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# **$\gamma$ -Tocotrienol Reversal of Epithelial-to-Mesenchymal Transition in Human Breast Cancer Cells is Mediated through a Suppression of Canonical Wnt and Hedgehog Signaling**

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Additional information is available at the end of the chapter

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## **Abstract**

$\gamma$ -Tocotrienol, a natural isoform within the vitamin E family of compounds, displays potent antiproliferative, apoptotic and reversal of epithelial-to-mesenchymal transition (EMT) activity against breast cancer using treatment doses that have little or no effect on normal cell viability. EMT is a route by which epithelial cells undergo various biochemical alterations leading to the acquisition of mesenchymal traits. Several aberrant signaling pathways are involved in EMT-dependent cancer metastasis. Specifically, dysregulation of the canonical Wnt and Hedgehog pathways are intimately involved in promoting breast cancer EMT and metastasis. Therefore, studies were conducted to examine effects of  $\gamma$ -tocotrienol on Wnt and Hedgehog signaling. Results from these studies demonstrate that  $\gamma$ -tocotrienol significantly inhibits canonical Wnt and Hedgehog signaling by inhibiting receptors, co-receptors and ligand expression, as well as inhibiting expression of cytosolic and nuclear signaling proteins within these pathways. Additional studies showed that  $\gamma$ -tocotrienol treatment increased the expression of negative regulators of both the Wnt and Hedgehog pathways. These findings demonstrate that  $\gamma$ -tocotrienol reversal of EMT is mediated, at least in part, through the inhibition of canonical Wnt and Hedgehog signaling, and strongly suggest that this form of vitamin E may provide significant benefit in the prevention and treatment of metastatic breast cancer.

**Keywords:**  $\gamma$ -tocotrienol, epithelial-to-mesenchymal transition, canonical Wnt pathway, canonical hedgehog pathway, breast cancer

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## 1. Introduction

Breast cancer is the second leading cause of death in women, and it originates from malignant breast cancer cells displaying unregulated growth to produce a tumor mass [1, 2]. Several cellular mechanisms are dysregulated in breast tumor cells, including the canonical Wnt and Hedgehog signaling pathways, which play an important role in promoting oncogenic proliferation, survival, motility, invasion, and epithelial-to-mesenchymal transition (EMT) [3]. Although these events are complex and poorly understood, recent findings show that specialized cell membrane microdomains known as lipid rafts are involved in mediating membrane receptor activation and signal transduction. Lipid rafts are solid platforms in the plasma membrane that consist of cholesterol and sphingolipids. Lipid rafts are essential for cellular signaling by recruiting transmembrane receptors with adaptor and signaling proteins from non-rafts to the raft area of the cell membrane [4–6]. In the case of canonical Wnt and Hedgehog signaling, low-density lipoprotein receptor-related protein 6 (LRP6) and patched (PTCH2), the main receptors for activation of these signaling pathways, were shown to be primarily located in the lipid raft microdomain [7–9]. Lipid rafts have been shown to be essential for Hedgehog signal transduction [10].  $\gamma$ -Tocotrienol is a natural vitamin E isoform that displays potent anticancer activities [11–13]. Previous reports have clearly shown that  $\gamma$ -tocotrienol exerts antiproliferative and apoptotic activity against neoplastic mammary epithelial cells at treatment doses that had little or no effect on normal cell growth and viability [14, 15]. The anticancer effects of  $\gamma$ -tocotrienol appear to be mediated through a variety of intracellular signaling mechanism [16–18]. Recently,  $\gamma$ -tocotrienol was found to disrupt lipid raft integrity and attenuation of receptor signaling transduction [19]. This chapter will focus of experimental evidence demonstrating  $\gamma$ -tocotrienol reversal of EMT is mediated through the inhibition of the canonical Wnt and Hedgehog signaling pathways.

## 2. Vitamin E and breast cancer

Epidemiological studies have shown that diet and nutrition can play a major role in cancer development and progression. It has been suggested that approximately 30–35% of cancer morbidity and mortality might be prevented with suitable adjustment of nutrition, and up to one third of all the cancers in the United States can be avoided by increasing the consumption of fruits and vegetables in the daily diet [20]. Vitamin E is a generic term that includes a family of eight naturally occurring compounds that are further divided into two subgroups known as tocotrienols and tocopherols. Tocotrienols are relatively rare and found only a few natural sources, such as palm oil, rice bran oil, and annatto bean, while tocopherols are much more abundant and found in a wide variety of foods, such as nuts, whole grains, dark green vegetables, egg yolk, and various vegetable oils [21–24]. The relative levels of tocopherol and tocotrienol in various dietary oil and fats are shown in **Table 1**.

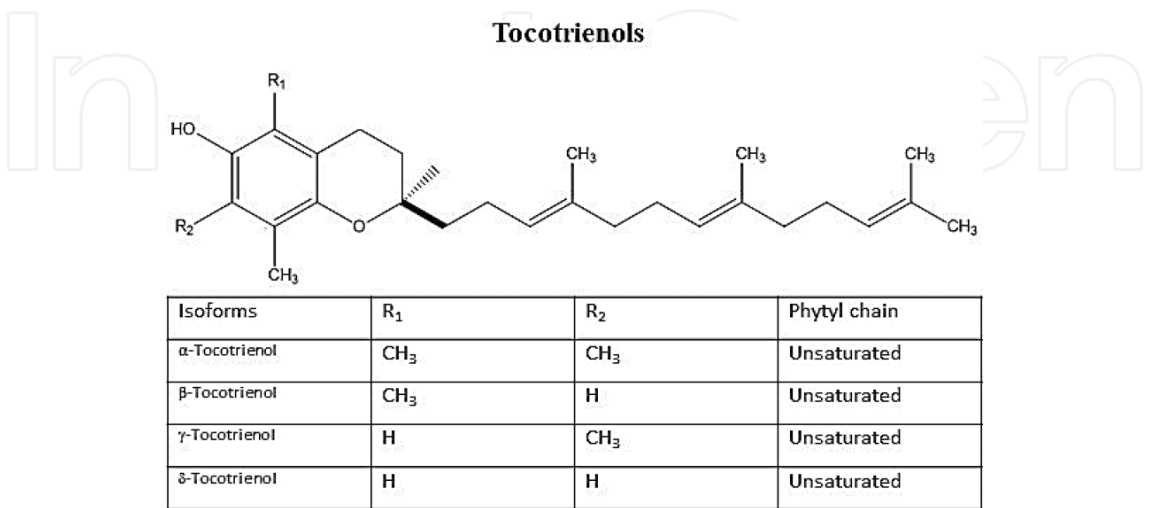
The chemical structure of all vitamin E isoforms are very similar and characterized by a long phytyl chain linked to a chroman ring structure methylated to varying degrees at the 5, 7, and 8 positions. The four isoforms in each subclass are classified as  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocotrienol

Dietary oil	Tocopherol	Tocotrienols			Total tocotrienol
	α	α	γ	δ	
Palm	152	205	439	94	738
Rice brain	324	236	349	–	586
Wheat germ	133	26	–	–	26
Coconut	5	5	1	19	25
Palm-kernel	12	21	–	–	21
Coco butter	11	2			2
Corn	112	–	–	–	0
Cottonseed	389	–	–	–	0
Peanut	130	–	–	–	0
Olive	51	–	–	–	0
Safflower	387	–	–	–	0
Soybean	101	–	–	–	0
Sunflower	487	–	–	–	0

**Table 1.** Vitamin E levels (mg/L) in common dietary oils [21].

or tocopherol. Tocotrienols differ from tocopherols only in that they contain an unsaturated, whereas tocopherols contain a saturated phytyl tail. **Figure 1** shows the chemical structures of different isoforms of tocotrienols.

Interestingly, numerous studies have shown that tocotrienols, but not tocopherols, have selective antiproliferative and apoptotic effects against various forms of breast cancer, while have little effect on normal mammary epithelial cell growth or function [14, 15, 25]. The anticancer



**Figure 1.** General chemical structure of the different tocotrienols isoforms.

potency of different tocotrienol isoforms was determined to be characterized as  $\delta$ -tocotrienol  $\geq \gamma$ -tocotrienol  $> \alpha$ -tocotrienol  $> \beta$ -tocotrienol [11, 15, 26]. The anticancer effects of tocotrienols were discovered in nutritional studies that investigated the role of high-dietary fat consumption on the development of mammary tumorigenesis in laboratory animals. These studies showed that diets containing high levels of palm oil inhibited the carcinogen-induced mammary cancer in rats [27]. Additional studies showed that palm oil diets stripped of tocotrienol no longer displays their protective effect against mammary tumorigenesis.

During the past decade, tocotrienols have received a great deal of attention because of their potential value in the prevention and treatment of breast cancer. Tocotrienols have been shown to inhibit multiple intracellular signaling pathways in cancer cells [15, 28]. Specifically, tocotrienols have been found to suppress EGF-dependent mitogenic signaling in neoplastic and normal mammary epithelial cells by significantly inhibiting activity of the phosphatidylinositol-4, 5-bisphosphate-3-kinase/protein kinase B (PI3K/Akt) pathway [29]. Other studies have shown that  $\gamma$ -tocotrienol treatment induced a dose and time-dependent inhibition of EGF-dependent Akt phosphorylation (activation) in mammary tumor cells, and these effects were not found to be associated with an increase in tensin homolog (PTEN) or protein phosphatase 2 A (PP2A) activity [30].  $\gamma$ -Tocotrienol was also found to decrease activity of signaling proteins downstream of Akt, such as inhibiting the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cell (NF $\kappa$ B) by suppressing the activation of inhibitor of nuclear factor kappa kinase alpha and beta (IKK $\alpha$  and IKK $\beta$ ), enzymes associated with induction of the NF $\kappa$ B activation [30]. Inhibition of NF $\kappa$ B transcription is associated with a suppression in cell proliferation and survival [31]. Additional studies have shown that the antiproliferative effects of tocotrienols is associated with an inhibition of protein kinase C alpha (PKC $\alpha$ ) activation in breast cancer cells [32]. In addition, mitogen activated protein kinase (MAPK) has also been shown to be a target of  $\gamma$ -tocotrienol anticancer activity. Studies have indicated that  $\gamma$ -tocotrienol induced inhibition of EGF-dependent proliferation of preneoplastic CL-S1 mouse mammary epithelial cells resulted from an inhibition of G-protein-mediated activation of adenylyl cyclase, cyclic adenosine monophosphate (cAMP) production, as well as a reduction in phosphorylated (activated) extracellular signal-regulated kinase 1/2 (ERK1 and ERK2) [33]. In addition to the inhibition of mitogenic signaling,  $\gamma$ -tocotrienol is known to inhibit numerous vital cellular functions including inhibition of cell cycle progression [13], mevalonate pathway [34, 35], glycolysis [12], angiogenesis [36], and epithelial mesenchymal transition (EMT) [37].

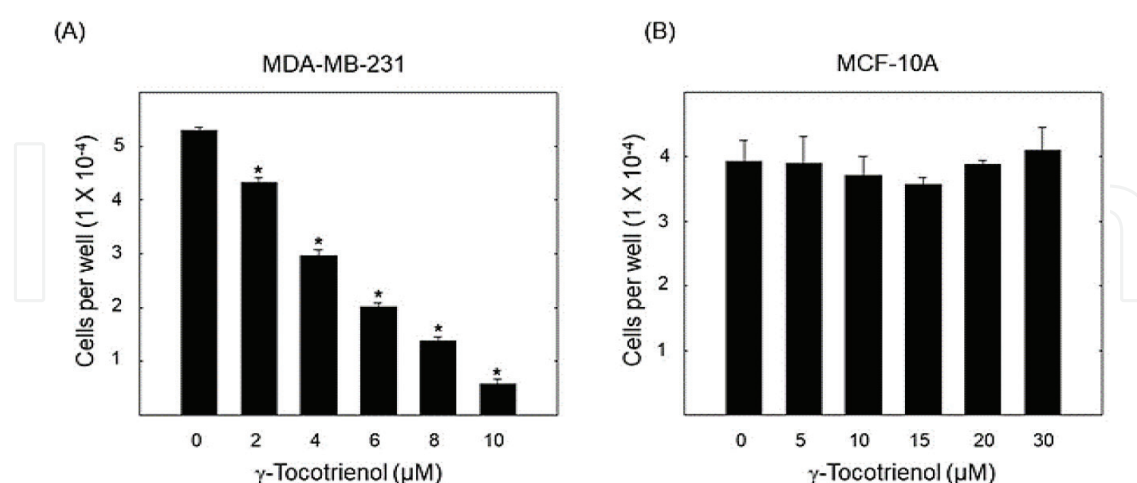
Lipid rafts are distinct structures within the cell membrane that are enriched with sphingolipids, cholesterol, and acyl fatty acid chains that act to form a very rigid microdomain. Lipid rafts exist in two different forms: "planar lipid rafts," which are referred to as "non-caveolar" and caveolae lipid rafts. Planar rafts are characterized as non-invaginated microdomains lacking specific morphological features. In contrast, caveolae lipid rafts are tube-like invaginations of the plasma membrane characterized by specific scaffolding proteins or caveolins [4]. Some proteins are essential to membrane raft development and their role can be seen as constitutive components of rafts. One of the important proteins serving scaffolding functions in the caveolar raft is caveolin 1 (Cav1), a classical hairpin protein that plays a role in caveolae-mediated signaling, endocytosis, and transport [4]. Recent studies have shown that tocotrienols act to disrupt lipid raft integrity and disrupt plasma receptor membrane receptor activation and signal transduction. These findings provide evidence to explanation the wide range of inhibitory

effects γ-tocotrienol has on numerous signaling pathways [19]. Molecular targets associated with tocotrienol anticancer activity are shown in **Table 2**.

**Figure 2** shows the effects of γ-tocotrienol treatment on the growth of malignant and normal human breast cancer cells. Results show that exposure various doses of γ-tocotrienol induced a dose-dependent inhibition in the growth of the highly malignant MDA-MB-231 breast cancer cells, as compared to cells in the vehicle-treated control group in **Figure 2A**. The  $IC_{50}$  dose γ-tocotrienol in these studies was found to be approximately 5 μM. However, treatment with similar or even higher doses of γ-tocotrienol on immortalized normal MCF-10A mammary epithelial cell line was found to have little or no effect on cell growth or viability (**Figure 2B**) [14].

Molecular target	References
PI3K/Akt	[29]
PKCα	[32]
MAPK	[33]
Cell cycle	[13]
Mevalonate pathway	[34, 35]
Glycolysis	[12]
Angiogenesis	[36]
EMT	[37]
Lipid rafts	[19]

**Table 2.** Summary of some of the molecular targets associated with mediating the anticancer effects of tocotrienols.



**Figure 2.** γ-Tocotrienol effects on the growth of the highly malignant MDA-MB-231 human breast cancer cells and the immortalized normal MCF-10A human mammary epithelial cells. MDA-MB-231 and MCF-10A cells were initially seeded at a density of  $1 \times 10^4$  cells/well (6 wells/group) in 96-well culture plates and maintained on serum-free defined media containing 0–30 μM doses of γ-tocotrienol over a 4-day culture period. The viable cell number was determined by using the MTT colorimetric assay. Vertical bars show mean cell number  $\pm$  SEM in each treatment group. (\* $P < 0.05$ ) as compared with cells in their respective vehicle-treated control groups.



### 3. Epithelial-to-mesenchymal transition (EMT)

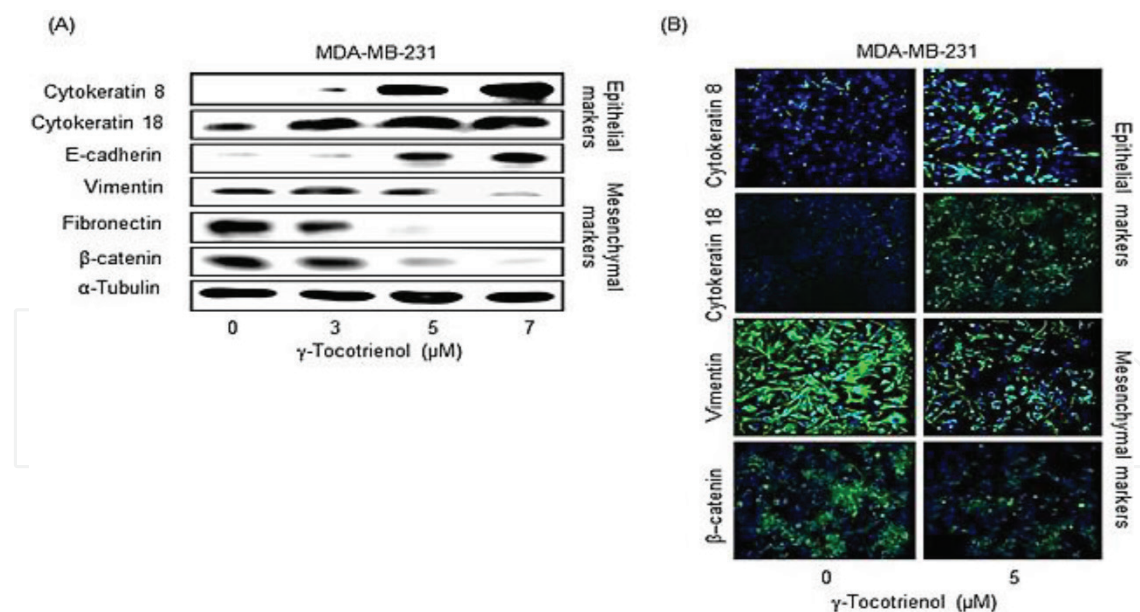
EMT plays a major role in organogenesis, angiogenesis and cancer metastasis [38, 39]. EMT was first observed and defined in the late 1980s at Harvard University by Elizabeth Hey [40]. EMT was defined as a differentiation program by which epithelial cells lose their attachment with other epithelial cells to become more mesenchymal-like, and are able to become mobile and invade their surrounding extracellular matrix [41, 42]. Because this process is reversible [43], epithelial cells displaying a mesenchymal phenotype, are also able to re-differentiate back into their epithelial phenotype [44]. Epithelial cells displaying their normal epithelial phenotype are well-structured in single layers of cuboidal or columnar cells. They are closely attached to surrounding cells by intercellular adhesion complexes. These cells also display an apicobasal polarity with a characteristic basal basement membrane that separates the epithelium from other tissues. In contrast, epithelial cells with a mesenchymal phenotype are characterized by the absence of polarity and intercellular adhesion junctions, hallmarks that have come to define EMT [45]. During the EMT process, cells lose the attachment of  $\beta$ -catenin and E-cadherin, which act to tightly link and attach surrounding epithelial cells together. This loss of attachment leads to a disruption of the adherens junctions [46]. These events then allow the mesenchymal phenotype to move freely and invade the surrounding extracellular matrix. In normal conditions, EMT provides a necessary function during embryogenesis, growth and wound healing. However, aberrant EMT can result in pathological conditions such as organ fibrosis and cancer metastasis. EMT mediated metastasis of malignant breast cancer epithelial cells can often form secondary tumors in the bone or lung [46]. EMT that occurs under normal conditions, such as embryogenesis, is referred to as type 1 EMT or classical EMT [47]. However, EMT that develops during inflammation, wound healing, tissue regeneration, and organ fibrosis is referred to as type 2 EMT, whereas EMT associated with cancer metastasis is termed type 3 EMT and plays an important role in the development, growth and progression of breast cancer [47].

Transcription factors also play a role in the initiation of EMT. Receptor activation by various growth factors, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) are involved in the activation of various transcription factors involved in EMT. Growth factor-induced activation of transcription factors include zinc finger protein snail 1 (SNAIL1), (SNAIL2), zinc finger e-box-binding homeobox 1 (ZEB1), (ZEB2), twist, forkhead box protein 1 (FOXC1), (FOXC2), transcription factor 3 (TCF3), also known as (E47), and homeobox protein goosecoid (GSC) [37, 43, 45]. EMT also plays a role in the restructuring of extracellular matrix proteins by up-regulating fibronectin, collagen, proteases like MMPs, and other remodeling enzymes. In addition, autocrine and paracrine secretion of growth factors, cytokines, and extracellular proteins can modulate cancer cells phenotype and promote EMT [37, 43, 45].

Epigenetic modification, such as acetylation or methylation of the DNA, also can play a role in the EMT activation. For example, methylation of arginine (R531) by protein arginine methyltransferases 7 (PRMT7) plays a crucial role in inducing the EMT and the promotion of migratory and invasive behavior of breast cancer cells [48]. EMT can also be activated by expression of certain miRNAs, such as micro-RNA200/205 family (miRNA200) and (miRNA205), whose

prominent targets are the ZEB1 and ZEB2, known as specific repressors of E-cadherin. Likewise, members of the ABC family of transporters, such as ABCB5, plays a major role in the activation of EMT [49]. Studies have shown that several signaling pathways, including the canonical Wnt pathway, the canonical Hedgehog pathway, Notch pathway, Janus kinase (JAK)/STAT pathway, and TGFβ pathway are involved in the activation of EMT [50]. Activation of these EMT-inducing signaling pathways leads to the disruption of adherens junctions (desmosomes), tight junctions, and gap junctions through suppression of several proteins, such as partitioning defective 6 homolog alpha or ZEB1, which represses plakophilin, an important junctional adhesion protein [43]. These pathways can act separately or together through cross-talk to increase cancer cell migration, invasion, drug resistance, stemness, and self-renewal potential [51, 52]. Taken together, it is clearly evident that EMT is an extremely complex process and a great deal more information is required to fully understand this phenomenon.

**Figure 3** shows the effects of γ-tocotrienol on the expression of EMT cellular biomarkers in the highly malignant MDA-MB-231 human breast cancer cells. Western blot analysis shows that MDA-MB-231 cells in the vehicle-treated control group displayed relatively low levels of expression for the epithelial cell markers cytokeratin 8, cytokeratin 18 and E-cadherin, and corresponding high levels of expression for the mesenchymal cell markers vimentin, fibronectin and total β-catenin (**Figure 3A**). Treatment with 3–7 μM γ-tocotrienol (MDA-MB-231) induced a dose-responsive reversal in epithelial versus mesenchymal cell marker expression (**Figure 3A**). Immunocytochemistry was then performed to confirm the finding in **Figure 3A**.



**Figure 3.** γ-Tocotrienol effects on epithelial versus mesenchymal cell markers expression. (A) Whole cell lysates were prepared from cells in each treatment group for subsequent separation by polyacrylamide gel electrophoresis (35 μg/lane) followed by western blot analysis. (B) Immunocytochemical analysis was done to confirm the finding shown in A. Cells in the various treatment groups were fixed, blocked, and incubated with specific primary antibodies for cytokeratin 8, cytokeratin 18, vimentin, and total level of β-catenin followed by incubation with Alexa Fluor 488-conjugated secondary antibody. Green staining in the photomicrographs (magnification 200×) indicates positive fluorescence staining for target proteins and the blue color represents counter staining of the cell nuclei with DAPI.



MDA-MB-231 cells in the vehicle-treated control group displayed a relatively low level of positive immunofluorescence staining for the epithelial cell marker cytokeratin 8, cytokeratin 18, and a relatively high level of positive immunofluorescence staining for the mesenchymal markers vimentin and  $\beta$ -catenin (**Figure 3B**). Treatment with 5  $\mu$ M  $\gamma$ -tocotrienol resulted in a reversal of positive immunofluorescence staining of epithelial versus mesenchymal cell markers in MDA-MB-231 cells (**Figure 3B**) [14].

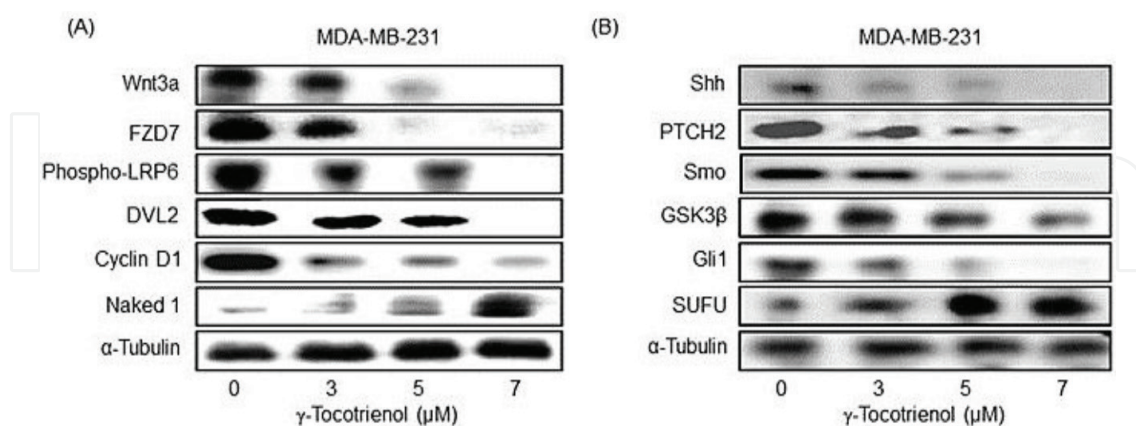
#### 4. Canonical Wnt pathway

The canonical Wnt pathway is one of the fundamental pathways that is overexpressed in cancer metastasis that is involved in the initiation of EMT [53, 54]. Wnt is an acronym derived from two proto-oncogene wingless and intel 1. At present, 19 Wnt ligands have currently been identified [55]. Those ligands form a large group of secreted glycoprotein that are secreted through autocrine and/or paracrine mechanisms. After DNA transcription and translation takes place, protein is translocated to the endoplasmic reticulum (ER), where a lipid tail is attached to the ligand by porcupine enzyme. Then, the ligand is transported to Golgi apparatus by Wntless/evensen enzymes. At the Golgi, a sugar moiety is linked to the ligand, facilitating its translocation to the ECM and binding to the receptors, respectively. Once the Wnt ligand is in the ECM, numerous proteins, such as dickkopf (DKK1), sclerostin (SOST), secreted frizzled-related protein (SFRP), and Wnt inhibitory factor 1 (WIF1), play a role to block the signal. In contrast, other proteins, such as R-spondin (RSPO) and norrin can stimulate Wnt signaling [56]. Wnt pathway co-receptors are located in the lipid rafts which are microdomains in the cell membrane needed for the stimulation of signal transduction [7]. A number of proteins, such as glycosaminoglycan, dally and dly, are responsible for handling the Wnt ligand to the lipid rafts [55]. Ligand then bind to the co-receptors and induce activation of the signaling pathway [55].

The Wnt pathway can be stimulated canonically and non-canonically [57]. Nevertheless, the critical and most studied pathway is the canonical Wnt pathway, known to have a role in triggering EMT [58]. When there is no need for any developmental process, this pathway remains inactive and the receptor ligand is sequestered in the extracellular matrix by the action of number of specific binding proteins. Bound ligand to the Wnt receptor is inactive and prevents to a reduction in the phosphorylation of the disheveled protein (DVL), which is known to inhibit the cytosolic complex. The cytosolic complex composed of several proteins, such as glycogen synthase kinase 3 beta (GSK3 $\beta$ ), axin 1, adenomatous polyposis coli (APC), and casein kinase 1 alpha (CK1 $\alpha$ ). The kinases in this complex remain active to phosphorylate the majority of the  $\beta$ -catenin, a biomarker for the canonical Wnt pathway activation. Phosphorylated  $\beta$ -catenin is then targeted for degradation by proteasomal enzymes [59]. In the nucleus,  $\beta$ -catenin is translocated out of the nucleus by the action of APC, Ran, and Manchette-associated binding adaptor protein 3 (BP3). The T-cell factor/lymphoid enhancer factor (LEF/TCF) area in the DNA is the binding location of  $\beta$ -catenin and is hidden by Groucho, histone deacetylase (HDAC), and glucose transporter-binding protein (GtBP) as a mechanism to rid the cell of  $\beta$ -catenin activity. Finally, the rest of  $\beta$ -catenin in the nucleus is sequestered by Chibby (CBY) and inhibitor of  $\beta$ -catenin and TCF 4 (ICAT) [60]. The summation of these events results in the blockade of Wnt signaling and downstream gene expression and mitogenesis.

However, during conditions of Wnt activation, such as during wound healing, the pathway becomes acutely active during the healing process. During this time, Wnt ligands are translocated to the extracellular matrix where they bind to their receptor and co-receptors, which ultimately leads to phosphorylation of DVL. Phosphorylated DVL block GSK3 $\beta$  activity in the cytosolic complex. As a result,  $\beta$ -catenin will not be phosphorylated and no longer targeted for degradation. The stabilized  $\beta$ -catenin can now be translocated from the cytosol into the nucleus and induce transcription [61]. There are also numerous other proteins such as CREB-binding protein (CBP), polymerase associated factor 1 (PAF1), and Brahma (Brm), which work together as transcription factors to potentiate the Wnt signaling pathway [60]. Activation of this pathway leads to increase cyclin D1 expression, which is associated with cell cycle progression and growth. Similarly, an increase in myelocytomatosis (c-Myc) expression as a result of Wnt activation leads to increased cell proliferation and increase in MMP9 expression, which is involved in the disruption of the tight junctions [62]. An increase in snail and slug expression leads to a loss of the attachment of  $\beta$ -catenin and E-cadherin and the progression of EMT [63]. However, after the wound is healed and Wnt signaling is no longer needed, a negative feedback effect can occur by the action of certain proteins, such as DKK1 and axin 2, and represents highly controlled gene expression and cell growth [64]. However, cancer cells are characterized by an increased expression of Wnt ligands, as well as has numerous proteins in the cytosolic complex, such as  $\beta$ -catenin, APC, or axin 1, that can become mutated. These factors lead to the continuous activation of the Wnt pathway and is associated with increased tumor growth, motility, invasion and metastasis [7, 65].

**Figure 4** shows the effects of  $\gamma$ -tocotrienol treatment on the relative levels of signaling and regulatory proteins within the canonical Wnt and Hedgehog pathways. Total levels of the Wnt3a, FZD7 receptor, phosphorylated-LRP6 (active form), DVL2 and cyclin D1 were highly expressed in the vehicle-treated MDA-MB-231 cell line with corresponding relatively low expression of



**Figure 4.** Western blot analysis of  $\gamma$ -tocotrienol effects on the canonical Wnt and Hedgehog major regulatory proteins. (A) Highly malignant MDA-MB-231 human breast cancer cells were initially seeded at density of  $1 \times 10^6$  cells/100 mm dish and maintained on serum-free defined media containing different doses of  $\gamma$ -tocotrienol over a 4-day culture period. Following treatment exposure, whole cell lysates were prepared from MDA-MB-231 in each treatment group for consequent separation by polyacrylamide gel electrophoresis (35  $\mu$ g/lane) followed by western blot analysis for the major regulatory proteins of the Wnt pathway. (B) Whole cell lysates were prepared then subjected to polyacrylamide gel electrophoresis (30  $\mu$ g/lane) and western blot analysis for detection of Shh ligand, PTCH2, Smo, GSK3 $\beta$ , Gli1 and SUFU levels within the Hedgehog pathway.

Naked 1 (a negative regulator of Wnt pathway (**Figure 4A**). Treatment with 3–7  $\mu\text{M}$   $\gamma$ -tocotrienol (MDA-MB-231) induced a dose-dependent decline in Wnt3a, FZD7 receptor, phosphorylated-LRP6, DVL2, cyclin D1 levels, and a corresponding increase in Naked 1 level as compared to cells in their respective vehicle-treated control groups (**Figure 4A**). These findings indicate that  $\gamma$ -tocotrienol inhibition of EMT is mediated in part by a suppression of canonical Wnt signaling. Similar results were observed in T47-D breast cancer line (data not shown). Previous studies have shown that inhibition of Wnt signaling resulted in a reduction in nuclear factor erythroid 2-related factor 2 (Nrf2) activity, a transcription factor associated with the promotion of EMT [66–68]. At present, it is not known if  $\gamma$ -tocotrienol reversal of EMT involves a corresponding decrease in Nrf2 activity. Additional studies are required to determine if Nrf2 plays a role in the anticancer effects of  $\gamma$ -tocotrienol. In summary, experimental evidence strongly suggests that  $\gamma$ -tocotrienol therapy may provide therapeutic value in the treatment of highly malignant breast cancer that is characterized by aberrant canonical Wnt signaling.

## 5. Canonical Hedgehog pathway

The canonical Hedgehog pathway is characteristically over active in many forms of metastatic breast cancer and is associated with enhanced migration, invasion, stemness and self-renewal of cancer cells [69–72]. Over activity of the Hedgehog pathway is also associated with playing a role in promoting EMT [71]. The Hedgehog ligand was first discovered in the *Drosophila* fruit fly [73]. Several human Hedgehog ligands have also been identified that are involved in cell growth and controlled organ formation by insuring that the tissue reaches the accurate size and position. In the adult, this pathway normally remains quiescent. However, activation of the Hedgehog pathway may be triggered during tissue maintenance and regeneration [74]. A link between Hedgehog signaling and developmental defects was first discovered in 1996, and later that year a link between Hedgehog signaling and cancer was found when the tumor suppressor gene patched (PTCH) was discovered [67]. Soon afterwards, the Hedgehog cell service signaling transducer, smoothened (Smo), was discovered and found to have the potential to function as an oncogene. These findings lead to the development of the Hedgehog pathway inhibitor, cyclopamine, and successful clinical trials using cyclopamine and similar agents followed [75]. Hedgehog ligands are produced by three different genes. The first gene is the Indian Hedgehog (Ihh) and is found in gut, skeletal muscle, and chondrocytes [76]. The second gene is the Desert Hedgehog (Dhh) and is expressed in the testis [76].

The third gene is the Sonic Hedgehog (Shh) and is involved in many developmental processes [76]. Shh is called a morphogen since the signal of this ligand relies on its concentration [77]. The Shh is produced from zone of polarizing activity (ZPA), which is located on the posterior side of the limb bud in the embryo [78]. The Hedgehog pathway has a link with the formation of specific types of humans cancer [74]. After the transcriptional and translational process occur, this ligand is secreted as a precursor protein, and the ligand is subsequently subjected to several post translational modifications [73]. Autocatalytic cleavage then splits the ligand into two parts. One part is the signaling molecule, while the other part appears to have no function. A cholesterol molecule and palmitic acid moiety are then added to C-terminal and

N-terminal of the signaling piece, leading to an increase in its hydrophobicity, localization and binding to the receptor [73]. The canonical Hedgehog signaling pathway has several vital components which play a role in modulating signal intensity. Most of the components within the Hedgehog pathway include the Hedgehog ligand, the PTCH receptor, Smo, and the cytosolic complex and downstream effectors, which consist of suppressor of fused (SUFU) and Gli family of proteins. The Gli family is an important component of the Hedgehog pathway which is divided into three forms known as Gli1, Gli2, and Gli3. Gli transcription factors can activate the signal, have dual function to stimulate or impede the signal [79]. A number of kinases, such as GSK3 $\beta$ , CK1 $\alpha$ , and protein kinase A, are known to be essential in the regulation of Hedgehog signaling [80]. The PTCH receptor of this pathway is located in the lipid raft microdomains of the plasma membrane [8].

Activation of the Hedgehog pathway can be blocked in the absence of ligand expression or a lack of mutation in PTCH and/or Smo [79]. In such cases, the inhibitory effect of PTCH on Smo is intact and Hedgehog ligand transport to the cell membrane is prevented and receptor activation and signal transduction does not occur [79]. In contrast, activation of the Hedgehog pathway will result in conditions when the Hedgehog ligand is highly expressed, and/or when mutation of PTCH and/or Smo occurs [79]. In these conditions, inhibitory effect of PTCH on Smo is absent and Smo can freely travel to the cell membrane, leading to the phosphorylation of SUFU and the transcription factor in the cytosolic complex. Once this occurs, Gli separates from the cytosolic complex proteins and then translocates into the nucleus where it promotes an increase in the Hedgehog target gene expression [79]. Recent studies have shown a direct connection between EMT and stemness of breast cancer resulting that is directly associated with the activation of the canonical Hedgehog signaling and the development of tumor recurrence and metastasis [71].

**Figure 4B** shows the effects of  $\gamma$ -tocotrienol treatment on signaling protein levels and activation within the Hedgehog pathways. Results show that the Hedgehog Shh ligand is relatively high in MDA-MB-231 breast cancer cells in the vehicle-treated control group. Similarly, PTCH2 receptor, Smo, GSK3 $\beta$ , and Gli1 were highly expressed, while the inhibitor for Hedgehog signaling SUFU displayed a relatively low level of expression in the vehicle-treated MDA-MB-231 human breast cancer cells (**Figure 4B**). Treatment with  $\gamma$ -tocotrienol induced a dose-dependent decrease in Shh ligand expression, as well as a dose-responsive reduction in PTCH2 receptor, Smo, GSK3 $\beta$ , and Gli1, and a corresponding increase in SUFU protein levels, as compared to MDA-MB-231 cells in the vehicle-treated control group (**Figure 4B**). These data indicated that  $\gamma$ -tocotrienol inhibition of EMT is also mediated by a suppression of canonical Hedgehog pathway and provides further evidence that  $\gamma$ -tocotrienol treatment may provide significant benefit in the treatment of metastatic breast cancer.

## 6. Conclusion

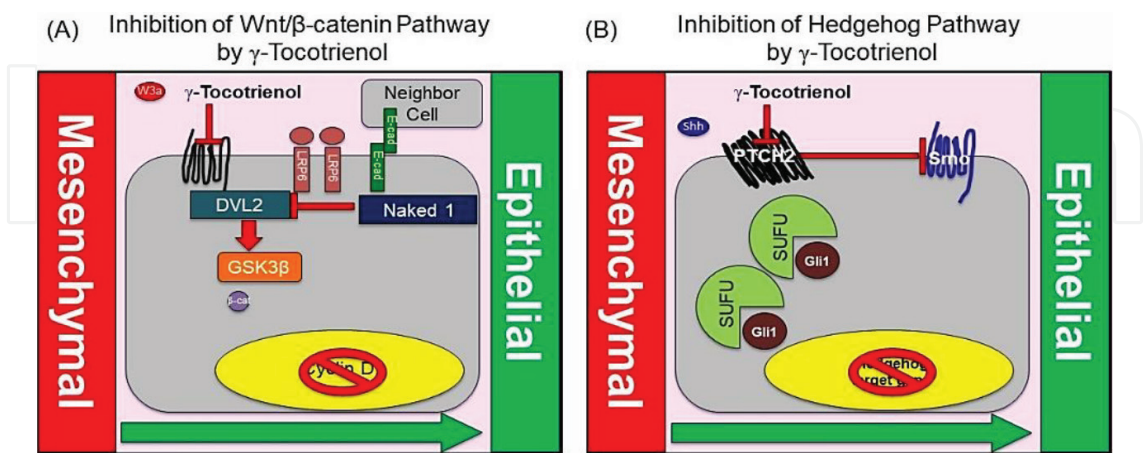
Results from these reports show that treatment with 0–5  $\mu$ M  $\gamma$ -tocotrienol induced a significant dose-dependent inhibition of highly malignant MDA-MB-231 human breast cancer



cell growth after a 4-day culture period. Furthermore, canonical Wnt and Hedgehog signaling are highly expressed in these triple negative breast cancer cells, and  $\gamma$ -tocotrienol growth inhibitory effects are associated with a reduction in Wnt and Hedgehog signaling and regulatory proteins. Since  $\gamma$ -tocotrienol also induces a reversal of EMT in these cells and canonical Wnt and Hedgehog signaling pathways are involved in promoting EMT, it can be concluded that  $\gamma$ -tocotrienol inhibition of EMT is mediated by a corresponding reduction in canonical Wnt and Hedgehog signaling in malignant MDA-MB-231 human breast cancer cells. This hypothesis is further evidenced by the finding that  $\gamma$ -tocotrienol inhibition of Wnt and Hedgehog signaling and reversal of EMT is associated with a significant decrease in migration, invasion and stemness of these cells [12].

Metastasis is still the primary cause for the mortality (90%) in cancer patients with cancer. While a great deal of progress has been recently achieved in the further understanding of the molecular and cellular mechanisms involved in the metastatic process, these mechanisms are not completely understood and clinical therapies for the management and treatment of metastatic cancer remains insufficient. Expanding knowledge in gene expression, cellular behavior, and biological events of cancer cells will provide important and novel insights for the treatment of metastatic breast cancer. New biomarkers in areas, such as EMT will provide innovative chances in predictive methods of the metastatic potential of a primary tumor and a novel target for therapy. Experimental results summarized in **Figure 5** indicates some of the key targets in the treatment of EMT and metastasis and the possible role of  $\gamma$ -tocotrienol in the prevention and treatment of these processes.

In summary, experimental evidence demonstrates that  $\gamma$ -tocotrienol reversal of EMT results, at least in part, through the inhibition of canonical Wnt and Hedgehog signaling. These findings also suggest that supplemental treatment with  $\gamma$ -tocotrienol may be effective in providing significant benefit in the prevention and treatment of metastatic breast cancer.



**Figure 5.** Schematic representation of  $\gamma$ -tocotrienol effects on the canonical Wnt and Hedgehog pathways and EMT.  $\gamma$ -Tocotrienol inhibits Wnt signaling by decreasing the expression of Wnt3a ligand, FZD7/LRP6 complex activation, DVL2 and cyclin D1 and a corresponding increase in Naked 1 level. Additionally,  $\gamma$ -tocotrienol inhibits Hedgehog signaling by decreasing the expression of Shh ligand, PTCH2, Smo, GSK3 $\beta$ , and Gli1 associated with a corresponding increase in SUFU levels. Several other cytosolic and nuclear proteins were minimized which can ultimately lead to a suppression in gene expression associated with EMT.



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## Conflict of interest

There is no conflict of interest.

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