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Inhibition of Cholesterol Biosynthesis Modulates Epithelial-Mesenchymal Transition in Primary Cicatricial Alopecia Through TGFβ and Angiotensin Receptors

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Abstract

Introduction: Primary Cicatricial Alopecia (PCA) is an autoimmune condition that affects the skin and causes hair loss in patients. In PCA the hair follicles of the patients are irreversibly damaged and replaced with fibrous tissue. This diseased condition lends relevance to our work since the fibrosis raises the potential that PCA may be affected in some way by the Epithelial Mesenchymal Transition (EMT). We used small interfering RNAs (siRNA) of TGFβ, AGTR and their regulators to identify the EMT modulation. Because these molecules mediate the induction of EMT. This study explores the idea of lowering PCA fibrosis by modifying EMT markers. **Methods**: We chose 7 DHC and BM15766 to investigate the function of cholesterol biosynthesis inhibition. We employed the HFORS in vitro and the mouse in vivo model system to examine EMT regulation PCA. Quantitative real-time PCR was utilised to examine the expression of genes in PCA scalp samples, compound-treated HFORS, and mouse tissues; immunohistochemistry was used to confirm the protein estimate in the scalp samples; and small interfering RNA (siRNA) transfection was used to identify the functional analysis of TGFβ and AGTR. **Results**: Reduced cholesterol production in PCA patients leads to permanent hair follicle damage. The in vitro and in vivo study using 7DHC and BM15766 revealed cells were positive for the EMT markers. PPARγ, AhR, and AGTR together can act as vital EMT regulators. As a result, the PPARγ agonist, AhR, and AGTR antagonist significantly downregulate the expression of CDH1, SNAIL1, and SMA. The markers of EMT are likewise deregulated by the transfection of siRNA for TGFβ and AGTR. **Conclusion**: We clarify how EMT is regulated in hair loss circumstances by suppressing cholesterol biosynthesis. We further confirm that EMT modulators (PPARγ, AhR, AGTR, and TGFβ) and siRNA can be employed as potentially effective strategies to slow the advancement of EMT. As a result, we propose these cholesterol and EMT modulators as potential inhibitors in PCA etiology.

Keywords: Cholesterol, Fibrosis, Hair, PPARγ, Pioglitazone, Transfection

1. Introduction

Primary Cicatricial Alopecia (PCA)[1–4] is an irreversible form of hair loss with retreating follicular ostia[1]. Depending on scar formation, alopecia has two forms: Scarring and nonscarring alopecia. The scarring alopecia is divided into lymphocytic, neutrophilic, and mixed. Lymphocytic is Central Centrifugal Cicatricial Alopecia (CCCA), Frontal Fibrosing Alopecia (FFA), and Lichen Planopilaris (LPP); neutrophilic include Tufted Folliculitis (TF) and Folliculitis Decalvens (FD); mixed type is Dissecting Cellulitis (DS)[2]. The pathogenesis of PCA is poorly known.

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Our study mainly engrossed in the pathological phase of alopecia. Fibrosis, itching, and pain are the pathophysiological trademarks of alopecia. Therefore, we focused on Epithelial-Mesenchymal Transmission (EMT) and various molecular modulators which plays an important role in phenotypic changes like apical-basal polarity,cell-cell adhesion, and finally, pathologic fibrosis, and physiologic repair.

Karnik, *et al*.[3] introduced the unsuspected role of the Peroxisome Proliferator-Activated Receptor (PPARγ). The target deletion of PPARγ in the isthmus or bulge of follicular epithelium shows LPS-like pathophysiology and LPP patients' manifest defects in peroxisome biogenesis and lipid metabolism. Histologically, the PCAs' lesioned Hair Follicles (HF) are eventually destroyed and replaced with fibrous tissue[4]. In addition to this, numerous hormones have a significant impact on both the hair cycle and the architecture of the hair follicle. In particular, the effects of androgens have been thoroughly examined and discussed in earlier studies. Depending on where on the body the hair is, androgens have an impact on hair follicles. The binding of androgen-to-androgen receptors on dermal papilla cells is the primary mechanism by which androgen affects the hair follicle.

Additionally, studies reveal where androgen is produced concerning the enzymes that make up the hair structure. These hormonal imbalances can result in female pattern hair loss and other types of alopecia[5]. The inflammation around the hair follicle is the aetiology of hair loss in PCA, but the details about the pathogenesis are unclear[6]. Lichen Planopilaris (LPP) is an archetypal PCA with sudden hair loss and clinical symptoms such as burning, itching, and scalp pain. The marginal areas of the affected scalp consist of perifollicular erythema and scale[7]. Frontal Fibrosing Alopecia (FFA) is considered a variant of LPP with an overlap of histologic features. Both entities illustrate T-cell-dependent lymphocytic inflammation and perifollicular fibrosis[8]. Another important condition in scarring alopecia is Dissecting Cellulitis (DC) which causes aberrant scalp purulence and is characterised by excruciating nodules[9]. The treatment strategies for PCA are minimal, and its diagnostics are based on clinical observations and histological studies[10]. Unfortunately, the current treatment strategies are inadequate to stop skin scarring and permanent hair loss[11]. Recent studies by Najeeb, *et al.,* suggested that an altered cholesterol biosynthetic pathway leads to the functional elevation of TGFβ- the SMAD pathway, which acts as a positive regulator of tissue fibrosis in PCA conditions. Similarly, the experimentally executed inhibited sterol regulatory pathways under *in vitro* and *in vivo* studies using 7DHC and BM15766 shows a surge in the expression of fibrotic TGFβ, AhR, AGTR1[12].

The influx of immune cells and inflammation is the hallmark of PCA. The study conducted by Panicker, *et al*., suggested that inhibition of the cholesterol biosynthetic pathway and the accumulation of cholesterol intermediates play a vital role in early pathogenesis[2]. The addition of exogenous 7-Dehydrocholesterol (7DHC) resulted in the upregulation of the interferon signalling network and Toll-like receptors *in vitro* and *in vivo*. Studies in murine models showed abnormal HF cycling and growth. Evidence of epidermal thickening and follicular plugging was also found after cholesterol inhibition. Similarly, an increased catagen induction and inactivation of stem cell markers were observed after cholesterol alteration. Previous studies reported that inflammatory responses are set in due to the accumulation of cholesterol precursor, and recruits macrophages, leading to hair follicle destruction[3]. The upregulated TGFβ is a well-established catagen and fibrosis inducer[13,14]. Therefore, we used the 7DHC (cholesterol precursor) and BM15766 (pharmacological inhibitor of DHCR7) to identify the EMT modulation via inhibitory cholesterol biosynthesis under *in vitro* and *in vivo* treatment conditions.

There is a well-established understanding of PPAR's regulatory role in PCA. The expression of PPARγ was found to be lower than that of the other two PPARγ isoforms in scalp samples from people with LPP and healthy controls. Peroxisomal gene expression was found to be suppressed in LPP. At last, evidence accumulated to show that PPARγ played a critical role as a master regulator in LPP pathogenesis[15]. Previous research indicates that PPARγ agonists can reduce skin and lung fibrosis caused by experimental means[16,17]. Additionally, the anti-inflammatory functions of PPARγ are well established[18–20]. The patients affected with psoriatic plaques utilize the pro-differentiating, anti-proliferative and immunomodulatory activities of the PPARγ[17]. The regulatory actions on PPARγ might modulate EMT activities. A previous clinical study indicates that Pioglitazone was used in treating LPP. Also, the clinical trials of 22 LPP patients administered with Pioglitazone showed reduced medical implications[7,21]. In association

with FFA, Aryl Hydrocarbon receptor (AhR) activity with alkyl phenolic compound interfered with the action of PPARγ[22,23].

Earlier studies on LPP and FFA proposed that perifollicular fibrosis is due to EMT[24,25]. In EMT, the epithelial cells lost their cell-cell polarity and acquired the properties of mesenchymal cells[26–28]. Clinical studies on FFA show that lesional sites are abundant in SNAIL activation, leading to EMT activation in PCA[24]. Herein we focused on modulating EMT to reduce the pathological implications of PCA, thereby reducing the severity of disease complications. Current evidence supports that cell-surface proteins (Cadherins, Integrins), cytoskeletal markers (α SMA, Vimentin, β Catenin), Extra Cellular Matrix proteins (Collagen, Fibronectin, Laminin), and Transcription Factors (SNAILfamily, TWIST, LEF-1) contribute to EMT[29].

In the HF morphogenesis of mice, SNAIL1 mRNA and protein are transiently expressed at the hair bud stage. In contrast, some studies suggest that SNAIL1 is limited in mesenchymal cells of the skin, especially below the hair bud of embryonic skin[30,31]. Thus, the Hair Follicle Stem Cells (HFSCs) are located at the bulge area responsible for the physiological hair cycle and regeneration. The cross-talk between specialized dermal cells and HFSCs regulates the induction of HF. Therefore, the well-organized epithelial-mesenchymal interaction is crucial for HF formation[32–34].

Studies conducted on LPP and FFA showed lose of cell-cell polarity and high level of SNAIL activation in lesional sites[25]. Here, we focused on modifying EMT to lessen the severity of disease complications brought on by PCA and its pathological ramifications. The evidence described that transcription factors (SNAIL family, TWIST, LEF-1), cell-surface proteins (Cadherins, Integrins), cytoskeletal markers (αSMA, Vimentin, Catenin), Extracellular Matrix proteins (Collagen, Fibronectin, Laminin), and other factors all play a role in EMT[29].

Other skin and organ fibrosis studies showed that TGFβ plays a crucial role in EMT induction. This induction is mediated by the TGFβ/Smad pathway, stimulating the epithelial cells to undergo EMT[38,39]. The clinical trials showed the suppressive activity of Losartan in EMT, Renal Hypertrophy and, finally, attenuated Renal Fibrosis[40]. Some previous pieces of literature provide for the idea of modulating the EMT by regulating the TGFβ[13,41] and AGTR. The relationship between TGFβ with Pioglitazone in LPP has already been studied. The downregulation of PPARγ causes the upregulation of TGFβ, SMAD2, and SMAD3. Therefore, the TGFβ signalling is the crucial mechanism for EMT in LPP[13]. In tissue repair and carcinogenesis, the SNAIL family proteins are the direct target of TGFβ and have a role in EMT[26]. We planned to interfere with TGFβ and AGTR to identify how the EMT markers are regulated. RNA interference is the standard method for the target gene knockdown[11]. Therefore, we used small interfering RNAs (siRNA) of TGFβ and AGTR to inhibit their activity, thereby modulating the EMT marker genes.

Our primary objective is to reduce fibrosis and associated scarring in PCA by modulating EMT. Cholesterol production is dramatically downregulated in PCA, which results in inflammation and fibrosis. Therefore, controlling cholesterol biosynthesis and inflammatory genes could lower patients' fibrosis severity. We planned to use PPARγ agonists and antagonists, Resveratrol, Angiotensin II, and Losartan, as therapeutic options to reduce fibrosis and eventually rescue the hair follicle. In addition, the inhibitory effect of TGFβ and AGTR on EMT was found, and these target molecules likely lowered the disease.

2. Materials and Methods

2.1 Human Scalp Tissue

Experiments on human subjects were conducted with the approval of the Human Ethical Committee, University of Kerala (ULECRIHS/UOK/2018/35). Informed written consent was collected from the participants. The neutrophilic and lymphocytic PCA were diagnosed through clinical observations. The patients selected for this study had active lesions and 4mm^2 scalp biopsies were collected from the affected and unaffected areas of the diseased individuals. The affected sites show inflammation but with retained HF. Scalp biopsy from the healthy individual was considered a control and confirmed that they had no evidence of skin or hair disorders. All patients had effective symptoms like itching, pain, and progressive hair loss. The collected samples were stored at -80^0 C for further evaluation.

2.2 Animals

Experiments on animals were conducted under approval from the Kerala University Institutional Animal Ethics Committee (IAEC 1-KU-12/21-ZOO-SRP(4). The animals were reared at the Animal House facility at the University of Kerala.C57BL/6 mice, aged seven weeks, were randomly grouped into four. Each group consisted of six animals. Depilation helped synchronize the hair growth, retaining the hair follicle in the telogen phase. The scalp hairs of the animals were removed using depilatory agents. The depilated scalp was treated with 25 mM 7DHC and 4 mM BM15766 for 15 days. Ethanol and DMSO were used to dissolve the compounds and applied topically. The treated scalp skin harvested was stored at -80°C for qRT-PCR analysis, and the samples were fixed in paraffin for histological sectioning (H and E staining).

2.3 Human Hair Follicle Outer Root Sheath Cells

Human Hair Follicle Outer Root Sheath Cells (HHFORS) (ScienCell Research Laboratories, USA) a gift from Dr Karnik P, cultured in Mesenchymal Stem cell media supplemented with FBS and Penstrip (according to manufacturer's instructions). Cells were seeded in 100mm Plates with a density of $0.6x10^6$ [4,8]. DHC, BM15766, Pioglitazone, GW9662, TCDD, Resveratrol, Losartan and Angiotensin II and their combinations were treated in HHFORS cells. Ethanol and DMSO were used as vehicles. The final vehicle concentrations never exceeded 0.1%. The treatment concentrations have been published in our previous study[4]. The cells were further used for RNA isolation, qRT-PCR, and immunofluorescence analysis.

2.4 Quantitative Real-Time PCR

RNA isolation was conducted with previously described protocols[2]. RNeasy Mini Kit was used for RNA extraction. SYBR Green-Labelled PCR primers for all targeted genes were purchased from G-Biosciences made in the USA (Cat # 786-5062). Expressions of all targeted genes in PCA, normal scalp samples, HHFORS, and mice samples were quantified.

2.5 Immunohistochemistry

Normal and PCA scalp tissues were fixed in 4% paraformaldehyde for 24 h. H and E staining and immunohistochemical staining were performed using the standard protocols. EMT-associated markers were detected using specific Rabbit polyclonal primary antibodies, including CDH1(1:100), α SMA (1:100), and SNAIL 1 (1:100) purchased from ImmunoTag USA (Cat# ITT02816), Goat anti-Rabbit IgG (HRP conjugated) Secondary antibodies were purchased from Real Gene. Images were captured using a LABOMED Lx 500 binocular microscope for immunohistochemical sections.

2.6 siRNA Transfection

The HHFORS cell with 70-90% confluency was used for siRNA transfection. The cells were incubated with liposome complex containing siRNA and Lipofectamine 3000 (Invitrogen) under serum and antibiotic-free conditions. The targeted siRNA of AGTR1 and TGFβ was obtained from (OriGene). After 8 h, a fresh mesenchymal stem cell medium with 10% FBS was added, and the cells were incubated for a further 16 h. After this 24 h incubation, the cells were harvested and used for RNA isolation, followed by qRT-PCR.

2.6 Statistical Analysis

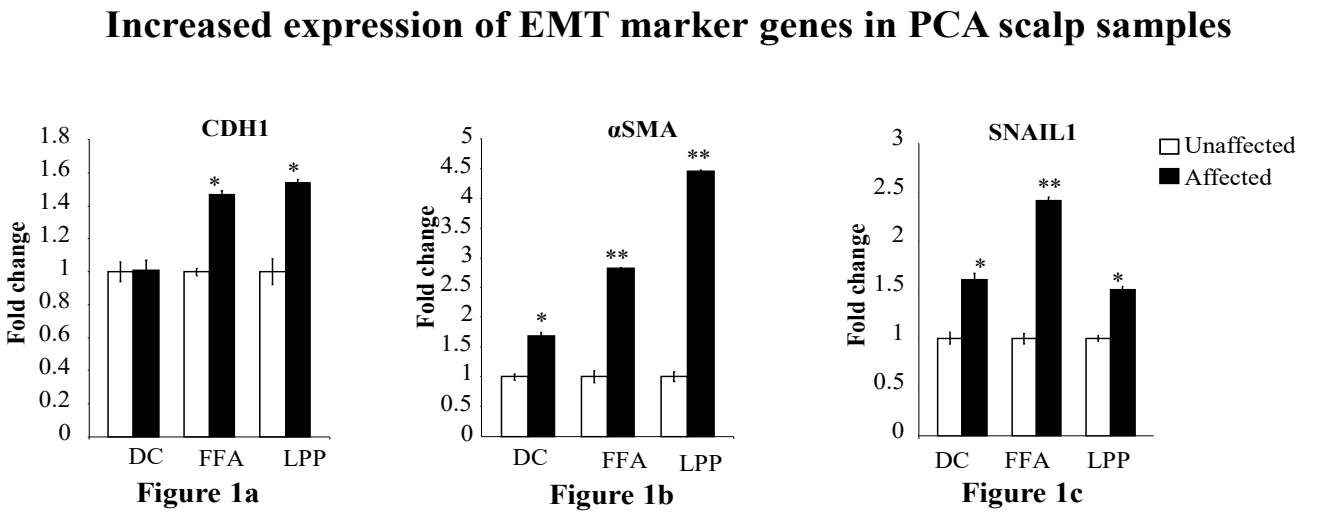
All experimental results were analysed by one-way analysis of variance ($p < 0.001$) using SPSS software (Ver. 22.0).

3. Results

3.1 Upregulated Expression of EMT Marker Genes and Proteins in PCA Scalp Samples

To find the involvement of EMT in PCA, the gene expression of EMT markers in PCA (DC, FFA, and LPP) samples were investigated (Figures 1(a)-1(c)). For transcriptome analysis, we selected the EMT markers, CDH1, SMA, and SNAIL1. Here, the affected scalp tissue samples are compared with healthy scalp tissue. The real-time PCR validation of PCA samples revealed significant overexpression of CDH1, αSMA, and SNAIL1 compared to the control.

Immunohistochemistry was used to look at how EMT marker proteins were expressed in PCA patient tissue samples (Figure 1(d)). CDH1, SNAIL1, and αSMA had increased expression, in affected samples. Additionally, the elevated expression of these EMT markers resulted in



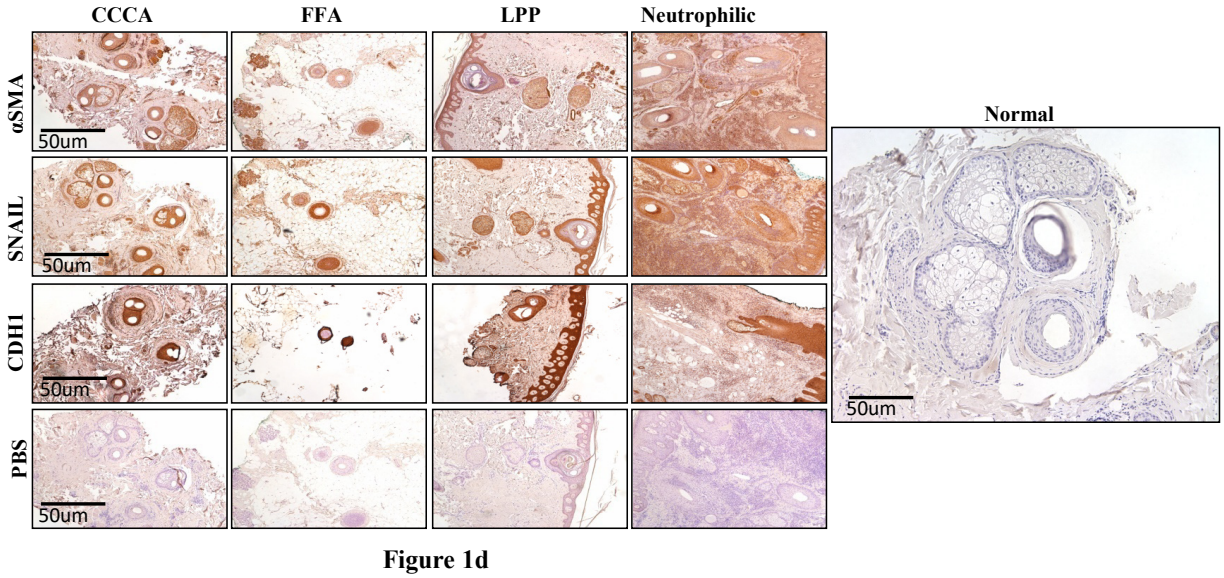


Figure 1. **(a-d)** Upregulated expression of EMT marker genes and proteins in PCA scalp samples. Real-time PCR validation of EMT marker genes, CDH1, α SMA, SNAIL1 in DC, FFA, and LPP (scalp samples) unaffected and affected. **(a)** The CDH1 expression upregulated FFA and LPP (*$p < 0.05$, **$p < 0.01$) compared with the unaffected tissue. **(b) (c)** The gene expression of SNAIL 1 (*$p < 0.05$, **$p < 0.01$) upregulated in the three PCA scalp samples (DC, FFA, and LPP) compared with the unaffected. **(d)** Immunohistochemical localization of αSMA, SNAIL1, and CDH1 to the human scalp samples of PCA affected (CCCA, FFA, LPP, Neutrophilic) and unaffected (Control sample).

increased cell-cell adhesion and thickening of the fibrotic tissue. Here, both neutrophilic and lymphocytic cell types were receptive to EMT markers. These findings imply that the EMT genes and proteins, which were mostly involved in fibrosis, are significantly upregulated in the PCA samples.

3.2 Cholesterol Precursors and Inhibitors Modulate EMT Marker Genes *In Vitro* and *In Vivo*

EMT markers were upregulated in affected PCA scalp samples. Therefore, we tried to identify the effect of inhibitory cholesterol biosynthesis and the intermediates on EMT markers. We analyzed the gene expressions after treating with 7DHC and BM15766 (Figures 2(a-c)). HHFORS was selected for the *in vitro* treatment. Both 7DHC- and BM15677-treated HHFORS showed significant activation of αSMA, SNAIL1, and CDH1, compared with the untreated sample. These data revealed that the accumulation of cholesterol precursors or inhibiting endogenous cholesterol biosynthesis induces EMT.

To reemphasize the *in vitro* data, we performed *in vivo* analysis in mice. For this, mice were treated with 7DHC

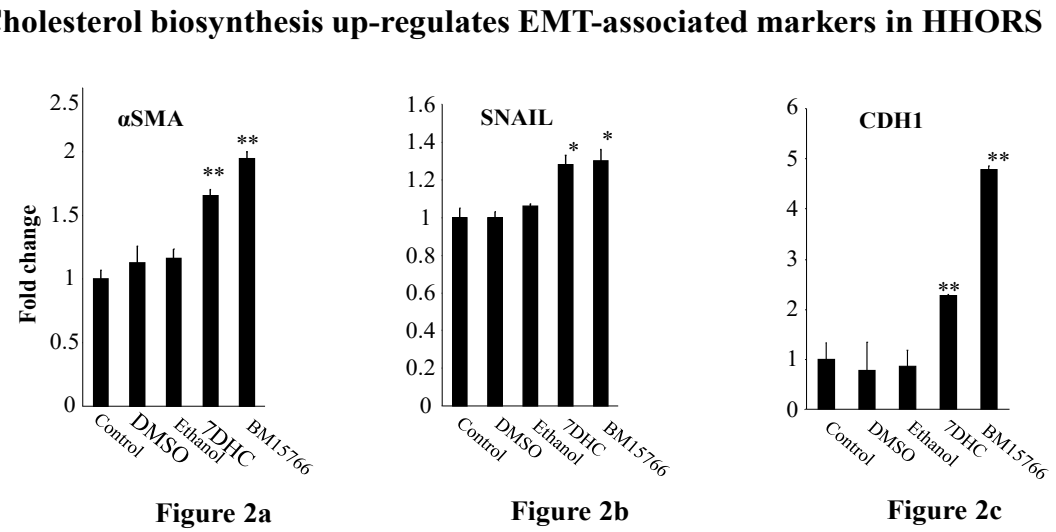


Figure 2a Figure 2b Figure 2c

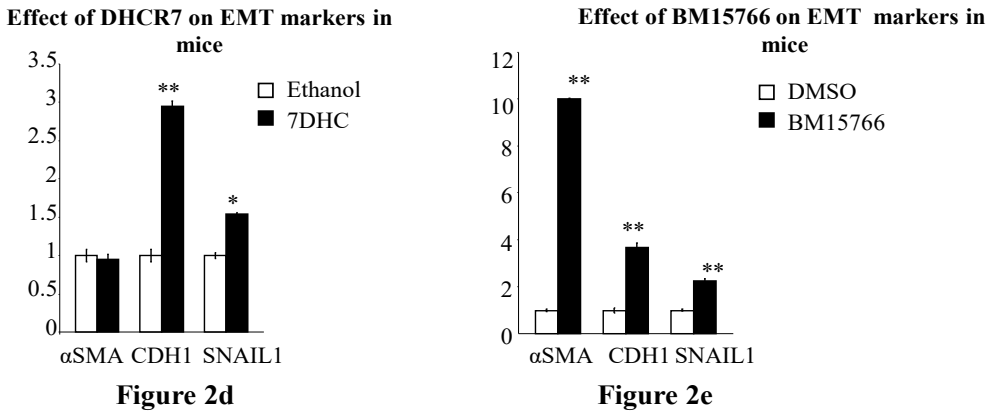


Figure 2d Figure 2e

Figure 2. **(a-e)** Cholesterol biosynthesis upregulates EMT- associated markers in HHORS and Mice. The real-time PCR validation of. **(a)** α SMA. **(b)** SNAIL1. **(c)** CDH1 gene expression in 7-DHC and BM15766-treated samples, HHFORS (*p<0.05, **p<0.01) is shown. **(d)** Treated mice samples show significant upregulation of CDH1 and SNAIL1. The effect of BM15766. **(e)** On EMT markers in mice. Compared with the vehicle (DMSO) control, the treated samples show significant upregulation of αSMA, CDH1, and SNAIL1.

and BM15766 (Figures 2(d)-2(e)). *The* research revealed the physiological significance of reduced cholesterol production during EMT activation.

3.3 EMT Modulated by PPARγ, AhR, and AGTR Agonist and Antagonist

The prevalence of decreased cholesterol biosynthetic pathway and immune responses in the scalp samples of PCA is a recently described entity. The loss of PPARγ can cause a pro-inflammatory response in PCA[1–4]. To elucidate the mechanism behind the EMT modulation via PPARγ and AhR, we treated Pioglitazone (PPARγ agonist), GW9662 (PPARγ antagonist), 2, 3, 7, 8 -Tetrachlorodibenzo-p-dioxin (TCDD, Ahr Agonist) and Resveratrol (a natural AhR antagonist) on HHFORS. Real-time PCR confirmed that the EMT marker genes (Figures 3(a)-3(c)) αSMA, SNAIL1, and CDH1 substantially downregulated their expressions on treatment with pioglitazone. These results

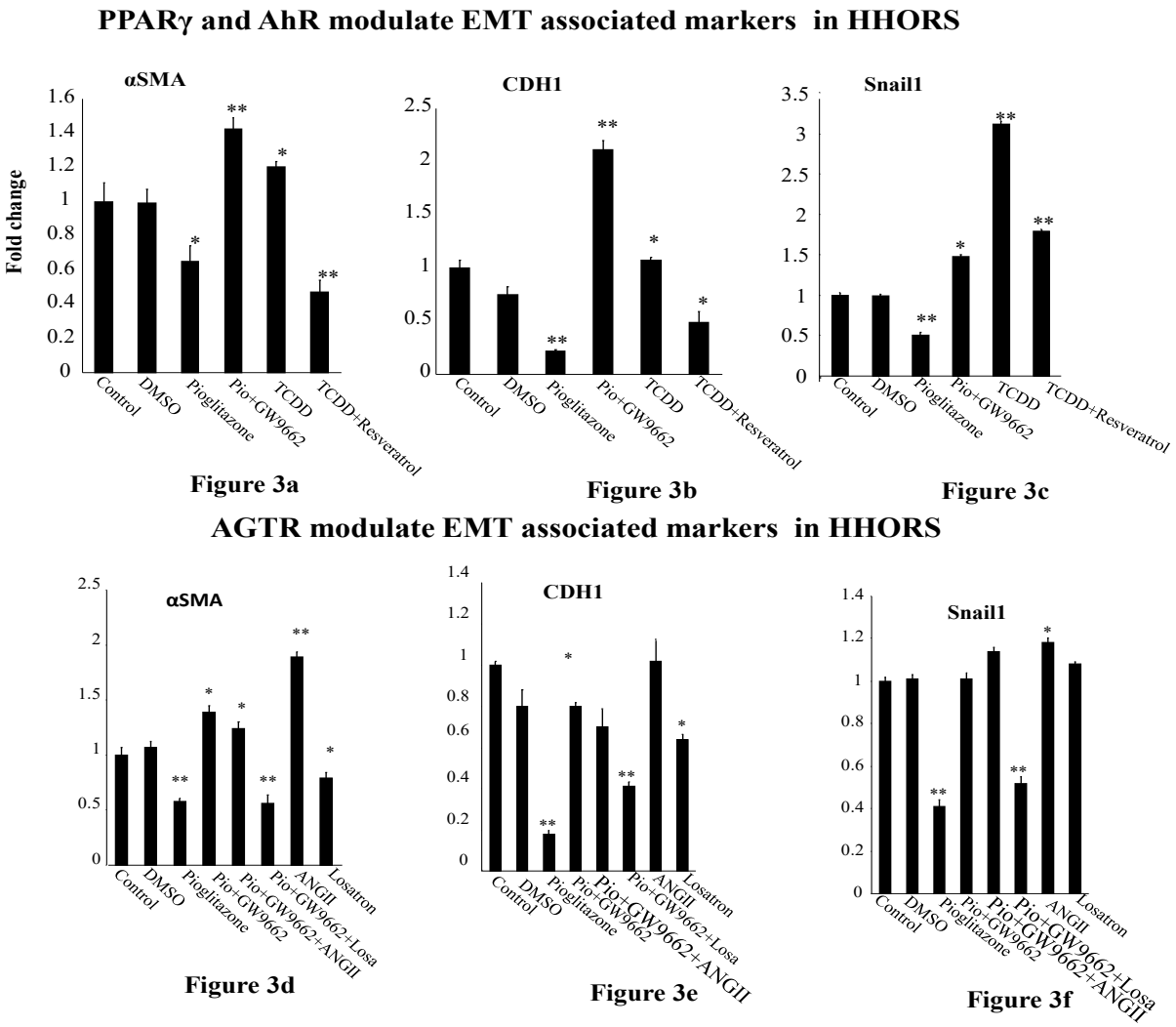
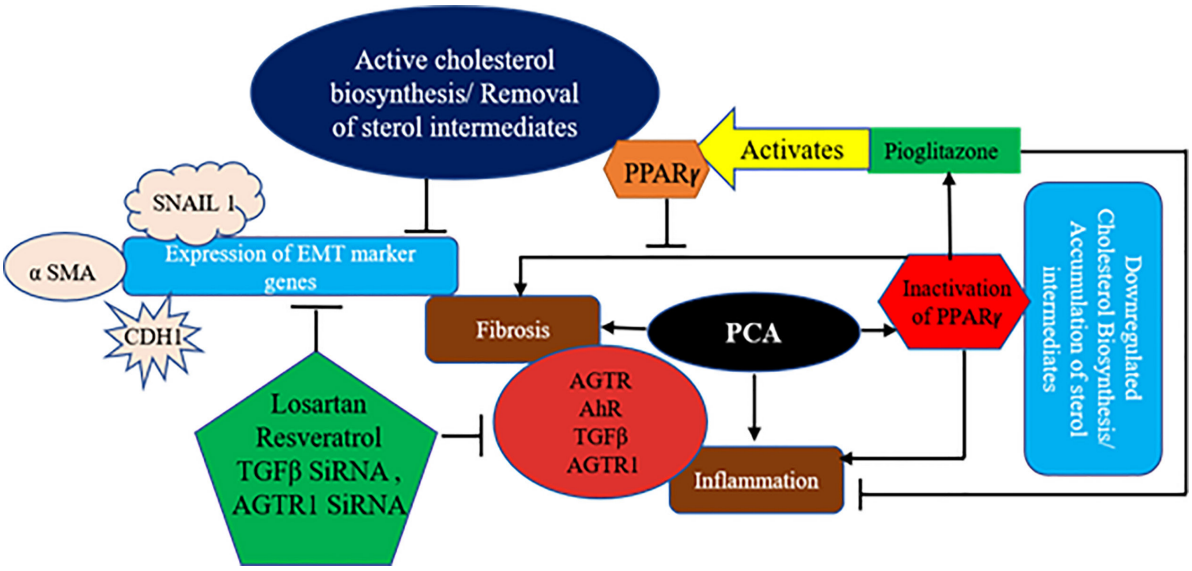


Figure 3. **(a-f)** PPARγ, AhR, and AGTR modulate EMT-associated markers in HHORS. HHFORS treated with Pioglitazone, (Pioglitazone and GW9662), and TCDD, (TCDD and Resveratrol). Real-time PCR shows the EMT marker genes α SMA. **(a)** CDH1. **(b)** SNAIL1. **(c)** On treatment with these drugs. **(d)** CDH1. **(e)** SNAIL1. **(f)** Expressions on treatment with these drugs. EMT marker genes are upregulated on treatment with (Pioglitazone and GW9662) and Angiotensin II compared with the control.



suggest that the activated PPARγ can downregulate the EMT, which helps to reduce the pro-inflammatory responses and fibrosis in PCA. While in combination with GW9662 (PPARγ antagonist), the expressions of EMT markers got amplified. It confirmed that the upregulated PPARγ could reduce the EMT-related clinical implications in PCA. Here the TCDD has a high affinity to AhR and hindered PPARγ. On treatment with TCDD, the EMT markers got amplified. In contrast, TCDD with Resveratrol deregulates them. So, the downregulated AhR and increased PPARγ activity can modulate EMT.

After treating HHFOS cells with Angiotensin II and losartan, we investigated the relationship between EMT and PCA pathogenesis (Figures 3(d)-3(f)). We added GW9662, Pioglitazone, and Angiotensin II, but the gene expressions did not change noticeably. According to this finding, the EMT is favoured by the enhanced AGTR activity. As a result, for regulating EMT PCA, we combined, as a cocktail, GW9662, Pioglitazone, and Losartan (a blocker of AGTR). Here, the expression of EMT markers was dramatically downregulated. Therefore, it is evident that AGTR can modulate EMT in hair loss conditions.

We treated Angiotensin II and its antagonist (Losartan) separately to identify the independent action of agonist and antagonist. Treatment of Angiotensin II alone showed activation of gene expressions, while the antagonist significantly downregulated the functions of αSMA and CDH1 and SNAIL1.

3.4 Small Interfering RNAs of TGF β and AGTR1 Modulate EMT

To check the functional analysis of EMT regulation in PCA through AGTR and TGFβ, we used small interfering RNAs (siRNAs). Here, the gene expressions of TGFβ and AGTR1 were inhibited, thereby reducing inflammatory responses (Figures 4(a)-4(c)). With increasing concentrations of TGFβ siRNA (20 nM, 30 nM, 40 nM, 50 nM), the expression of αSMA, SNAIL1, and CDH1 was inhibited. In AGTR1, we utilised two distinct siRNA clones. AGTR1-siRNA1 treated samples demonstrated concentration-dependent downregulation of SNAIL1 and αSMA expression, whereas the second (AGTR1-siRNA2) had variable effects. The results confirmed that TGFβ and AGTR siRNA inhibits the expression of EMT markers in HHFOR. TGFβ and AGTR1 play an essential role in PCA inflammatory responses and pathogenesis.

4. Discussion

Usually, PCAs are a challenge to treat and are associated with painful scalp symptoms and marked psychological impact. Here we present the PCA that shares the phenomenon of HF depletion and EMT. A more systematic study of the regulatory factors of PCA helps to determine the clinical manifestations and future therapeutic targets. The previous research suggests that the affected HF of LPP/FFA shows progressive perifollicular

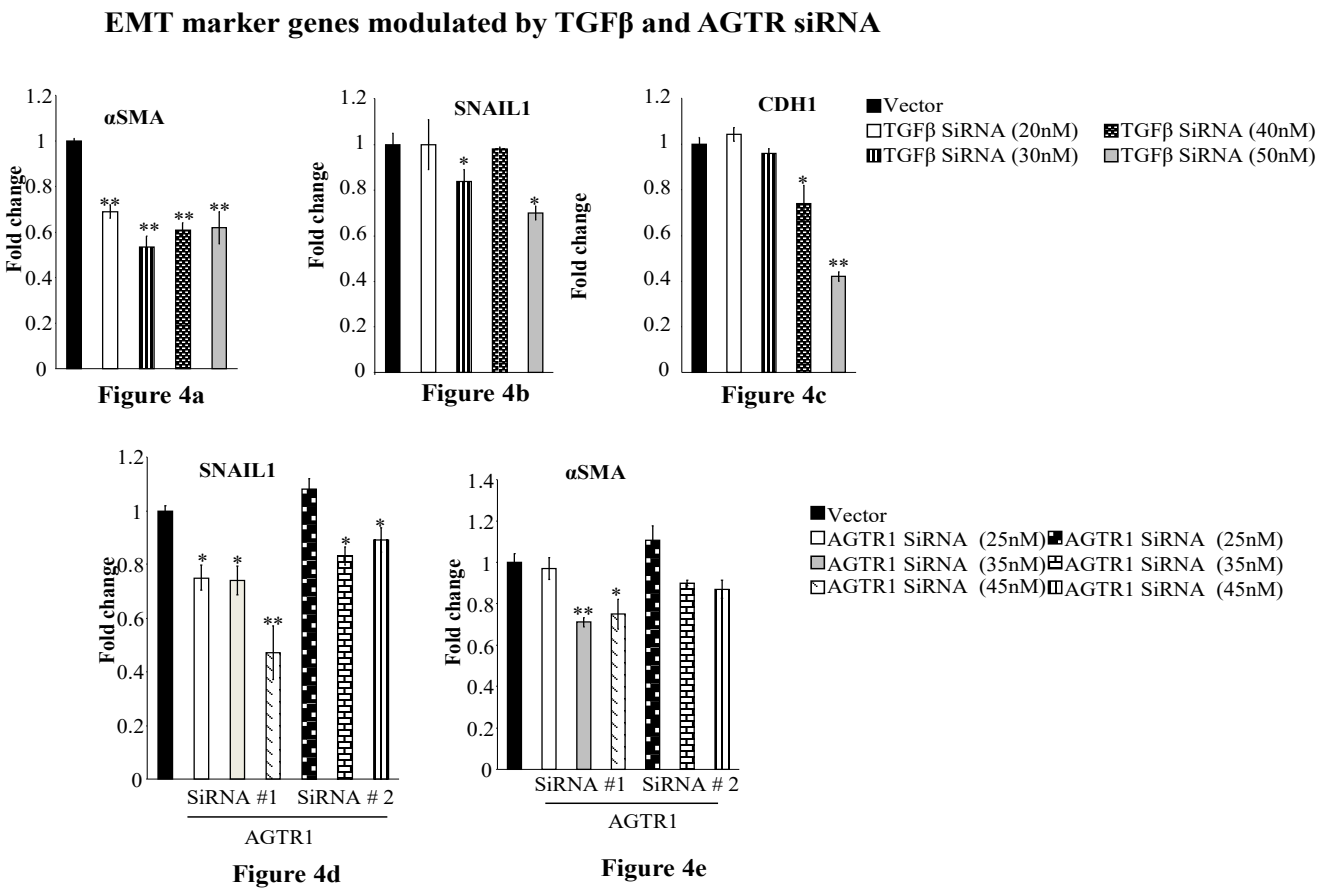


Figure 4. **(a-e).** EMT marker genes modulated by TGFβ and AGTR1. Small interfering RNAs(siRNAs) of TGFβ treated with HHFORS (*p<0.05, **p<0.01). The real-time PCR validation of. **(a)** αSMA. **(b)** SNAIL1. **(c)** CDH1 on treatment with TGFβsiRNA. Two different clones of siRNA AGTR1 (AGTR 1- siRNA1 and AGTR 1- siRNA 2) were used for the EMT marker gene. **(d)** SNAIL 1. **(e)** αSMA expression analysis.

fibrosis leading to the replacement of HF with fibrous tissue. The explanation for fibrosis is EMT. The FFA lesions positive for the SNAIL1–EMT marker and the HF of LPP show upregulated gene and protein expressions of mesenchymal markers[42]. Thus, our data clearly show that HFs affected by DC, FFA, and LPP, undergo EMT, which actively bestows extensive scars in PCAs. The EMT marker genes are significantly upregulated here. The immunohistochemical findings of protein expressions (CDH1, αSMA, and SNAIL1) align with the recent report on reducing or losing terminal hair follicles by fibrosis in FFA[24]. Therefore, future PCA therapies should effectively manage EMT progression in hair follicles. Thus, it is tenable that EMT modulatory therapies are applicable in PCAs with extensive scars. Recent findings proposed that Lymphocytic (LPP) and mixed (Acne Keloidalis Nuchae) diseased types of PCA show EMT at the bulge region. This leads to the loss of the HFSC population, and a cluster of fibroblast cells contributes to follicular fibrosis[13,43].

Compared to normal skin, the LPP-affected skin shows a downregulated expression of PPARγ[43], and the PPARγ agonist was recommended as a treatment for LPP[44]. Karnik and colleagues pioneered the concept of inhibition of fatty acid metabolism, peroxisome and cholesterol biogenesis in PCA[3]. Next, we examined the role of this inhibited cholesterol biosynthetic pathway in EMT modulation. To explore this more holistically, we treated the HHFORS with 7DHC and BM15766. The real-time PCR validation identifies the significantly inflated activation of EMT markers compared with the control. Panicker, *et al.*, also confirmed the role of altered cholesterol biosynthetic pathways in early PCA pathogenesis. The PCA-affected

scalp samples show significant downregulation of EBP and DHCR7. The results suggest that cholesterol biosynthesis is significantly downregulated in PCA. The microarray data of BM15766 or 7DHC-treated HHFORS showed significant activation of the expression of inflammatory genes. In addition, several pathways, like immune cell trafficking, inflammatory and humoral immune response, and cell-mediated immune responses were affected[2]. In our results, the 7DHC- and BM15766-treated samples show activated EMT markers, so inhibitory cholesterol biosynthesis might contribute to the fibrotic gene expressions in PCA pathogenesis. We confirmed the vital role of altered cholesterol biosynthesis and the accumulation of sterol intermediates in EMT. Here the EMT marker genes are significantly upregulated with the hindered cholesterol biosynthesis.

Next, we explored the possibility of reducing experimentally induced EMT using a cocktail of compounds with a regulatory role in PPARγ, AhR, and AGTR. The choice of treatment in PCAs is based on the severity of symptoms, progression of hair loss, and clinical activity[10,45]. A previous clinical study recommends oral administration of PPARγ agonist, Pioglitazone (15mg/day), for LPP patients. The patients tolerated the medication without scalp itching during the first month of treatment[44]. Therefore, we re-examined by treating the HHFORS with Pioglitazone. The investigated effect of Pioglitazone in our study could reverse the EMT markers. Antecedent studies suggest that the Pioglitazone dose could not change the EMT while treating the drug 72 h after EMT induction[13]. Clinical trials of 22 LPP patients with Pioglitazone effectively reduced disease progression and inflammation[21]. One retroactive cohort study on Pioglitazone showed equilibration of hair loss and mild hair regrowth in 75% of treated patients[46]. Our findings the Pioglitazone's inhibitory effect in tissue fibrosis[47]. We tried to block the effect of this knowledge by treating it with GW9662 (PPARγ antagonist). The co-treatment with the PPARγ antagonist elevated the gene expression. We show that CDH1, αSMA, and SNAIL1 are upregulated, while PPARγ is abrogated.

Adding further support for the modulation of EMT using AhR modification, we treated with dioxin called TCDD. TCDD is the most widely accepted ligand of AhR (a cytosolic receptor)[48]. The physiologically activated AhR was used to check out the EMT modulation. The results showed that the gene expressions are significantly upregulated. The activity of AhR promotes and also inhibits the EMT based on the cell type and the system[22]. As expected, however, the AhR modulates the EMT, and a balance of factors is at play. Former studies put forward that the TCDD impedes the PPARγ mRNA synthesis[35]. In a mouse model, the AhR agonist inhibited the TGFβ induced EMT[49]. Other data suggest that TCDD-activated AhR can cause pathological dysregulation in keratinocyte differentiation[50]. In contrast, recent studies emphasize the favourable impacts of AhR activation in skin and tissues as a therapeutic target[51,52].

Then, we went on to the treatment of TCDD in conjunction with Resveratrol, a therapeutic antagonist of AhR. The information available is that they can affect lipid metabolism[36]. We investigated all potential mechanisms for modulating EMT by experimentally inducing AhR with its antagonist. The results demonstrate the deregulation of EMT marker genes and the function of PPARγ and AhR in EMT modulation. As a result, we hypothesise that the EMT in alopecia can be manipulated to incorporate these elements into its therapeutic strategies.

The results further encourage the exploration of AGTR activators and suppressors as complementary therapeutics in PCA. We search for the suspected role of AGTR in EMT modulation. Previous investigations on local fibrosis in renal interstitial fibrosis show that Angiotensin II contributes to EMT[37]. The Renin-Angiotensin-Aldosterone System (RAAS) interacts with the pro-fibrotic pathway (TGFβ) in the fibrosis of many cells and tissues[53]. Here also we used a cocktail of compounds for the treatment. The combination of Pioglitazone, GW9662, and Angiotensin II showed no substantial decrease in EMT gene activity, while in combination with Losartan, the genes significantly reduced expression. The renal cell studies delineate Losartan's role in reducing renal hypertrophy, EMT, and renal fibrosis[40]. The functional activity of this cocktail (Pioglitazone, GW9662, and Losartan) prompted us to examine the effect of Angiotensin II and Losartan, individually. Losartan proved to be a potent inhibitor of EMT markers via AhR inactivity, while in Angiotensin II, the EMT becomes highly expressed. The reason for the hyperactivity might be the activated AGTR. Some clinical investigations show that Angiotensin-Converting Enzyme (ACE) was significantly elevated in alopecia

areata patients. This elevated serum ACE activity in this study may show that the RAAS is involved locally in the aetiology of AA[54]. These results suggest that active manipulation of PPARγ, AhR, and AGTR can modulate the EMT in PCA.

Existing literature suggests modulation of the EMT by regulating the TGFβ[43] and AGTR. Finally, we understand that the siRNAs of TGFβ and AGTR1 could inhibit EMT. In both cases, a concentration-dependent reduction in the EMT marker genes was observed. In clinical research studies, TGFβ plays a pivotal role in cancer-associated fibrosis and acts as a potent inducer of EMT[55].

In conclusion, our study shows that EMT signature can be experimentally induced and therapeutically modulated in PCAs. Cholesterol plays a vital role in EMT modulation because the inhibitory cholesterol biosynthetic pathway and sterol intermediate accumulation (7DHC and BM15766) activated the EMT markers *in vivo* and *in vitro*. Therefore, the cholesterol biosynthesis activation and relevance of various molecules such as PPARγ, Resveratrol, Losartan, AGTR, and TGFβ siRNA effectively downregulate the expression of these fibrotic genes. Thus, we can control the clinical complications due to EMT in PCA. The inflammatory genes can also regulate the EMT signature. In our results, the siRNA-based inhibition of TGFβ and AGTR downregulates the EMT marker gene expressions. Therefore, PPARγ agonist, Resveratrol, and Losartan play a pivotal role in EMT via PPARγ, AhR, and AGTR. Our results are well appropriate for a screening strategy to determine the candidate drug that may therapeutically counteract EMT. Apposite functional regulation of EMT and fibrotic genes in PCA pathogenesis can act as an anti-fibrotic therapy. A deeper understanding of the cross-talk between PPARγ, AhR and the nexus of TGFβ, and AGTR1 in PCA represents an opportunity for a broadly effective treatment strategy.

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