor-bearing hosts. Cell Mol Immunol 7:306–315. https://doi.org/10.1038/cmi.2010.11
18. Chang YF, Chuang HY, Hsu CH, Liu RS, Gambhir SS, Hwang JJ (2012) Immunomodulation of curcumin on adoptive therapy with T cell functional imaging in mice. Cancer Prev Res (Phila.) 5:444–452. https://doi.org/10.1158/1940-6207.capr-11-0308
19. Luo F, Song X, Zhang Y, Chu Y (2011) Low-dose curcumin leads to the inhibition of tumor growth via enhancing CTL-mediated antitumor immunity. Int Immunopharmacol 11:1234–1240. https://doi.org/10.1016/j.intimp.2011.04.002
20. Lu Y, Miao L, Wang Y, Xu Z, Zhao Y, Shen Y, Xiang G, Huang L (2016) Curcumin micelles remodel tumor microenvironment and enhance vaccine activity in an advanced melanoma model. Mol Ther 24:364–374. https://doi.org/10.1038/mt.2015.165
21. Farag SS, Caligiuri MA (2006) Human natural killer cell development and biology. Blood Rev 20:123–137. https://doi.org/10.1016/j.blre.2005.10.001
22. Postow MA, Callahan MK, Wolchok JD (2015) Immune checkpoint blockade in cancer therapy. J Clin Oncol 33:1974–1982. https://doi.org/10.1200/JCO.2014.59.4358
23. Zhang H, Ye Z-l, Yuan Z-G, Luo Z-Q, Jin H-J, Qian Q-J (2016) New strategies for the treatment of solid tumors with CAR-T Cells. Int J Biol Sci 12:718–729. https://doi.org/10.7150/ijbs.14405
24. Scharton-Kersten T, Afonso L, Wysocka M, Trinchieri G, Scott P (1995) IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. J Immunol 154:5320–5330
25. Huang Z, Peng S, Knoff J, Lee SY, Yang B, Wu T-C, Hung C-F (2015) Combination of proteasome and HDAC inhibitor enhances HPV16 E7-specific CD8+ T cell immune response and antitumor effects in a preclinical cervical cancer model. J Biomed Sci 22:7
26. Brantley EC, Guo L, Zhang C, Lin Q, Yokoi K, Langley RR, Kruzel E, Maya M, Kim SW, Kim S-J, Fan D, Fidler IJ (2010) Nitric oxide-mediated tumoricidal activity of murine microglial cells. Transl Oncol 3:380–388
27. Chakravarti N, Myers JN, Aggarwal BB (2006) Targeting constitutive and interleukin-6-inducible signal transducers and activators of transcription 3 pathway in head and neck squamous cell carcinoma cells by curcumin (diferuloylmethane). Int J Cancer 119:1268–1275
28. Ito S, Ansari P, Sakatsume M, Dickensheets H, Vazquez N, Donnelly RP, Larner AC, Finbloom DS (1999) Interleukin-10 inhibits expression of both interferon a– and interferon gamma–induced genes by suppressing tyrosine phosphorylation of STAT1. Blood 93:1456–1463
29. Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC, Balkwill FR (2008) "Re-educating" tumor-associated macrophages by targeting NF-kB. J Exp Med 205:1261–1268
30. Hagemann T, Biswas SK, Lawrence T, Sica A, Lewis CE (2009) Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB. Blood 113:3139–3146. https://doi.org/10.1182/blood-2008-12-172825

31. Ohmori Y, Hamilton TA (2001) Requirement for STAT1 in LPS-induced geneexpression in macrophages. J Leukoc Biol 69:598–604
32. Vakkila J, Demarco RA, Lotze MT (2008) Coordinate NF-kB and STAT1 activation promotes development of myeloid type 1 dendritic cells. Scand J Immunol 67:260–269
33. Marotta LLC, Almendro V, Marusyk A et al (2011) The JAK2/STAT3 signaling pathway is required for growth of CD44+ CD24– stem cell–like breast cancer cells in human tumors. J Clin Investig 121:2723–2735. https://doi.org/10.1172/JCI44745
34. Schroder K, Hertzog PJ, Ravasi T, Hume DA (2004) Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75:163–189. https://doi.org/10.1189/jlb.0603252
35. Bontkes HJ, Kramer D, Ruizendaal JJ, Meijer CJ, Hooijberg E (2008) Tumor associated antigen and interleukin-12 mRNA transfected dendritic cells enhance effector function of natural killer cells and antigen specific T-cells. Clin Immunol 127:375–384
36. Hamza T, Barnett JB, Li B (2010) Interleukin 12 a key immunoregulatory cytokine in infection applications. Int J Mol Sci 11:789–806. https://doi.org/10.3390/ijms11030789
37. Kuge S, Watanabe K, Makino K, Tokuda Y, Mitomi T, Kawamura N, Habu S, Nishimura T (1995) Interleukin-12 augments the generation of autologous tumor-reactive CD8+ cytotoxic T lymphocytes from tumor-infiltrating lymphocytes. Jpn J Cancer Res 86:135–139
38. Michel T, Hentges F, Zimmer J (2012) Consequences of the crosstalk between monocytes/macrophages and natural killer cells. Front Immunol 3:403. https://doi.org/10.3389/fimmu.2012.00403
39. Morrison BE, Park SJ, Mooney JM, Mehrad B (2003) Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. J Clin Invest 112:1862–1870. https://doi.org/10.1172/jci18125
40. Gertsch J, Guttinger M, Heilmann J, Sticher O (2003) Curcumin differentially modulates mRNA profiles in Jurkat T and human peripheral blood mononuclear cells. Bioorg Med Chem 11:1057–1063
41. Kang TH, Lee JH, Song CK et al (2007) Epigallocatechin-3-gallate enhances CD8+ T cell-mediated antitumor immunity induced by DNA vaccination. Cancer Res 67:802–811. https://doi.org/10.1158/0008-5472.CAN-06-2638
42. Bellora F, Castriconi R, Dondero A, Reggiardo G, Moretta L, Mantovani A, Moretta A, Bottino C (2010) The interaction of human natural killer cells with either unpolarized or polarized macrophages results in different functional outcomes. Proc Natl Acad Sci USA 107:21659–21664
43. Verma R, Foster RE, Horgan K, Mounsey K, Nixon H, Smalle N, Hughes TA, Carter CR (2016) Lymphocyte depletion and repopulation after chemotherapy for primary breast cancer. Breast Cancer Res 18:10. https://doi.org/10.1186/s13058-015-0669-x

PERCEIVED STRESS SCALE

by Sheldon Cohen

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If you find the **Perceived Stress Scale** useful, you might be interested in these other great Mind Garden instruments.

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by Charles D. Spielberger

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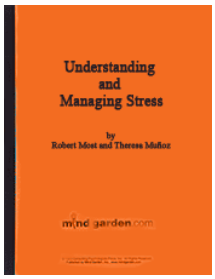

Understanding and Managing Stress

by Robert Most and Theresa Muñoz

This forty-page workbook offers individuals a comprehensive approach to managing stress. The workbook includes basic strategies for: managing daily on-the-spot stress; problem and emotion focused coping skills; and improving personal and work lifestyle; as well as resources for further exploration.

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These instruments measure anxiety or stress in a variety of situations including test anxiety, school-related stress, and anxiety as a state-like and trait-like construct. Many of these instruments are complimented by reports or workbooks that provide tips and exercises to manage stress and anxiety.

We offer such instruments as **Hassles & Uplifts** and the **Psychological Distress Profile**.

PERCEIVED STRESS SCALE

by Sheldon Cohen

The *Perceived Stress Scale* (PSS) is the most widely used psychological instrument for measuring the perception of stress. It is a measure of the degree to which situations in one's life are appraised as stressful. Items were designed to tap how unpredictable, uncontrollable, and overloaded respondents find their lives. The scale also includes a number of direct queries about current levels of experienced stress. The PSS was designed for use in community samples with at least a junior high school education. The items are easy to understand, and the response alternatives are simple to grasp. Moreover, the questions are of a general nature and hence are relatively free of content specific to any subpopulation group. The questions in the PSS ask about feelings and thoughts during the last month. In each case, respondents are asked how often they felt a certain way.

Evidence for Validity: Higher PSS scores were associated with (for example):

- failure to quit smoking
- failure among diabetics to control blood sugar levels
- greater vulnerability to stressful life-event-elicited depressive symptoms
- more colds

Health status relationship to PSS: Cohen et al. (1988) show correlations with PSS and: Stress Measures, Self-Reported Health and Health Services Measures, Health Behavior Measures, Smoking Status, Help Seeking Behavior.

Temporal Nature: Because levels of appraised stress should be influenced by daily hassles, major events, and changes in coping resources, predictive validity of the PSS is expected to fall off rapidly after four to eight weeks.

Scoring: PSS scores are obtained by reversing responses (e.g., 0 = 4, 1 = 3, 2 = 2, 3 = 1 & 4 = 0) to the four positively stated items (items 4, 5, 7, & 8) and then summing across all scale items. A short 4 item scale can be made from questions 2, 4, 5 and 10 of the PSS 10 item scale.

Norm Groups: L. Harris Poll gathered information on 2,387 respondents in the U.S.

Norm Table for the PSS 10 item inventory

Category	N	Mean	S.D.
Gender			
Male	**926**	**12.1**	**5.9**
Female	**1406**	**13.7**	**6.6**
Age			
18-29	**645**	**14.2**	**6.2**
30-44	**750**	**13.0**	**6.2**
45-54	**285**	**12.6**	**6.1**
55-64	**282**	**11.9**	**6.9**
65 & older	**296**	**12.0**	**6.3**
Race			
white	**1924**	**12.8**	**6.2**
Hispanic	**98**	**14.0**	**6.9**
black	**176**	**14.7**	**7.2**
other minority	**50**	**14.1**	**5.0**

PERCEIVED STRESS SCALE

The questions in this scale ask you about your feelings and thoughts during the last month**. In each case, you will be asked to indicate by circling *how often* you felt or thought a certain way.**

Name _____ Date _____

Age _____ Gender (*Circle*): **M F** Other _____

0 = Never 1 = Almost Never 2 = Sometimes 3 = Fairly Often 4 = Very Often

1. In the last month, how often have you been upset because of something that happened unexpectedly?	0	1	2	3	4
2. In the last month, how often have you felt that you were unable to control the important things in your life?	0	1	2	3	4
3. In the last month, how often have you felt nervous and "stressed"?	0	1	2	3	4
4. In the last month, how often have you felt confident about your ability to handle your personal problems?	0	1	2	3	4
5. In the last month, how often have you felt that things were going your way?	0	1	2	3	4
6. In the last month, how often have you found that you could not cope with all the things that you had to do?	0	1	2	3	4
7. In the last month, how often have you been able to control irritations in your life?	0	1	2	3	4
8. In the last month, how often have you felt that you were on top of things?	0	1	2	3	4
9. In the last month, how often have you been angered because of things that were outside of your control?	0	1	2	3	4
10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

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References

The PSS Scale is reprinted with permission of the American Sociological Association, from Cohen, S., Kamarck, T., and Mermelstein, R. (1983). A global measure of perceived stress. *Journal of Health and Social Behavior, 24,* 386-396.

Cohen, S. and Williamson, G. Perceived Stress in a Probability Sample of the United States. Spacapan, S. and Oskamp, S. (Eds.) *The Social Psychology of Health.* Newbury Park, CA: Sage, 1988.