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Androgenic Alopecia: Cross-Talk Between Cell Signal Transduction Pathways

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Abstract

Signaling pathways that control coordination of hair follicle cells genetic program are the convenient model for explaining the hierarchy of intercellular interactions governing cyclic growth of hair. This chapter describes models of molecular signaling pathways specific to dermal papilla cells from balding human scalp in hair follicle cycle. These models include already published data, as well as information inferred from pathway analysis of microarray data and protein-protein interaction database. Interplay of androgenic-alopecia-related signaling pathways FGF, TGFB, BMP, and WNT, as well as cyclin-dependent kinases signaling, is shown.

Keywords: hair follicle, dermal papilla, catagen, androgen receptor, alopecia, microarray, pathway analysis, SNEA

1. Introduction

Androgenetic alopecia (AGA) is the state of progressive cessation of hair growth on the human frontal area of the scalp, most likely inherited and androgen-dependent (OMIM locus numbers are 109200, 612421, 300710). In women, as well as in men with androgenic alopecia, the growth of frontal scalp hairs slows down, and the transition occurs from terminal (pigmented and thick) hair type to vellus (nonpigmented and thin) hair type. The duration of fast growth periods (anagen) gradually reduces with each hair cycle, hair follicles become narrower and shorter, and progressively miniaturized [1]. Peak of the disease in most male patients occurs at the age of 40–50 years. Male pattern hair loss ultimately leaves narrow bands of hair on temples and occiput, where hair never fall off during AGA [2, 3].

It was thought that AGA is associated with high levels of androgens circulating in blood. However, currently prevalent firm opinion is that it is not increased levels of androgens cause

this disease, but rather altered androgen cell sensitivity [4]. There is evidence that in AGA, in dermal papilla cells of hair follicles from the frontal and parietal scalp skin, 5 α -reductase-driven synthesis of topical androgens is increased just as well as activation of androgen receptor complex [5]. The paradoxical situation occurs with accepting the androgen-dependent paradigm of AGA: when the activity of androgen-related signaling stimulates beard growth, while the same signaling arrests hair growth from frontal area of the scalp [5–8].

Nevertheless, failures in treatment of AGA using anti-androgens or 5 α -reductase blockers suggest that additional causative factors do take part in the development of the disease. Present day search for beginnings of AGA is in fact search for answers to the following questions: (a) To which extent do cell-specific signaling pathways contribute to the initiation and maintenance of hair growth cessation in a frontal scalp skin? (b) What are the features of hair follicles from a beard or an occipital scalp and from a frontal scalp that manage baldness in first two cases and hair growth in another? One of the accepted answers is that despite great variety of causes, the basic mechanism of hair growth pathologies consists in discoordination of cross-talk between canonical signaling pathways within hair follicle dermal papilla and hair follicle outer root sheath stem cells [9, 10].

Signaling pathways are the convenient model for explaining the hierarchy of intercellular proteins interactions, which control the hair follicle cells genetic destiny. The pathway models described in the chapter include specific to cells of hair follicle already published data, as well as information inferred from publicly available results of microarray essays and ResNet® Mammalian database [11].

2. Hair follicle-related signaling pathways in androgenic alopecia

Pathway is the term from molecular biology which denotes the way or the mechanism by which a cell reacts to stimuli. In general, pathway is a simplified model of any process in a cell. More accurate scientific definition of the pathway could be as follows: Pathway is a biological process, which initiates when extracellular signaling molecule activates a specific receptor located on the cell surface or inside the cell. In turn, this receptor triggers a chain of events inside the cell, creating a response. Usually, a pathway is visualized as a graphical map. Besides graphical information, a signaling pathway model also includes annotations and references about entities and relations between entities from curated database. There are software applications that allow a researcher to use such signaling pathway models in microarray data analysis and for dynamic simulation of signal propagation [12, 13].

Hair follicle is a multicellular organ that has strictly organized structure and that follows distinct patterns in its development [14, 15]. As opposed to other human organs that grow constantly, hair growth occurs in a cyclic manner. A normal hair cycle of scalp hair includes a fast growing period (anagen phase, 2–6 years), regression period (catagen phase, 2–3 weeks), and resting period (telogen phase, 10–12 weeks). The duration of the anagen phase defines hair length and pigmentation. Normally, 80–90% of hairs in hairy part of scalp are in active growth phase—*anagen* [1]. Numerous reviews are focused on cyclic growth of hair follicles [15–19].

The earliest sign of AGA is increased amount of frontal scalp area skin hair follicles at telogen phase [20]. Developing the alopecia is a long-term process. There is progressive reduction of the duration of anagen phase of hair follicles on the frontal scalp area with each passage through the hair cycle. Other discovered physiological changes in AGA: dermal papilla cell reduction; thinning of the dermis; the sebaceous glands enlargement; connective tissue streamers presence; number of mast cells increased; and T-cell infiltration of follicular stem cell epithelium [21–23]. There are strong evidences that AGA is androgen signaling-dependent disease. Pattern of AGA inheritance is related with an autosomal dominant trait. Castration in men stops progression of hair loss while administration of testosterone restores balding [2]. Men with type 2 5 α -reductase deficiency syndrome do not develop AGA balding phenotype [24]. Finasteride (type 2 5 α -reductase inhibitor) reduces dihydrotestosterone (DHT) level in scalp skin and increases hair growth in more than 60% of men with AGA [25]. Androgen receptor (AR) gene is the only risk gene for AGA phenotype confirmed in gene association studies [26]. Androgen receptors level was higher in cultured dermal papilla cells derived from balding scalp hair follicles, compared to dermal cell from nonbalding follicles [5, 8]. The concentration of DHT has been shown to be higher in the balding scalp than in the nonbalding scalp [27]. Both type 1 and type 2 5 α -reductase have higher level in frontal than occipital follicles from AGA scalp [5]. Expression of enzymes involved in DHT synthesis (STAR and HSD3B1) was enhanced in the bald frontal area, in comparison with occipital areas of AGA patients [28].

Another layer in research of androgen-dependent hair growth covers seeking differences in androgen sensitivity of distinct areas of skin. Follicles from frontal and parietal areas of the human scalp are considered to be androgen-sensitive. There are also two more types of hair follicles: androgen-independent hair follicles, such as eyelashes, and androgen-dependent pigmented terminal hair follicles, such as beard [26, 29, 30]. Why do hair follicles have different sensitivity to androgens is still open question. There are few studies towards difference between androgen-dependent and androgen-independent hair follicles [8, 30–35].

Thus, from the point of view of signaling pathways research, studying AGA mainly focuses on DHT synthesis in the skin and AR signaling affecting cell processes that cause arrest of anagen phase in frontal scalp. Precisely, these include diminishing of dermal papilla cell proliferation and hair bulge stem cell inactivation. Also, there are important pathways of sebocyte and melanocyte maintenance, keratinocytes apoptosis, and macrophages migration.

2.1. Dermal papilla-specific signaling pathways

The entire hair develops from the niche of activated dermal papilla (DP) cells—group of mesenchymal cells in lower part of hair follicle adjacent to dermis, which provide the regulation of hair keratinocyte differentiation [36]. The changes in molecular signaling which follow the dermal papillae burst in balding scalp of a person with AGA result in suppressed growth of hair keratinocytes derived from DP cells [29]. Dermal papilla in developed hair follicle in anagen is surrounded by a proliferative zone within the hair bulb named matrix. Dermal papilla and matrix include heterogenic cell populations with even multi-lineage potential [37, 38]. For example, adult DP cells were induced to differentiate into adipogenic and osteogenic lineages, as well as in pluripotent stem (iPS) cells [39]. The activity of alkaline phosphatase (ALP) can be used both in mice and human hair follicles as a marker for detecting DP activity [40].

Androgen synthesis is the first fragment of AGA-related signaling pathway in DP cells (**Figure 1**). DP cells can synthesize androgens themselves to stimulate androgenic receptor. All types of hair follicle DPs (beard, pubic, and scalp) have testosterone and androstenedione. But normally, only beard cells produce more potent androgen 5-alpha-dihydrotestosterone produced by SRD5A2. In AGA, DP cells from androgen-sensitive frontal scalp contain more SRD5A2 than those from androgen-insensitive occipital scalp [5]. Steroid sulfatase (STS) in the dermal papillae of human terminal hair follicles hydrolyzes dehydroepiandrosterone sulfate (DHEAS) to dehydroepiandrosterone (DHEA). In healthy human occipital scalp, as well as in the beard, STS activity was significantly higher in the hair dermal papillae than in the outer root sheaths [41].

AR signaling is the key fragment of AGA-related signaling pathway in DP cells. Dermal papilla appears to be the site of androgen action in hair follicle, as androgen receptors are only found in DP cells. In AGA, dermal papilla cells from balding scalp express higher levels of androgen receptors than nonbalding scalp cells [5, 35]. AR gene isoforms or functional mutation near the androgen receptor gene may explain the aberrant expression of the AR protein in the balding scalp of AGA patients. Of the many AR gene polymorphisms known, G allele of StuI and polyQ polymorphisms has the most significant association with AGA, especially in white populations [42]. DNA methylation of the AR promoter is increased in hair follicles from

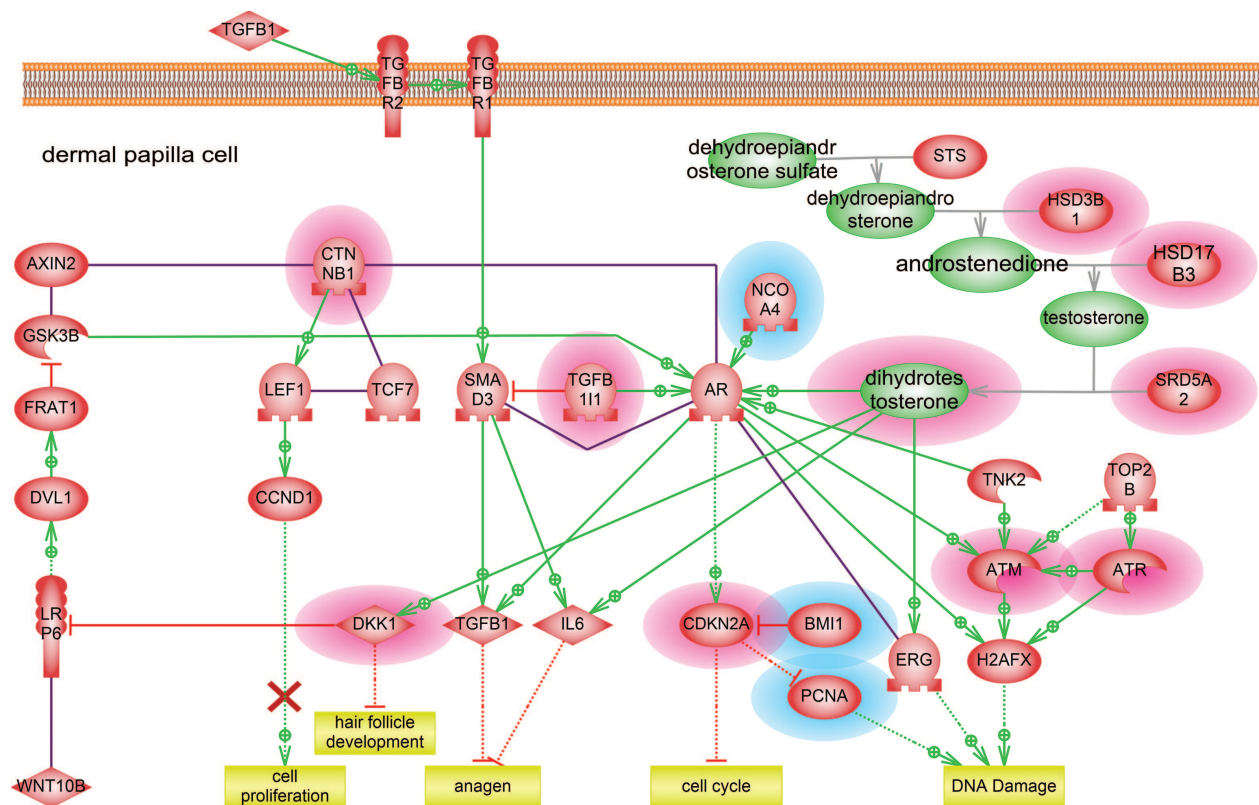


Figure 1. Androgens promote scalp dermal papilla cells dysfunction in AGA. Highlighted entities are underexpressed genes (blue) or overexpressed genes (red) in AGA, according to previous studies. Pathway was built in Pathway Studio environment. The legend for the pathway is provided in Methods section (**Figure 7**).

occipital scalp compared with those from vertex AGA scalp, indicating efficacy of the DNA methylation for protecting occipital hair follicles against AR expression [43]. Human DP cells from AGA express dihydrotestosterone-driven TGFB1, DKK1, and IL6 proteins. TGFB1, DKK1, and IL6 were able to promote apoptosis in outer root sheath keratinocytes derived from dermal papilla cells, which results in hair growth arrest [44–46].

Intersections between WNT, TGF, and AR signaling comprise the next important fragment of AGA-related signaling pathway in DP cells. DHT suppresses WNT signaling (LEF/TCF-mediated transcriptional activity) in DP cells from bald scalp. However, these phenomena could not be observed in DP cells of non-AGA males. It was shown that AR and beta-catenin 1 (CTNNB1) interact with each other [47]. In response to DHT, high expression level of dickkopf 1 (DKK1) was shown in balding dermal papilla cells in AGA [48]. DKK1 acts as inhibitor of canonical WNT/CTNNB1 signaling and hair follicle growth in mice [49]. In mice, in cells of outer root sheath layer of hair follicle, recombinant human DKK1 as inhibitor of WNT induced the pro-apoptotic protein BAX, resulting in apoptosis [45].

TGFB1 can enhance androgens sensitivity in balding dermal papilla cells through SMAD3 signaling, in cooperation with TGFB1I1 as a coactivator of the androgen receptor [50]. In beard and bald frontal scalp dermal papilla cells, expression of *TGFB1I1* mRNA was higher than in cells from the occipital scalp [39]. Also the common fact is that increased expression of cytokines (TGFB1, IL6) promotes apoptosis [44].

IL6 is expressed in DP cell, whereas its receptors IL11RA and IL6ST are expressed in the inner and outer root sheaths of human hair follicles [46]. It was shown that DHT induces IL6 expression in the balding DP of human cells compared with nonbalding DP cells. This results in anagen-to-catagen transition in hair follicle and hair growth arrest [51]. Direction of the IL6 expression change by androgens is cell-specific. In several studies, testosterone and DHT were found to inhibit production of IL6 by human peripheral blood monocytes and osteoblasts. In study of acute wound healing, DHT reduced the expression and secretion of *Il6* (and *Tgfb1*) in mice dermal fibroblasts [52].

Expression of NCOA4, AR coactivator, and a cell growth promoter was found to be reduced in dermal papilla of balding areas [53]. The dermal papilla and hair bulb expressed only the short (beta) but not the long (alpha) form of NCOA4. NCOA4 is capable to enhance AR transactivation induced by ligands such as estradiol, androstenediol, and the phytoestrogen daidzein. Studies indicate that AR competes with VDR and PPARG/RXR for NCOA4 availability [54].

Finally, PTGDS (prostaglandin D2 synthase) is elevated at the mRNA and protein levels in bald scalp, compared to haired scalp of men with AGA. Prostaglandin D2 (PGD2) is similarly elevated in bald scalp. PGD2 is a potent vessel contractor and may contribute to AGA development throughout deterioration of blood supply of hair follicle. PGD2 inhibits hair follicle regeneration through the PTGDR2 receptor [55].

Induction of androgen-accelerated premature senescence (loss of proliferative capacity) in bald scalp dermal papilla cells of AGA patients is one more fragment of the pathway. Loss of proliferative capacity of balding DPC was associated with higher expression of CDKN2A and lower expression of BMI1 and PCNA. CDKN2A protein levels were upregulated in response

to androgen, and knockdown of the AR diminished the effects of androgen [56]. This suggests that balding DP cells are sensitive to environmental stress. Three important DNA damage sensors, H2AX, ATM, and ATR, were detected only in balding DP cells. Expression levels of H2AX protein were further increased with AR overexpression [57].

In general, the hypothesis of the pathway is that increase in AR signaling promotes regression of dermal papilla in AGA scalp, and the phase of hair follicle degeneration (catagen) gets initiated earlier than it normally should. AR signaling stimulates catagen likely through (a) TGFB1/HIC5/ARA55 cross-talk [57]; (b) increased level of DNA damage and loss of proliferative capacity induction, which is associated with higher expression of CDKN2A [56]; (c) DKK1 expression, which inhibits cell growth and WNT signaling [47]. However, none of these mechanisms alone is sufficient for the development of AGA, highlighting the combinatorial causes in the disease progression (**Figure 1**).

2.2. Bulge stem cells renewal pathway

Defect in differentiation of hair follicle stem cells (keratinocyte stem cells) could play a key role in the pathogenesis of AGA. New anagen phase and hair follicle arise due to activity of stem and stem-like cells of hair follicle which activate and fill the dermal papilla slow-cycling stem cells (pluripotent/multipotent cells) locate in outer root sheath of hair follicle (bulge region). Other pool of cells with features of “stemness” (secondary germ cells or hair germ cells) becomes morphologically defined between dermal papilla and bulge area in the end of telogen phase [58]. Moreover, in mice, mesenchymal self-renewing cell populations (hair follicle dermal stem cells) were found within the surrounding of DP dermal sheath [59].

Different molecular markers were suggested for distinguishing populations of hair follicle stem cells [58, 60]. Mice cells from bulge area express such specific marker as CD34. Human bulge cells could be found with CD34 as negative marker and CD200 as best positive marker [60, 61]. Mouse bulge cells distinct from secondary germ cells with expression of markers S100A6 and S100A4. Human hair follicle stem cells were categorized into nine subpopulations in Ref. [62] according to different expression of cell-specific markers. CD34 and CD271 expression in human hair follicle is confined to stem cells from sub-bulge area in outer root sheath of the hair follicle [63]. The sub-bulge area is the area between the bulge and dermal papilla, which become distinct in the telogen phase [64]. Stem cells in bulge (and sub-bulge) areas and secondary germ cells are considered to be functionally related cell populations. The CD34+ cells from sub-bulge area are considered to be a reservoir to the secondary hair germ cells upon the end of anagen. Also, secondary germ cells appeared to transform back (or send proliferative signals) into bulge cells in the telogen phase in mice [58, 64].

In bald areas in patients with androgenic alopecia, K15+ITGA6+ cells were present, but CD200+ITGA6+ cells and CD34+ cells were greatly diminished. The authors remarked that AGA likely results from diminished conversion of hair follicle stem cells in outer root sheath to progenitor secondary germ cells [65]. A decrease in second germ generation progenitor cells population attached to dermal papilla in telogen, possibly due to a lack of replenishment from sub-bulge cells, could potentially lead to AGA phenotype, when the each next anagen phase is characterized by new hair follicle smaller than its predecessor. Although these results can

explain decrease in amount of hair follicles in anagen phase in balding scalp in AGA, they do not point at what causes the hair fallout, since human cells from the outer root sheath of hair follicles display CD34 only during anagen phase [63]. To address this question, the detailed research of stem cells maintenance in hair follicle cycle in AGA involving modern techniques is required. The paper that summarizes the data on human hair follicle cycle and proposes standardized approach in this field was recently published [1].

Signaling pathways in stem cells during hair follicle cycle currently lack human-specific information and are mostly based on data from animal models. According to “bulge activation hypothesis,” stem cells in bulge area are activated through interactions with the adjacent dermal papilla before the anagen phase [66]. Activation of new anagen and new hair growth can be described in two steps [64]. First, secondary germ cells become activated at the end of telogen. This activation characterized by WNT signaling and subsequent stabilization of beta-catenin. Second, secondary germ cells stimulate activation of stem cells in bulge area of outer root sheath, which in turn maintain the pool of matrix and dermal papilla cells. The initial pre-first step of anagen phase activation is depended on inductive signal coming from DP. It is supposed that the close proximity of the secondary germ cells to the DP in telogen results in their prior than bulge area activation [64, 67].

In AGA, anagen activation appears to fail, and telogen phase lasts longer [23]. In the next cycle, anagen phase onset is often absent at all [29].

Telogen is the resting phase for the cells and major proliferative signaling pathways within. In healthy mouse, bulge area cells WNT signaling is silent in catagen and telogen [68]. Microarray data suggest that mice bulge cells overexpress inhibitory factors for WNT signaling (*sFRP1*, *Dkk3*, *Wif*) [69]. In human bulge cells, increased expression of the WNT inhibitors *WIF1* and *DKK3* was also shown [60]. In patients with AGA, dermal papilla cells were shown to produce excessive amounts of factors inhibiting WNT signaling (for instance, *DKK1*) [45].

Balance between BMP and WNT signaling was discovered to be important in regulating the transition between quiescence and activation state of murine bulge stem cells [70]. High levels of BMP signaling during early (refractory) telogen are considered the cause of bulge stem cells retention in the quiescent state. In contrast, bulge stem cells activation in the late telogen is thought to require inhibition of BMP signaling. Both BMP and WNT signaling can regulate each other. Quiescence happens due to reciprocal negative feedback loops in these signaling pathways [71, 72]. In mice, *Bmp2* and *Bmp4* gene expression as well as expression of *Dkk1* and *Sfrb4* genes inhibit the expression of WNT family genes and thus limit hair follicle development to the stage of refractory telogen [70]. BMP signaling pathway suppresses itself by triggering the expression of *Noggin* [73, 74].

In human bulge stem cells, the BMP signaling antagonist FST was found overrepresented in the bulge in outer root sheath (ORS) [60]. Also, TGFb2 counteracted BMP signaling in mice bulge stem cells by enhancing the expression of *Tmeff1*, another antagonist of the BMP pathway. TGFb signaling needs to be activated for the hair cycle renewal [75].

Normally, telogen ends and anagen begins when the balance shifts towards WNT signaling is activated [70]. What signaling pathways normally initiate stem cells proliferation and subsequent

anagen, and whether such signals are missed in case of AGA remains unknown. Perhaps, the external signals from adipocytes or fibroblasts in sebaceous gland surrounding hair follicle could be the origin of this initial stimulus [19].

3. Pathway analysis of microarray experimental data in AGA

Microarray technology allows detecting simultaneously the expression of thousands of genes in cells. Data acquired using microarray technology expose differences in gene expression between samples, such as cells from balding and nonbalding human scalp skin.

There are a lot of statistical and bioinformatics methods that can be used to identify cellular processes and signaling pathways with high activity in various cell types in hair bulge. Pathway analysis is the common term for these methods [12, 76]. We used sub-network enrichment analysis (SNEA) method [77] for pathway analysis of AGA hair follicle-related public microarray data. SNEA is based on the Gene Set Enrichment Analysis algorithm [78]. It uses a variation of a nonparametric statistical tests and considers “sub-network” as gene sets. SNEA uses Mann-Whitney statistical enrichment test to find sub-networks statistically enriched with nearest neighbors of the testing entity (“seed”) that are most differentially expressed in the microarray experiment. In context of Pathway Studio data model, a seed can be a protein, complex, protein functional class, metabolite or drug, cellular process, disease, or a cell type. SNEA algorithm uses existing relationships in Pathway Studio ResNet® database to build “sub-networks” based on user-specified criteria. Significant seeds calculated by SNEA can be further used to identify the pathways or gene ontology groups that are significantly enriched with seeds. This analysis can point on pathways activated or repressed in different cell types or diseases. SNEA algorithm implemented in Pathway Studio® software iterates through the database of six million relations between proteins, molecules, cell processes, and diseases extracted from biomedical research literature by natural language processing technology. SNEA implementation in Pathway Studio allows to determine major transcription factors, receptors, and hormone upstream of differentially expressed genes in an experiment and identify small molecules that are associated with the diseases through changes in the proteins activity.

There are several public microarray datasets measuring expression of genes in the bald scalp of patients with AGA. Datasets with accession numbers GSE6664 [31] and GSE36169 [55] [65] could be uploaded from Gene Expression Omnibus (GEO) database [79]. Two more experiments were described by Midorikawa et al. [34] and Kwack et al. [48]. Besides oligonucleotide microarray experiments, microRNAs differential expression analysis was conducted in balding and nonbalding dermal papilla cells [32]; the expression of sex-determining genes in bald frontal scalp area was examined by real-time RT-PCR [80]. Also, microarray analysis to find differences in expression between beard and scalp dermal papilla cells is available [30].

The results of gene expression studies conducted for bald scalp in AGA patients are often controversial. For example, two gene microarray analyses with DP cells from AGA frontal and occipital scalp areas reveal either high level of DKK1 expression in DP cells from bald frontal scalp [48] or diminished DKK1 expression [31]. Most probably, this mismatch is due to

differences in methods for DP cells isolation and culture or different levels of DHT treatment between experiments. It is also not clear from the corresponding articles if the DP cell donors were taking hair loss medications at the time of biopsy. In one more microarray experiment [81], higher levels of DKK1 were shown in human palmoplantar fibroblasts (human skin area without hair follicles) compared to nonpalmoplantar fibroblasts. So, one more putative cause of the mismatch could be the presence of mRNA from other cell type in the sample.

Nevertheless, some genes still have similar differential expression trends in DP cells from balding scalp in several experiments. The following genes were found to be overexpressed: TGFb2, ODC1, RYBP, FGF7, and CDKN2B/A. Genes BMI1, MCL1, SERPINE2, NCOA4, and others were found to be underexpressed. All results from human balding scalp from patients with AGA are summarized in **Table 1**.

Gene	Level	Source	Method	Treatment	Pathway	References
ARA70b (NCOA4b)	Down	DP cells	Hybridization		AR signaling	[55]
BMI1	Down	DP cells	ELISA		Cell senescence	[86]
CLDN11, CDKN1A, POSTN, KBTBD11, PSG5	Down	DP cells	cDNA array	1–10 nM DHT		[87]
SCF	Down	DP cells	ELISA		Impeding bulbar melanocyte pigmentation	[4]
SERPINE2	Down	DP cells	PCR	10 nM DHT	Extracellular matrix turnover	[88]
TIMP2, MCL1, IL6, TYRO3, RHOB	Down	DP cells	cDNA array			[36]
ACTG2, DNER, NPTX2, BEX1, GDF15	Up	DP cells	cDNA array	1–10 nM DHT		[87]
AR	Up	Early-passage DP cells			Induces apoptosis in ORS cells	[50]
ARA55 (TGFB1I1)	Up	DP cells	PCR		AR signaling	[89]
CDKN2A, SOD1, CAT, ATRIP	Up	DP cells	ELISA		Cell senescence	[86]
DAX1 (NR0B1), SRY, WT1, SOX9	Up	Scalp	RT-PCR, Western blotting			[84]
DKK1	Up	DP cells, scalp	cDNA array, immunoblotting	50–100 nM DHT	Induces apoptosis in ORS cells	[50]
DNPH1, CASP4, MOG, CDKN2B, ICAM1	Up	DP cells	cDNA array			[36]
IL6	Up	DP cells	ELISA	10–100 nM DHT		[53]
MIR221, MIR125b, MIR106a and MIR410	Up	DP cells	microRNA APM			[33]
PTGDS	Up	Scalp, HF in catagen	cDNA array		Inhibit hair growth	[82]

Gene	Level	Source	Method	Treatment	Pathway	References
StAR, HSD3B1	Up	Scalp	RT-PCR			[90]
TGFB1	Up	DP, keratinocytes	ELISA	Androgen	Induces apoptosis in ORS cells	[91]
TGFB2	Up	DP cells	Protein level	10 nM DHT	Induces apoptosis in ORS cells	[92]
YARS, SGK, ADAMTS5, SLC19A2, SLC2A3	Up	DP cells	cDNA array, immunoblotting	50–100 nM DHT		[50]
SRD5A1, SRD5A2	Up	HF in anagen	Northern blotting			[5]
H2AX						[58]
SOX9						[84]

For cDNA arrays, only top five significant genes ranked by fold change are shown.

Table 1. Genes with altered levels of mRNA expression in bald scalp from patients with AGA.

3.1. In silico analysis of microarray experimental data on dermal papilla cells

Dermal papilla (DP) is the key part of hair follicle that defines growth rate, thickness, and shape of hair shaft. GEO microarray dataset with accession number GSE66664 [31] compared gene expression in cultured immortalized human DP cells from balding (frontal) and nonbalding (occipital) scalps in response to DHT treatment. DP immortalized cell lines were obtained from two white 26-year-old men with Hamilton–Norwood grade IV AGA. The authors reported underexpression of vasculature-related genes in DP cells of balding hair follicles. Overexpressed genes were mostly enriched by genes from gene ontology groups related to cell cycle and mitosis. DP cells from both bald and nonbald scalp samples included classical DP signature genes, such as ALPL, WIF1, LEF1, and VCAN, expression of which correlated with inductive role of DP cells in hair morphogenesis [40].

Using gene array downloaded from GEO GSE66664 dataset, we found overexpressed and underexpressed genes in balding versus nonbalding immortalized dermal papilla cells cultured with 1 and 10 nM DHT. We also found differentially expressed (DE) genes in balding versus nonbalding dermal papilla cells cultured without DHT treatment. These genes were chosen with 0.05 p-value and twofold change of the expression cutoff. Both lists of differentially expressed (DE) genes were used for further pathway analysis as datasets 1 and 2, respectively.

The differential expression between genes in balding DP cell samples cultured with DHT versus balding DP cell samples cultured without DHT has founded to limit the number of external factors affecting the experiment. As result, we obtained 2 datasets with DE genes in bald DP cells—one for 1 nmol/L and the second for 10 nmol/L (nM) of DHT treatment's level. Each dataset contains 11 DE genes lists with values as results of calculation the ratio between values of genes in sample without DHT treatment (0 min) and 11 samples with DHT treatment periods from 15 min to 48 h. Two equivalent datasets for nonbald DP cell samples were also calculated. Lists of DE genes from all datasets were used for further pathway analysis (**Table 2**).

Dataset	Samples	DHT treatment	DE ratio ³
Dataset 3	bDP ¹	1 nM	0 h vs 15 min–48 h
Dataset 4	bDP ¹	10 nM	0 h vs 15 min–48 h
Dataset 5	nbDP ²	1 nM	0 h vs 15 min–48 h
Dataset 6	nbDP ²	10 nM	0 h vs 15 min–48 h
Dataset 1	bDP, nbDP (15 min–48 h)	1 and 10 nM	bDP vs nbDP
dataset 2	bDP, nbDP (15min–48 h)	–	bDP vs nbD P

¹Bald dermal papilla cells sample.

²Nonbald dermal papilla cells sample.

³Two samples between which differential expressions of genes were calculated.

Table 2. Datasets with differentially expressed genes were used in current analysis based on gene array GSE66664.

3.2. AR-related differentially expressed genes in microarray with AGA DP cells

In response to DHT, AR completely translocates to the nucleus after 30 min [82]. Considering additional 30–50 min for transcription, we concluded that the optimal time to find AR-related targets in studied microarray data is 1 h after of DHT treatment. All changes in expression after 1 h are likely related with paracrine signaling pathways following AR activation.

Total number of AR neighbors in ResNet13® database is 4931 genes. Among DE genes in bald compared to nonbald scalp DP cells samples with DHT treatment (dataset 1), we found 114 DE genes—neighbors of AR. We also found 126 DE genes—neighbors of AR in DP cells samples without DHT treatment (dataset 2) (**S. Table 1**). Out of them, only five genes (NOS3, EGR1, SMAD6, BTG2, and LATS2) were underexpressed in bald DP cells samples more in presence of DHT (dataset 1) while less in absent of DHT. Relations between these genes and AR are not specific for DP cell and were discovered mostly in cancer cells. However, reconstruction of the pathway model based on data from other tissues is routine practice in pathway analysis (**Table 3**).

BTG2 controls G1/S phase transition negatively by direct inhibition of cyclin D1 (CCND1) transcription in human cancer cells [83]. Inhibition of BTG2 (B-cell translocation gene 2) expression by DHT/TNF axis was shown in human keratinocytes [84]. LATS2 is also involved in the regulation of the G1/S transition of the cell cycle and degrades cyclin-dependent kinase inhibitor 1A (CDKN1A) by phosphorylation [85]. LATS2 was shown to modulate expression of AR target genes in cancer cells [86]. In rat immortalized dermal papilla cells, DHT also increased cell cycle arrest, increased *Cdkn1b* level, and decreased *cyclin E (Ccne1)*, *cyclin D1 (Ccmd1)*, and *cyclin-dependent kinase 2 (Cdk2)* levels [87].

There evidences that AR-related SMAD6, EGR1, and NOS3 genes founded in dataset 1 may play role in hair follicle functioning. SMAD complex is regulated by TGFB signaling was shown to induce the catagen phase of the hair cycle in mammals [87].

Early growth response-1 (EGR1) is induced by various stressor factors. EGR1 had found functionally interacting with the androgen receptor and promoting androgen receptor nuclear

Name	Log change (d. 1)	p-value (d. 1)	Log change (d. 2)	p-value (d. 2)	Relation with AR	Localization
NOS3	-1.0376	5.175E-78	-0.935	2.25E-07	Regulation, PMID: 20416296	Vessel endothelial cell
EGR1	-2.096	1.45E-36	-0.947	8.47E-04	Binding, PMID: 12890669	Prostate carcinoma cell
SMAD6	-1.0187	9.55E-34	-0.9821	2.77E-07	Binding, PMID: 16249187	Colon carcinoma cell
BTG2	-1.0740	6.587e-07	-0.8412	0.0003658	Binding, PMID: 21172304	Prostate cancer cell
LATS2	-1.2569	7.83E-39	-0.8982	0.01183	Binding, PMID: 20935475	Breast cell

Lowering of activity of AR target genes in bald DP cells after DHT treatment that we found in datasets 1 could be related with cell cycle dysregulation.

Table 3. AR neighbors underexpressed in bald DP cells samples (datasets 1–2).

translocation in prostate cancer cells [88]. EGR1 has been also reported to be involved with apoptosis and cell migration in several cell types [89, 90]. The lower EGR1 expression in the dataset 1 (bald DP cells samples) may perhaps be result of competing of EGR1 and AR coactivators and corepressors. SHP inhibits both androgen receptor and EGR1 by competing coactivators and inhibitory proteins [91]. In mice embryonic fibroblasts, TP53 was shown to inhibit Egr1 through angiogenesis-associated gene Elk3 [92]. Egr1 inhibition also may be modulated in part via the binding to mesenchymal-specific corepressors NAB1 and NAB2 [93].

NOS3 does not have direct connection with AR, but its expression in epithelial cells is shown to be regulated through AR signaling, as well as its phosphorylation [94]. NOS3 causes the NO-induced vascular relaxation and may trigger angiogenesis in bald scalp. NR0B1, PTGDS, and SGK1 were among other DE genes in dataset 1 that has relations with AR. These genes had been reported previously to play important role in scalp hair follicle development in AGA (**Table 1, S. Table 1**).

The examination of datasets 3–6 (in view of time impact and DHT level impact on the differential expression) reveals more genes with expression correlated with DHT/AR signaling in bald DP cells (**Figure 2**).

Among genes with highest differential expression values (± 2 -fold change values) in datasets 3–6, we found 32 genes neighbors of AR or DHT in ResNet12® database (**S. Table 1**). Several AR neighbors differ in expression by more than 20% between bald and nonbald DP cell samples (**Figure 2**). Differential expression of genes such as CYB1P1 or CTGF had positively increased in bald samples versus nonbald, whereas SPRY1 and CEPBD genes had negatively deepened expression trend in bald samples. A lot of genes reduced their expression in bald samples compare with nonbald. For example, EGR1 and EGR2 genes reduced their expression by more than 70% in balding versus nonbalding scalp. We noticed also that EGR1 is responsible gene to 1 nM level of DHT treatment in bald DP cells samples, while EGR2 (and S100A14)

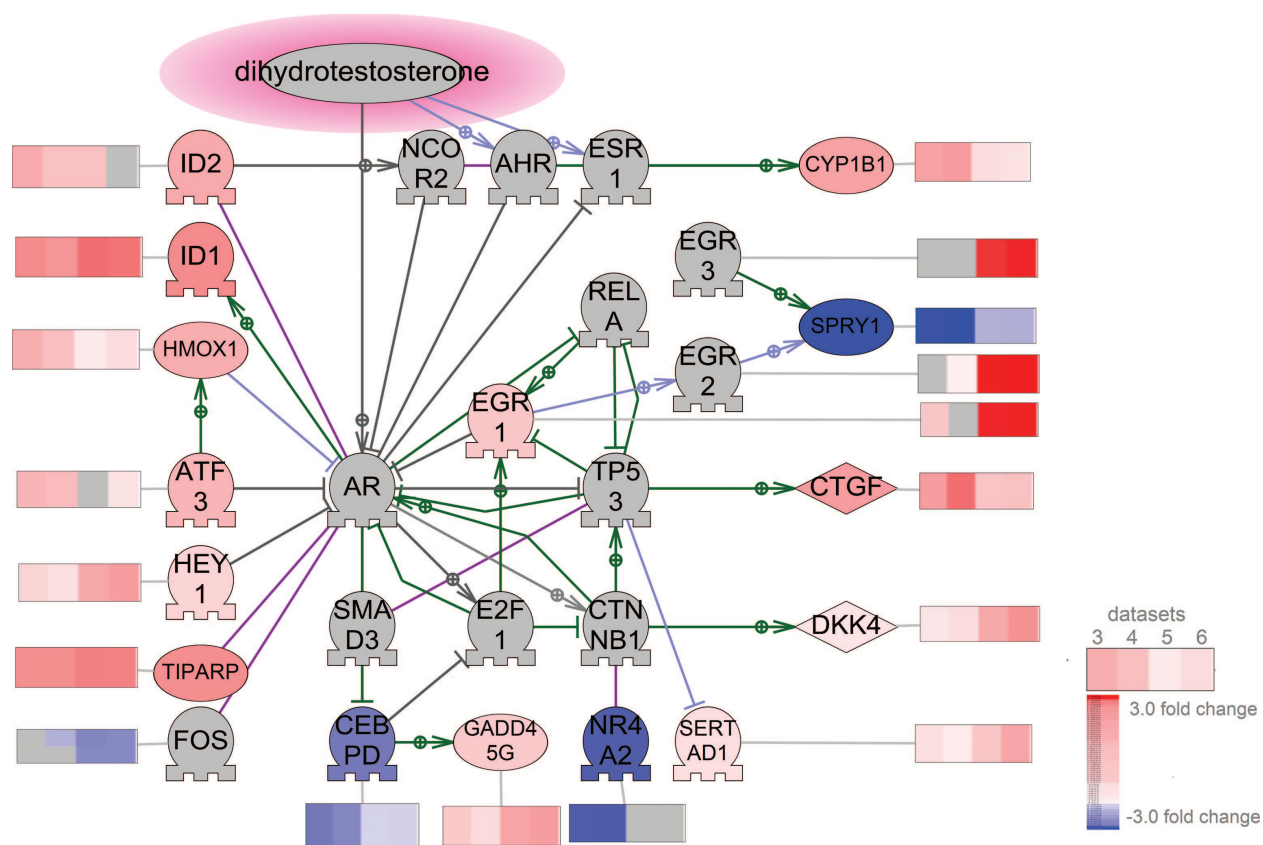


Figure 2. The impact of DHT and AR on DE genes with notably variances between bald versus nonbald dermal papilla cell samples after 1 h of 1–10 nM of DHT treatment. Highlighted proteins are corresponded to DE values in bald DP cell sample after 1 h of 1 nM DHT treatment (dataset 3). SPRY1, NR4A2 and CEBPD are underexpressed genes. ID1-2, CYP1B1, TIPARP and CTGF are overexpressed genes. Changing in DE values is represented by heat maps (rectangles near proteins).

are responsible genes to higher (10 nM) level of DHT. The 1 h of higher (10 nM) DHT level treatment correlated also with the lowering of SPRY1 differential expression in bald DP cells. The same conditions in nonbald DP cell samples correlated with slightly increasing of DKK4 and SERTAD1 differential expression (**Figure 3**).

Dickkopf WNT signaling pathway inhibitor 1 (DKK1) is a familiar target in AGA research. DKK4 also was shown to be able to inhibit WNT signaling in mice hair follicle [95]. AR could trigger DKK4 expression due to CTNNB1 activation [96]. Beta-catenin (CTNNB1) the central protein in WNT signaling in negative feedback loops manner activates DKK4 expression [97]. One more possible option how AR may impact on DKK4 expression in bald DP cells is through TRPS1. In the rat vibrissa follicles, AR could repress TRPS1, which in turn upregulates the expression of the Dkk4 [98, 99].

EGR1, EGR2, and EGR3 are early stress response transcriptional factors. In bald DP cell samples in dataset 1, EGR1 underexpressed severely. In datasets 3–6, EGRs rather downregulated expression in bald cell samples than higher differential expression in nonbald cell samples. Growth factors induce Egr2 and its target Igfbp5 expression, which are important for mice zigzag hair development [100]. Also Smad3 is involved in regulation of Egfr2 and Egr3 activation in mice skin [101]. The low expression of SPRY1 in datasets 3–4 could be associated with insufficient

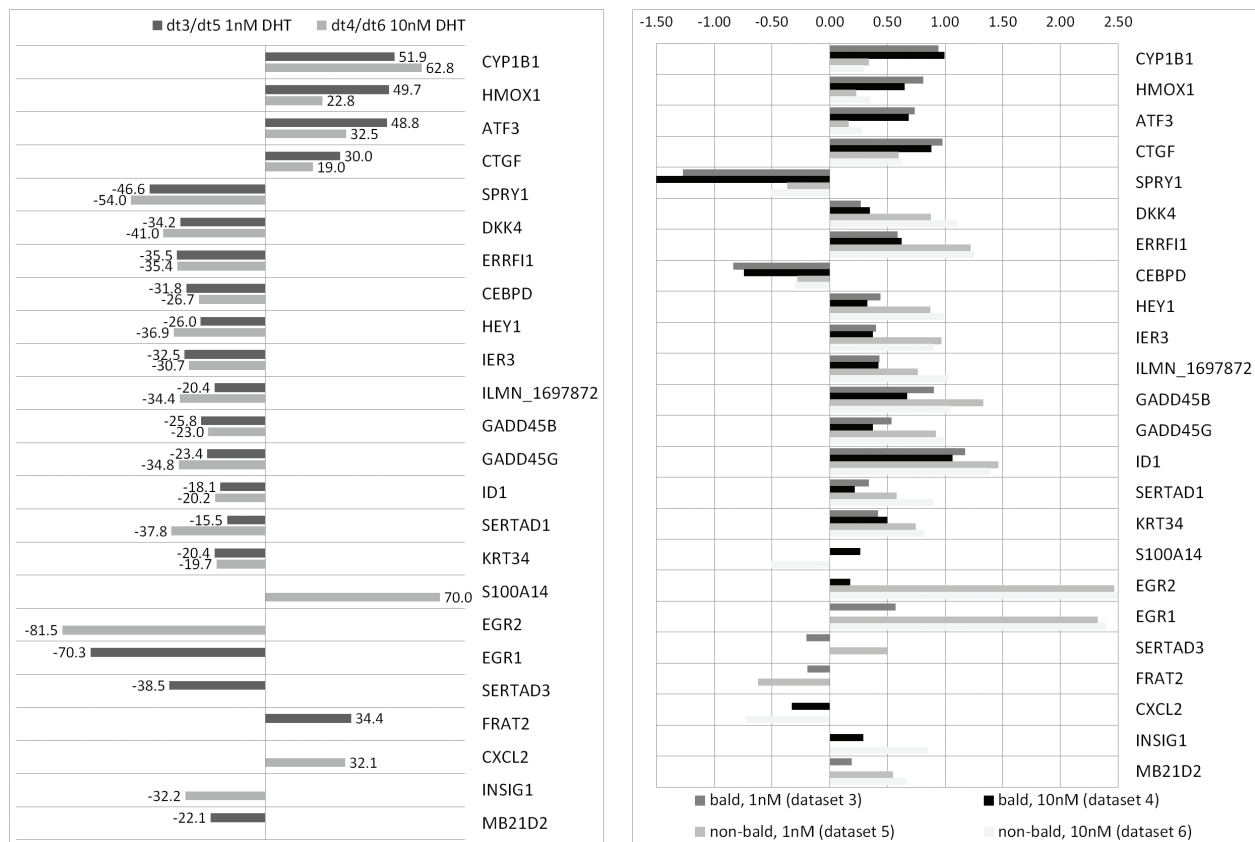


Figure 3. Genes with more than 20% distinguish in differential expression values between bald versus nonbald dermal papilla cell samples after 1 h of 1–10 nM of DHT treatment. The percent of ratio between DE values of genes in bald and nonbald samples (on the left). The comparison of DE values (log fold changes) of genes in datasets 3–6 (on the right).

EGR3 expression as *Spry1* is expression target of *Egr3* in mice T-cell [102]. In its turn, *SPRY1* may inhibit *EGR1* by disrupting the GRB2-SOS interaction in FGF or CTGF signaling [103]. *SPRY1* also was shown to be down-regulated by androgens in prostate cancer cells [104].

CYP1B1 is necessary for metabolizing intracellular oxidative stress molecules. In addition, *CYP1B1* catalyzes the generation 4-hydroxy estradiol which causes DNA damage in ovarian cancer [105]. *CYP1B1* contains an estrogen response element and addition to aryl hydrocarbon receptor response elements in its promoter [106]. *SERTAD1* expression level was significantly increased in conditions with low levels of nutrients [107]. Relation of this protein with AR signaling is unclear. *ID1* and *ID2* are key BMP signaling target transcriptional factors, and their important role was shown in mice hair follicle as well as in other organs [71]. Most probably, *ID1* and *ID2* overexpression marked the BMP signaling activation in DP cell samples at the time point 1 h after DHT treatment. AR-related activation of *ID1* was shown in cancer [108]. *ID2* was shown to interact with AR though *NCOR2* [109]. *CEBPD* and *NR4A2* both were underexpressed in bald DP cell samples, in datasets 3 and 4. *NR4A2* is required for neuronal differentiation and is also induced as an immediate early gene in dermal endothelial cells and melanocytes [110]. *NR4A2* binding to *CTNNB* disrupts co-repressor complexes, allowing co-activators to bind and activate *NR4A2* [111]. There is some evidence that androgens may participate in *NR4A2* activation [112]. Hypothetically, AR may be involved in *NR4A2* inhibition

with NR0B1 (nuclear receptor DAX-1) partnership. CEBPD is important for differentiation, survival, and cell death in many cell types including adipocytes and fibroblasts. The most important transcription factors regulating CEBPD transcription are CREB, SP1, and STAT3 [113].

There is an important note that, among others, such genes as HEY1, SPRY1, PTGS1, EGR3 are considered to be DP signatures genes [31].

3.3. Pathway and ontology analysis of AGA DP cells microarray data

3.3.1. Regulators and ontology analysis

SNEA processing of DE genes from GSE6664 array in Pathway Studio application revealed top 100 significant regulators of differentially expressed genes in datasets 1–6 (see Section 5). Regulators themselves are not genes with DE in the bald versus haired scalp, but the ones that have genes from DE list as expression targets (connected with them with expression or promoter binding relation types). SNEA regulators help to reveal activated signaling pathways that include genes with altered expression.

TGFB11I, TGFB2, CDKN2A, ATM/ATR, and MAP2K3 appeared in the list of regulators from dataset 1, which support previous data about their pivotal role in AGA development (S. Tables 2 and 1). TGFB11I, TGFB2, ATM/ATR, and MAP2K3 are found to be the regulators of underexpressed genes in bald DP cells. CDKN2A was found to be the regulator of overexpressed genes.

Anaphase-promoting complex (APC), AURKA, E2F2, and E2F7 are also found as regulators of overexpressed genes in bald DP cells. E2F transcription factors are well-known transcription factors in the cell cycle. Anaphase-promoting complex (APC/C) allows the sister chromatids at the metaphase plate to separate and move to the poles. AURKA (aurora kinase A) is required for spindle pole formation during chromosome segregation in mitosis [114].

Moreover, using SNEA, we found that MIR106A (median change -1.10844 , p-value $2.02e-03$) to be the first significant miRNA regulator of DE genes in balding DP cells. Overexpression of MIR106A was shown in DP cells from patient with AGA in [32]. Among genes that MIR106 could regulate, there are such specific for hair follicle development genes as VIM, SPP1, and IL6. SPP1 (osteopontin) is expressed only during catagen but not in anagen or telogen in cultured rat vibrissae dermal papilla cells [115]. IL6 (interleukin 6) gene is a thoughtful candidate for DHT inducible catagen phase and regression of human hair shafts [51]. BMP2 is also a direct target of MIR106A [116].

The most number of genes with differential expression more than 2-fold change values in datasets 3–6 come about 6–12 h' time point transition after DHT treatment compared to absent of DHT (data not shown). We are presume that genes with extremes of expression of these time points point to paracrine signaling pathways initiated afterward DHT/AR signaling. SNEA regulators of DE genes in 6/12 time point are representing AR signaling follow-on cell processes and pathways.

RUNX1, CRTIC1, FGF8, and others appeared in the list of regulators of expression which were common for bald DP cells samples (dataset 3 and 4) at 12 h after DHT treatment (S. Table 2).

Inductors of mesenchymal cell differentiation were the notable group in the regulator's list. A lot of detected regulators are important for mesenchymal cell differentiation in brain, bone, and hematopoietic cell lineage. For example, CREB-regulated transcription coactivator 1 (CRTC1) is a coactivator of NOTCH and mesenchymal cell differentiation and also regulates the expression of *KISS1* in normal melanocytes [117]. RUNX1 is the well-known transcriptional factor that drives the development of skin appendages as well as hematopoietic cell lineage [118]. In hair follicle stem cells, RUNX1 promotes proliferation and simultaneously represses cell-cycle-related *p21*, *p27*, *p57*, and *p15* transcription. Expression targets of RUNX1 and CRTC1 SNEA regulators include top DE genes in 6/12 h after DHT treatment in datasets 3–6 (Figure 4). The relations between regulators and their targets are not specific to dermal papilla cells (described for different cell models).

AR-related proteins, such as *PIAS2*, *LATS2*, and *URI1*, were found as regulators in dataset 4. *PIAS2* binds to the androgen receptor and inhibits in a testosterone-dependent manner AR transcription activity in testis [119]. *PIAS2* decreases N-cadherin protein expression during the neural crest development [120]. We found that at 12-h time point, N-cadherin expressed more intensively only in nonbald DP cell samples (dataset 5 and 6) versus bald DP cell samples. During epithelial-mesenchymal transition, *ID1* and *ID2* also repress E-cadherin promoter expression [121]. *URI1* (prefoldin RPB5 interactor) was revealed as repressor regulator of AR transcription in human cancer prostate cell lines [122]. *LATS2* already has been noticed as underexpressed DE gene in the dataset 1.

DP signature genes, such as *MYLIP*, *SPRY1*, *FABP5*, and *GPM6B*, were found to be underexpressed after 12 h of DHT treatment in bald DP cell samples.

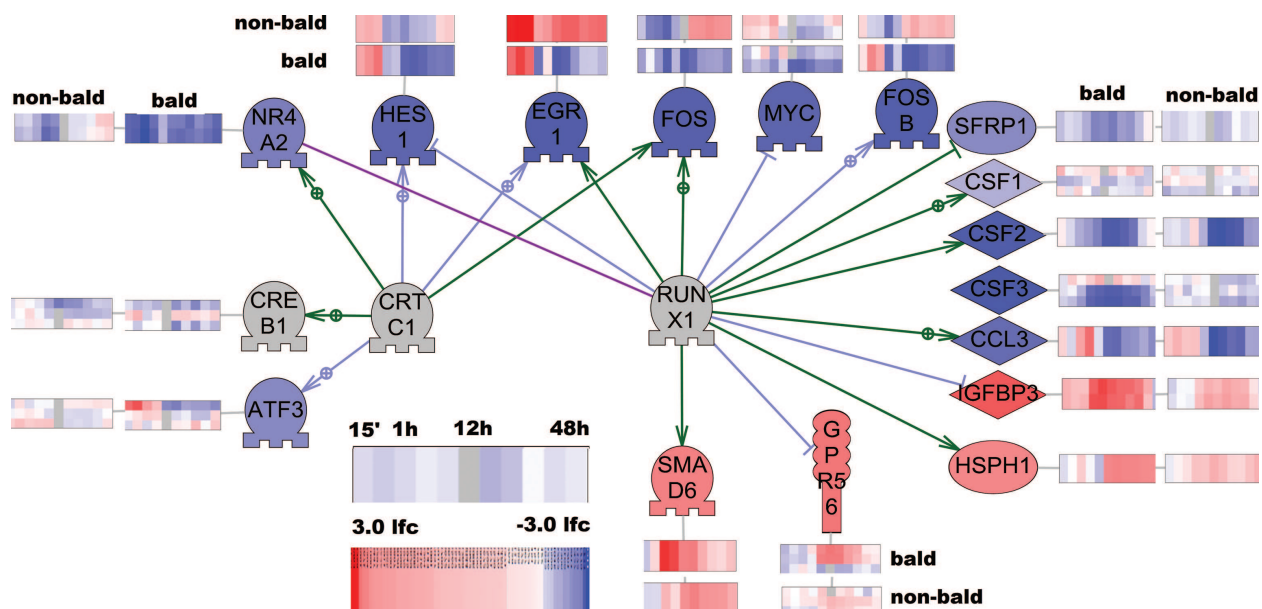


Figure 4. Expression targets of SNEA regulators RUNX1 and CRTC1 associated with DE genes in bald and nonbald DP AQ15 cell samples after 12 h of 1–10 nM DHT treatment. Highlighted proteins are corresponded to DE values in bald DP cell sample after 12 h of 1 nM DHT treatment (dataset 3). *CREB1*, *CRC1* and *RUNX1*—genes without significantly DE in the dataset 3 (12 h time point). *ATF3*, *NR4A2*, *HES1*, *EGR1*, *FOS*, *MYC*, *FOSB*, *SFRP1*, *CSF1*–3 and *CCL3*—underexpressed in bald DP cell sample genes. *IGFBP3*, *HSPH1*, *GPR56* and *SMAD6*—overexpressed genes. Changing in DE values is represented by heat maps (rectangles near proteins). Chemokines, cytokines, and proteins involved in hematopoietic cell differentiation are widely presented in the list of regulators in datasets 3–6 (*CSF1*, *TYROBP*, *L6ST*, and others).

Statistical methods in the gene expression analysis such as Fisher's exact test and SGEA help to quickly reveal general picture of actual genes group, cell processes, and signaling pathways preceded the experiment. Several GO (Gene Ontology) groups and cell processes enriched with top DE genes from datasets 1–6 have revealed with GSEA. Specific biological cell processes such as cytokine-mediated immune response, regulation of apoptotic process, angiogenesis, and other similar to those in original research [31] were associated with underexpressed DE in bald frontal DP cells in dataset 1. Cellular response processes related to overexpressed DE genes were also similar to results in [31] and included DNA damage, collagen fibril organization, mitotic spindle organization, telomere maintenance, skin development, and others. Regulators of overexpressed DE genes from dataset 1 were significantly associated with "cell cycle," "response to DNA damage," and "apoptosis" cell processes. Regulators of underexpressed DE genes were associated with "apoptosis," "cell adhesion and migration," and "angiogenesis" (**S. Table 2**). Several ligands (TGFB2, THBS1, PTHLH, and ANGPT2) associated with cell process "catagen progression" also have been found to be regulators of underexpressed genes in bald DP cells from dataset 1.

We found stem cell and endothelial cells differentiation cell processes associated the list of regulators of DE genes after 12 h of DHT treatment for the datasets 3–6. Other types of GO and cell processes associated with regulators were "response to DNA damage stimulus," "induction of positive chemotaxis," and "angiogenesis." "Estrogen metabolic process" was found in the top of GO groups enriched with regulators for the datasets 3–6 in 12-h time point.

DE genes after 12 h of DHT treatment from datasets 3–6 associated themselves with "negative regulation of pathway-restricted SMAD protein phosphorylation," "regulation of WNT signaling pathway," "positive regulation of epithelial to mesenchymal transition" cell processes. DE genes after 12 h of DHT treatment characterized with "positive regulation of BMP signaling pathway," "regulation of angiogenesis," and other cell processes. (**S. Table 2**).

3.3.2. Pathways analysis

Pathway analysis with the lists of DE gene regulators from the dataset 1 on Elsevier PS Pathway Collection (PS Collection) reveals activation of cell cycle-related pathways. Pathways related to chromatin remodeling were most significantly enriched with DE genes in bald DP cells. G2/M and G2/S cell cycle phase transition pathway has 18 overlapping genes out of total 47 (p-value 2.66e-04). Kinetochore assembly pathway has 22 overlapping genes out of total 48 (p-value 5.10e-11). Metaphase/anaphase phase transition pathway has 13 overexpressed overlapping genes out of total 16 (p-value 2.07e-04). TGFB-/BMP-related pathways as well as chemokine related were also found to be activated in cultured bald DP cells in the dataset 1 (**S. Table 2**).

DNA repair is also activated process in bald DP cells. It is governed mostly by ATM/H2AFX double-strand repair mechanism. ATR/ATM is known as proteins that "recognize" DNA damage. In G1/S DNA damage checkpoint, these kinases phosphorylate TP53. TP53 stimulates the induction of CDKN1A, which results in CDKs inhibition and cell cycle arrest, preventing the replication of damaged DNA [123]. It is a probability that cell cycle arrest cannot be achieved in bald DP cells because CDKN1A strong underexpressed there, compared to nonbald ones as we found in datasets 1 and 2 (−4.94 log fold changed, p value 1.42e-88).

Though, underexpression of CDKN1A was not confirmed in analysis of differential expression in datasets 3–6. Androgen receptor could directly bind to the CDKN1A promoter [124]. AR was also involved in CDKN1A expression inhibition through LATS2 [85]. TGF β 2 or BMP signaling pathways could effect on CDKN1A expression through SMAD and RUNX2 transcriptional factors [125]. ID1 and ID2 were shown to also down-regulate CDKN1A and promote cell cycle in prostate epithelial cells [126].

Also, in pathway analysis of datasets 1–2, there were canonical pathways of apoptosis and general signaling like TNF, TGF β , and NF- κ B. Other groups of pathways were adiposal, bone, neuronal, or hematopoietic-specific pathways.

Pathway analysis with datasets 3–6 generally checked with the results of gene group's ontology analysis (**S. Table 2**). Cell-cycle-related pathway G0/G1 cell cycle phase transition (46% overlap, p-value 3.57093E-55) and single-strand nucleotide excision DNA repair (25% overlap, p-value 9.45E-10) were one of top pathways associated with bald DP cell samples. Apoptosis-associated TNF-alpha/TNFRSF1B signaling (64% overlap 1.80E-27) and TGF-beta signaling (32% overlap, p-value 3.04097E-27) also were on the top of the list. Estrogens/ESR1 nongenomic signaling (45% overlap, p-value 6.96E-06) and androgen receptor nongenomic signaling (14% overlap, 3.08E-05) also were found (**Figure 5**). A lot of pathways in the list were associated with canonical signaling pathways, such as ERK/MAPK canonical signaling or NF- κ B canonical signaling (**Figure 6**). Pathways associated with chemokines signaling, adipose tissue, bone tissue, neuronal tissue, and hematopoietic cells were present.

3.4. Cross-talk between pathways affected in AGA DP cells

All noticed differences in molecular signaling pathways between DP cells from frontal and occipital scalp should help to define the general model of AR-related frontal scalp area hair follicle cycle switch in AGA. The results of microarray analysis suggest that androgen synthesis and metabolism are not affected in DP cells from AGA patients. Differential expression of such ferments from the androgen metabolism pathways as STS, HSD3B2, HSD17B1, and SRD5A2 is not changed in any time point in datasets 3–6. Though, expression of several proteins involved in the metabolism synthesis of cholesterol and estradiol had elevated. CYP1B1 differential expression was slightly elevated in bald DP cells with compare to nonbald DP cells at 1-, 3-, and 6-h time points.

BMP signaling is seemed to be activated both in bald and nonbald DP cell samples in the first 6 h after DHT treatment (while in bald DP cell samples, the activation was stronger). During the length of DHT treatment, the markers of BMP signaling DE genes ID1 and ID2 became underexpressed in bald DP cell samples (datasets 3–4) while not in nonbald DP cell samples. BMP2 was the only BMP ligand which differential expression had slightly changed (increase) in bald DP cells samples with compare to nonbald in the analysis of datasets 3–6. In mice, *Bmp4* has strong expression in catagen and early telogen and weak expression only before new anagen [9]. It is considered that in hair follicle, lowered activity of BMP signaling correlates with generally upregulated WNT signaling activity in the end of telogen due to existed reciprocal inhibition between WNT and BMP signaling [64, 70] (**Figure 6**). In mice hair follicle, low activity of BMP signaling in DP cells led to loosing signature characteristics of DP cells and failure in generating

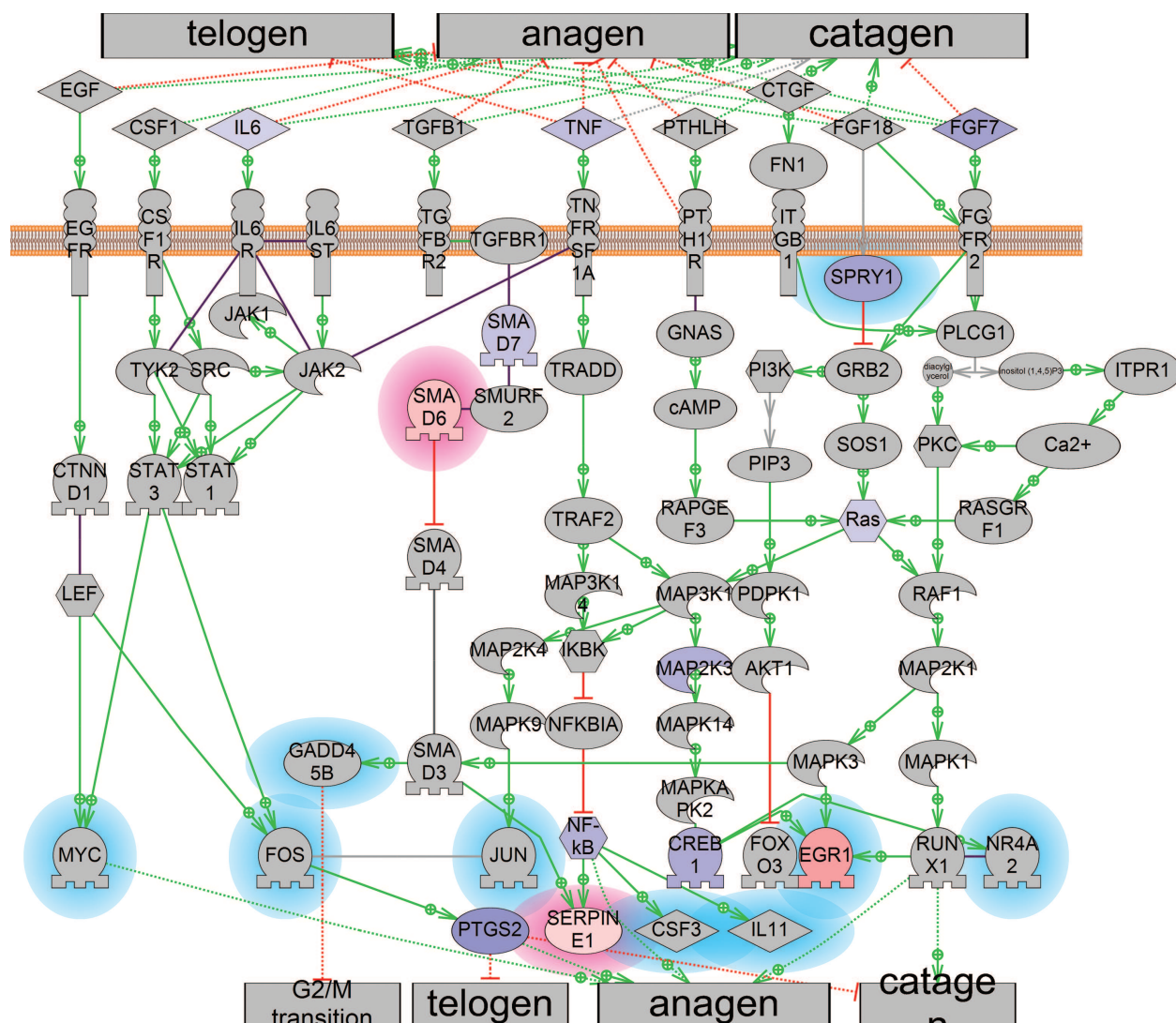


Figure 5. Signaling pathways of the regulation of transcription by growth factors and cytokines in AGA scalp. Pathways that include genes with changed expression in bald and nonbald DP cell samples after 12 hours of DHT treatment are shown. TNF α , CSF1, TGF β , EGF, FGF, PTHLH, CTGF, and IL6 ligand/receptor signaling pathways were the major affected pathways found in the pathway analysis. Only key proteins and relations of each ligand/receptor signaling are shown on the figure. Major transcription factors from dataset 3-4 (NF- κ B, JUN-FOS, MYC, CREB1, and RUNX1) are shown on the bottom of the figure. Highlighted proteins correspond to underexpressed genes (SPRY1, NRHA2, EGR1, IL11, CSF3, JUN, FOS, GADD45B, and MYC) or overexpressed genes (SMAD6 and SERPINE1) in bald DP cells 12 hours after DHT treatment (datasets 3-4). IL6, TNF, FGF7, SPRY1, SMAD7, PTGS2, and CREB1 – underexpressed genes in datasets 5-6 (nonbald DP cells 12 hours after DHT treatment). SMAD6, EGR1, and SERPINE1 – overexpressed genes in datasets 5-6. The color saturation reflects the intensity of the differential expression within $-0.5; 0.5$ log-change interval, and 0.05 p-value cutoff.

new hair follicle cells [127]. Thereby, we may assume that the activity of WNT signaling should be lower in bald DP cell samples in first 6 h of DHT treatment. In nonbald “normal” DP cell samples, we found that WNT signaling was lowered but still activated in bald DP samples. DKK1 was suggested as one of the key player of triggering catagen in AGA [45]. We could not detect significant DE of DKK1 in dataset 1. We detected slight DKK1 underexpression in nonbald DP samples (dataset 5–6) at 1/3 h’ time point, whereas is not in bald DP cells. DKK4 is, vice versa, overexpressed in nonbald DP cells in the first 1 h.

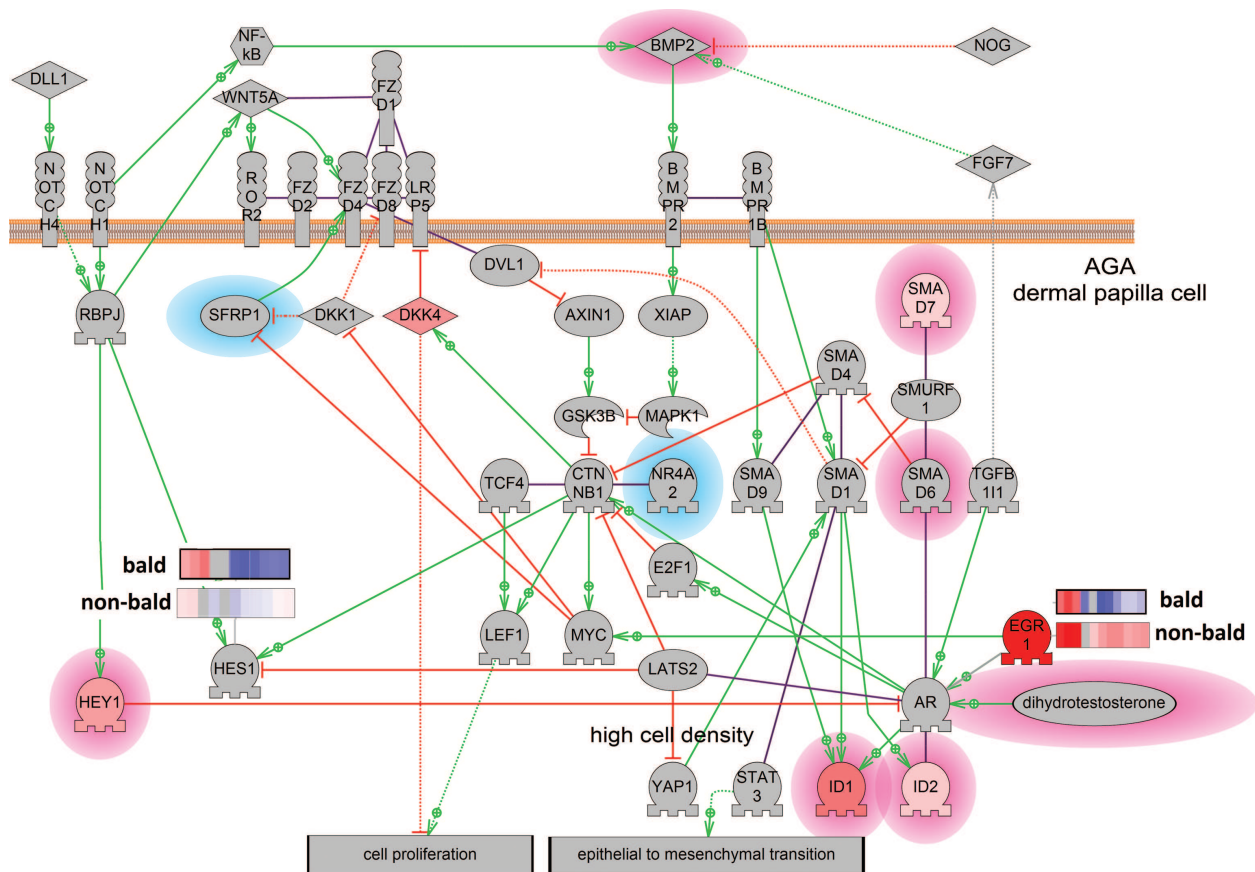


Figure 6. Model of BMP, WNT, NOTCH, and AR-related pathway cross-talk in AGA dermal papilla cell. DKK4, EGR1, HEY1 and ID1-2 are underexpressed genes in nonbald DP cells 1–3 h after DHT treatment (datasets 5–6). The higher color saturation reflects the higher degree of differential expression ([0.5; 0.5] log-change interval, 0.05 p-value cutoff). SFRP1 and NR4A2 are underexpressed genes in bald DP cells 1 and 3 h after DHT treatment (datasets 3–4). BMP2, SMAD6-7, HEY1, and ID1-2 are overexpressed genes. DKK1 and DKK4 inhibit WNT due to feedback loop in WNT signaling. AR regulates WNT pathway through LATS2, EGR1, and CTNNB1. BMP signaling inhibits activity of WNT pathway. BMP signaling in general inhibits WNT signaling. NOTCH pathway has negative effect on AR expression via HEY1. EGR1 and HES1 have significant changes in expression at all time points assessed after DHT treatment, so their expression is shown with heat maps.

Perhaps, larger amount of DHT is needed to stimulate AR-related DKK1 expression. High level of DKK1 was observed within 3–6 h after 50–100 nM DHT treatment previously [45]. In mice, DKK1 expression, similarly to BMP2/4 expression, is time-dependent in hair cycle and is changing according to hair follicle cycle phase [70]. We could not find severe relevant difference in the pattern of WNT signaling activity, which is not surprising, since fluctuations in these networks are closely associated with current hair cycle phase, which is, firstly, poorly studied in human, and secondly, impossible to verify in a culture of separated dermal papilla.

SFRP1 is one of the significant overexpressed in dataset 1 genes that could stimulate WNT pathway. Other speculations we developed on the basis of pathway analysis of current microarray data are shown (Figure 6).

As shown in Figure 7, numerous feedback loops regulate BMP/WNT crass-talk themselves, as well as intersections with AR and other signaling pathways, such as SHH, NOTCH, or Hippo/YAP1 [73].

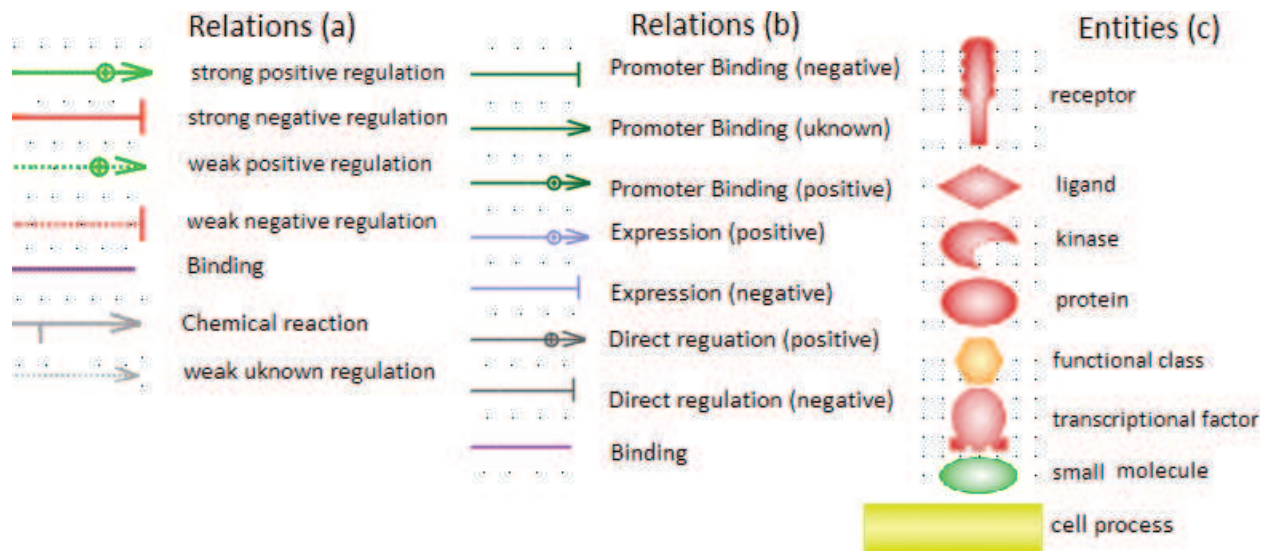


Figure 7. Legend for entities and relations displayed on pathways. Relations from ResNet® used in **Figures 1, 5, and 6 (a)** and in **Figures 2 and 4 (b)** are shown. Shapes of entities from ResNet® used in **Figures 1–6** are shown (c).

LATS2 is a DHT-responsible underexpressed gene in bald DP cell samples in current analysis. LATS2 phosphorylates and cause the degradation of the transcriptional co-activators Hippo/YAP1 and TAZ, which are in turn interact with RUNX1, SMAD7, and play role in TGF-beta/BMP/SMADs signaling inhibition [128]. SMAD7 and SMAD6 were detected slightly underexpressed in bald DP cell samples before 6-/12-h time point.

Overexpression of PTGDS in hair follicles during catagen was shown previously [55]. We detect overexpression of other type of synthase (PTGS2) of prostaglandins in current analysis (**Figure 5**). PTGS2 is DHT/AR-inducible expression target in skin [129]. Prostaglandin has different role, such as enhancing of the vascularization and chemotaxis.

In mice, FGF7 is one of the DP signature genes that elevated during telogen. FGF7 participates in instructing DP cells to proliferate and initiate the new hair cycle [64]. In current microarray, we did not detected significant overexpression of FGF7 in bald DP cells in datasets 3–6. FGF18 and its expression target RUNX1 were among SNEA regulators of underexpressed DE genes in dataset 3–4 at 12-h time point. SPRY1 may contribute to FGF signaling regulation in dermal cells. SPRY1 expression was shown to induce upon activation of the FGF receptor signaling pathway in cells of mesenchymal and epithelial origin [130]. In response to growth factors, SPRY1 binds GRB2 and inhibits downstream intracellular signaling. SPRY1 gene had negatively DE trend in bald samples after 1 h of DHT treatment (**Figure 5**).

4. Conclusion

Hair follicle miniaturization in androgenic alopecia is complex process induced by AR influence on dermal papilla cell function. Signaling pathways are useful models of complicated protein interactions in dermal papilla cell during hair follicle cycle. Cross-talks between AR

and TGFB, BMP, and WNT signaling are believed to promote shortage on amount and functional activity of dermal papilla cells in bald scalp in AGA. WNT signaling is considered to be the main trigger of telogen-anagen transition that drives repopulation of dermal papilla progenitor cells from bulge niche. Its impairment could potentially lead to AGA phenotype. Detailed understanding of molecular causes of hair loss in AGA is still has not been reached. New standardized studies of hair follicle cell cycle in human scalp are required to find human-specific information about dermal papilla and stem cells maintenance during their lifetime renewals.

Pathway analysis of cDNA microarray data from cultured immortalized human DP cells from balding (frontal) AGA scalps reveals activation of chromatin remodeling and metaphase-anaphase transition pathways. Observed slight up-regulation of cell cycle inhibitor protein CDKN2A confirms other studies and indicates up-regulation of DNA repairing pathways. Expression of AR-related genes NOS3, EGR1, SMAD6, BTG2, and LATS2 was significantly down-regulated exclusively after DHT treatment in frontal DP cells compared to occipital bald cells from AGA scalp. Differential expression of TGFB1I1, TGFB2, THBS1, PTHLH, and ANGPT2 was not changed dramatically, but they appeared among top 25 regulators of underexpressed genes in bald DP cells samples. TGFB1I1 is a reported AR cofactor. Ligands TGFB2, THBS1, PTHLH, and ANGPT2 launch receptors signaling related to catagen progression (and ligand CTGF to telogen). MIR106A was found among the top of significant miRNA regulators of DE genes in bald DP cells.

After 1 h of DHT treatment, EGR1 and EGR2 genes reduced their expression by more than 70% in balding versus nonbalding scalp. SPRY1 and CEPBD and NR4A2 genes had also negatively differential expression trend in bald samples. CYB1P1 and CTGF had positively increased in bald samples versus nonbald after 1 h of DHT treatment. DKK1 as WNT inhibitor was underexpressed in nonbald DP samples 1 h after DHT treatment. DKK4 was, vice versa, overexpressed in nonbald DP cells in the first 1 h. NR4A2, EGR1, HES1, and NR4A2 had remarkable difference in expression between bald and nonbald DP cell samples. Unfortunately, role of these proteins was acquired in cancer-related studies that make them hardly applicable to the hair follicle research. Further investigation is required.

The obtained results suggest that AGA dermal papilla in frontal scalp area differs from occipital ones unlikely due to downregulation of proliferation and increased expression of catagen triggers, but rather due to reduced expression of anagen triggers.

5. Methods

5.1. Pathway analysis

We chose several public studies about hair loss in men with AGA using microarray technology from GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). Data from GSE66664 dataset were log₂ transformed, quantile normalized, and then imported into Pathway Studio® software. We used Pathway Studio® version 9 with ResNet® database version 12.

Differentially expressed genes were identified with two-class unpaired T-test implemented in Pathway Studio®. Multiple probes were averaged by best p-value or maximum magnitude. Selection of differentially expressed genes was made using samples BAB (GSM1623702-72) and BAN (GSM1627372-441) after 0 min and after 15 min, 30 min, 3 h, 24 h, and 48 h after DHT treatment.

Cell processes and pathways enriched in over (under) DE genes were set up with GSEA algorithm or Fisher exact test. From DE list, significantly expressed genes were chosen with 0.05 p-value and 2-fold change of the expression (log change interval $[-1; 1]$) cutoffs. In GSEA, Mann-Whitney U-test with 0.05 p-value cutoff was applied, followed by expanding of the content of proteins groups. GO biological process groups and pathway collection from Pathway Studio were used as gene set. The pathway collection included Cell Process, Receptor Signaling, Expression targets, Canonical Signal Transduction Signaling, Immunology, Toxicity, and Disease sub-collections (2160 pathways in total). Moreover, 10 pathways were constructed specifically for analysis of hair follicle-specific genes expression. These are Dermal Papilla Cells Proliferation in Anagen; Dermal papilla Cell Regression in Catagen; Keratinocytes Apoptosis; Melanocytes Differentiation; Hair Stem Cells Maintenance; Androgen Synthesis in Sebocytes; Sebocytes Proliferation; Hair follicle in Telogen; Immune System Activation in AGA; Androgen Receptor Genomic, and Non-genomic Signaling (files with pathways available in Pathway Studio® or in XML format upon request). Total numbers of entities and relations in hair follicle-related pathways are 150 and 76, respectively.

To find statistically significant regulators of DE genes, SNEA algorithm was applied. SNEA is a sub-network analysis of DE genes or proteins based on GSEA method and 2 million biological relations in ResNet® database [71]. The choice of neighbors for sub-network generating was entities upstream of DE genes (proteins/genes, complexes, functional classes) connected by relations of “Expression” or “Promoter Binding” types. We used 0.05 p-value cutoff and limit of 100 generated sub-networks ranked by best p-value. We performed SNEA to find 100 significant miRNA regulators.

Pathways statistically enriched with discovered list of regulators were found with Fisher exact test. All images have been exported from Pathway Studio with special color specification (all overexpressed genes in experiment are red, all underexpressed genes are blue, entities not measured or filtered with p-value or log change cutoff are grey).

5.2. Signaling pathway reconstruction

Pathway Studio® software and ResNet® database from Elsevier were used for building hair follicle cells specific interactive model pathways. Model signaling pathways reconstruction was based on current scientific knowledge: settled facts and hypotheses (e.g., data acquired in animal models). Pathway creation relies on published papers and searching ResNet® database for neighbors, functions, and expression of chosen proteins and molecules.

Model of cell specific molecular interactions in this article consists in an interactive signaling pathway that is represented by the graphical scheme and the annotations. In the graphical representation, the members of interactions are shown linked together with functional relations. The annotations section includes the information about properties of pathway members

and relations pulled out from ResNet® database. ResNet® database stores dictionary and ontology of biological-related entities (proteins, small molecules, diseases, cell processes, cells, treatments, etc.) linked together by relations (such as protein 1-protein 2 “binding”) (**Figure 7**). Relations depict facts supporting by sentences (references) with scientific evidences which are extracted from more than 3 million articles and manually curated when adding to pathways. The relations indicate the effect of relation (negative or positive), the direction of the relation, mechanism of the relation (such as phosphorylation), etc. [71]. The method of the pathway reconstruction required looking for common relationships to entities in the database (common downstream cell process or disease, upstream regulators, downstream expression targets, etc.) and reviewing manually the results.

Supplemental Information:

Supplemental Table 1 and Supplemental Table 2 could be downloaded via link:

<https://data.mendeley.com/datasets/yfnrkc7r9x/1>

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