

Human hair follicle regeneration: from molecular mechanisms to therapeutic targeting

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“Every scientist dreams of doing something that can help the world.”

Tu Youyou - Nobel Prize in Physiology or Medicine in 2015

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“For an idea that does not first seem insane, there is no hope”

Albert Einstein, Nobel Prize in Physics 1921

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Resumo

A crescente sensibilização da sociedade atual para os padrões de beleza, em que um cabelo saudável está associado a boas condições de saúde em geral, juntamente com a crescente incidência da alopecia em pessoas mais jovens, tem levado a um aumento da procura de novas soluções terapêuticas. A limitada eficácia e os efeitos colaterais dos tratamentos atuais estimulam a procura crescente de novas e melhores soluções para esta doença.

Acredita-se que será possível curar a alopecia produzindo folículos *de novo* (multiplicação de folículos), o que permitirá obter um número ilimitado de folículos para serem transplantados para a região calva de um paciente. Por isso, grandes esforços têm sido colocados no desenvolvimento de metodologias de engenharia de tecidos e medicina regenerativa cada vez mais eficazes e eficientes.

As células mesenquimais da papila dérmica humana (DP) têm sido foco de estudo no tratamento desta doença, uma vez que representam um reservatório de células progenitoras adultas de fácil acesso e com elevado potencial terapêutico. Para além disso, são o centro de sinalização responsável por regular a indução e crescimento do cabelo. No entanto, ao contrário do modelo de ratinho, o maior obstáculo para uma terapia de bioengenharia para alopecia deve-se ao facto da cultura *in vitro* de células humanas da DP conduzir à perda das propriedades tricogénicas (capacidade de induzir formação de cabelo). Deste modo, o grande objetivo da medicina regenerativa para o tratamento da alopecia consiste em recuperar/estimular o potencial tricogénico das células da papila dérmica.

Deste modo, esta tese apresenta um estudo de análise e caracterização das células da papila dérmica do folículo capilar humano e do seu potencial regenerativo. Nesta tese, estudamos também a modulação de vias moleculares já estudadas (TGF β) e associadas á alopecia, de modo a restaurar a assinatura molecular tricogénica das células da DP. Para além disso, analisámos o efeito terapêutico da inibição da via do TGF β para o estímulo do ciclo de crescimento do folículo capilar e concluímos quando ao seu potencial terapêutico para a alopecia.

Por fim, apresentamos uma metodologia inovadora para rastrear compostos quanto ao seu potencial tricogénico. A metodologia desenvolvida permite prever a resposta folículo capilar humano a um grande número de fármacos e com grande eficiência. Com esta metodologia desenvolvida em células da papila dérmica de folículos humanos será possível descobrir novos fármacos com elevado potencial para o tratamento clínico da alopecia.

Palavras-chave: Alopecia; Folículo capilar; Engenharia de tecidos, Medicina regenerativa; Investigação translacional; Descoberta de fármacos.

Abstract

There is an increasing demand for new therapeutic solutions targeting alopecia as people are now more concerned about beauty standards, lifestyle and general health. The limited efficacy and the adverse side effects of the current treatments has fostered the need for new products and/or treatments for AGA.

The Human mesenchymal dermal papilla (DP) cells have been the focus of intense research as they modulate hair follicle development and growth during hair follicle cycle and represent a reservoir of easy-access adult progenitor cells. For long, they have been explored for hair follicle tissue engineering therapeutic strategies against hair loss. A successful bioengineering solution for alopecia would require an available amount of competent epithelial and inductive DP cells that, if properly combined, would be capable of generating numerous HFs *de novo* (hair cloning) that serve as an infinite source of material to cover patient's balding scalp. However, a major bottleneck in HF bioengineering has been the inability to obtain a large number of human trichogenic DP cells. The *in vitro* expansion of human DP cells aiming *de novo* hair growth in balding scalp has been extremely challenging due to the loss of trichogenic properties after cell culturing. Therefore, a major goal on regenerative medicine studies for hair loss cure consists in stimulating or recovering back the dermal papilla hair inductive potential.

This thesis studies and characterizes the molecular mechanisms controlling hair follicle dermal papilla cells' regenerative potential. Also, we validate the efficacy of a potent TGF β receptor I inhibitor in ameliorating DP culturing stress and retrieving inductive DP signature. Additionally, we further studied TGF β effects on hair growth cycle and validate it as a critical pharmacological therapy for hair regeneration.

Finally, we set out to develop a novel high throughput methodology to screen for hundreds of drug compounds and confidently predict on human hair follicle inductive capacity. We discuss promising new pharmacological players capable of inducing *de novo* hair follicle growth and their impact on medicine regenerative strategies for hair loss treatment.

Keywords: Alopecia; Hair follicle; Hair regeneration; Tissue engineering; Translational research; Drug discovery.

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List of abbreviations

AA	Alopecia Areata
ACTC1	Actin alpha cardiac muscle 1
ACTA2	Actin alpha 2, smooth muscle
AI	Artificial Intelligence
AGA	Androgenic Alopecia
ANGPT2	Angiopoietin-2
AKT	Serine-threonine protein kinase
APOE	Apolipoprotein E
AR	Androgen Receptor
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia telangiectasia and Rad3 related
ASCs	Adipose-derived Stem Cells
B2C	Business-to-consumer
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BP	Biological process
BSA	Bovine serum albumin
CBP	CREB-binding protein
CDH2	Cadherin-2
CNN1	Calponin 1
cDNA	Complementary deoxyribonucleic acid
cGMP	current Good Manufacturing Practice
ChIP-Seq	Chromatin Immunoprecipitation Sequencing
COVID-19	Coronavirus 19
COMP	Cartilage oligomeric matrix protein
COL7A1	Collagen type VII alpha 1 chain
COL8A2	Collagen type VII alpha 1 chain
COL5A1	Collagen type V alpha 1 chain
CT	Control
DAPI	2-(4-amidinophenyl)-1H -indole-6-carboxamide
DEG	Differentially expressed genes
DESM	Desmin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DNMT	DNA methyltransferase
DHT	Dihydrotestosterone
DIO2	Iodothyronine deiodinase 2
DP	Dermal Papilla
DPCs	Dermal Papilla Cells
DS	Dermal Sheath
DSC	Dermal Sheath Cup
DSG3	Desmoglein 3
DTC	Direct-to-costumer
EFNA5	Ephrin
EC	Extracellular
ECM	Extracellular Matrix
EC50	Effective concentration
EM	Epithelial-Mesenchymal
EN1	Engrailed1
EMI	Epithelial-mesenchymal interaction
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
FDP	Fresh dermal papilla
FGF	Fibroblast growth factor
FOXP1	Forkhead box protein N1
FOXP1	Forkhead Box S1
FUE	Follicle Unit Excision
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GL	Galunisertib
GMP	Good Manufacturing Practice
GO	Gene ontology
GREM1	Gremlin 1
GREM2	Gremlin 2
GSEA	Gene set enrichment analysis
HB	Hair bulb
HBSS	Hank's Balanced Salt Solution
HDACs	Histone deacetylase
HES1	Hes Family BHLH Transcription Factor 1
HES5	Hes Family BHLH Transcription Factor 5
HES2	Hes Family BHLH Transcription Factor 2
HEYL	Hes related family bHLH transcription factor with YRPW motif like

hESCs	Human embryonic stem cells
HF _s	Hair follicles
HFSCs	Hair follicle stem cells
hiPSCs	Human-induced pluripotent stem cells
HFOCs	Hair Follicle Organ Cultures
HIF-1	Hypoxia-inducible factor 1
HR	Hairless
hSKPs	Human Skin-derived precursors
HTS	High-throughput screening
IF	Immunofluorescence
IHC	Immunohistochemistry
iPSC	Induced Pluripotent Stem Cells
ITGAV	Integrin Subunit Alpha V
JAK2	Janus kinases
JAK-STAT	Janus kinase signal transducer and activator of transcription pathway
JMJD	Jumonji C domain-containing
KDM2/7	Lysine Demethylase 2/7
Lef1	Lymphoid Enhancer Binding Factor 1
LiCl	Lithium Chloride
LSD1	Lysine-specific demethylase 1A
MAPKs	Mitogen-activated protein kinases
MEM	Minimum Essential Medium
MeSCs	Melanocyte Stem Cells
MHC	Major histocompatibility complex
MMP2	Matrix metaloproteinase-2
MMP8	Matrix metaloproteinase-8
MMP3	Matrix metaloproteinase-3
MPZ	Myelin Protein Zero
MSK1	Mitogen- and stress-activated kinase 1
MSX2	Msh Homeobox 2
MSC	Mesenchymal Stem Cells
NES	Negative normalized enrichment score
NDNF	Neuron derived neurotrophic factor
NTN4	Netrin 4
NCAM1/2	Neural Cell Adhesion Molecule 2
OPN	Osteopontin
ORS	Outer Root Sheath

PAI-1	Plasminogen activator inhibitor
PARP	Poly (ADP-ribose) polymerases
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PDGFR α	Platelet-derived growth factor receptor α
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PGF2 α	Prostaglandin F2 α
PGE2	Prostaglandin E2
PHD2	Prolyl hydroxylase domain 2
PI3K	Phosphoinositide 3-kinase
Pim	Proviral insertion in murine
PMEL	Premelanosome protein
p0	Passage 0
p2	Passage 2
POSTN	Periostin
PPP1R15A	Protein Phosphatase 1 Regulatory Subunit 15A
PRL	Prolactin
PRMT5	Protein arginine methyltransferase 5
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
PRP	Platelet-rich Plasma
PTP	Protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase 1B
PTPMT1	Protein Tyrosine Phosphatase Mitochondrial 1
WNT	Wingless
KEGG	Kyoto Encyclopedia of Genes and Genomes
KGF	Keratinocyte growth factor
KRT82	Keratin 82
KRT85	Keratin 85
KRT84	Keratin 84
KTs	Keratinocytes
RNAi	Interference ribonucleic acid
RIN	RNA integrity number
RGD	Arginyl-glycyl-aspartic acid
SCID	Severe Combined Immunodeficiency
SEMA3B	Semaphorin 3B

SEMA6D	Semaphorin 6D
SERPINE1	Serpin Family E Member 1
SETD8	SET Domain Containing (Lysine Methyltransferase) 8
SHH	Sonic hedgehog
SgRNA	Single guide ribonucleic acid
SMA	Smooth muscle actin
SMAD	Mothers against decapentaplegic
SKP	Skin-derived precursors
SCs	Stem Cells
SCID	Severe combined immunodeficient
SCUBE3	Signal Peptide, CUB Domain And EGF Like Domain Containing 3
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SG	Sweat gland
SHT	Smart Hair Transplant
SIRT2	Sirtuin 2
SRD5A1/A2	Steroid 5 Alpha-Reductase ½
TACs	Transit-amplifying cells
TBS	Tris Buffered Saline
TET	Ten-eleven-translocation
TCF7	Transcription Factor 7
TGFβ	Transforming growth factor-β
TβRI	TGFβ receptor I
TNF-α	Tumor necrosis factor α
TF	Transcription factor
THBS1	Thrombospondin 1
TSA	Trichostatin A
3D	Three-dimensional
2D	Two-dimensional
UBQ	Ubiquitin
WB	Western blot
WIP1	Wild-type p53-induced phosphatase
WISP-1	WNT1-inducible-signaling pathway protein 1
VEGF	Vascular endothelial growth factor
VIM	Vimentin
VCL	Vinculin

I

State-of-the-art and literature review

Text extracts from the following publications were included in this chapter:

Castro AR, & Logarinho, E. (2020). “Tissue engineering strategies for human hair follicle regeneration: How far from a hairy goal? Concise review”, STEM CELLS Translational Medicine, vol. 9, pp. 342-350, 2019. <https://doi.org/10.1002/sctm.19-0301>. <https://doi.org/10.1002/sctm.19-0301>

Castro AR, Portinha C, Logarinho E. “The Emergent Power of Human Cellular vs Mouse Models in Translational Hair Research”, STEM CELLS Translational Medicine. <https://doi.org/10.1093/stcltm/szac059>

Chapter 1

State-of-the-art and literature review

The demand for an efficient therapy for alopecia disease has fueled the hair research field in recent decades. However, despite significant improvements in the knowledge of key processes of hair follicle biology such as genesis and cycling, translation into hair follicle replacement therapies has not occurred. Great expectation has been recently put on hair follicle bioengineering, which is based on the development of fully functional hair follicles with cycling activity from an expanded population of hair-inductive (trichogenic) cells. Most bioengineering approaches focus on *in vitro* reconstruction of trichogenicity by manipulating key regulatory molecular/physical features of hair follicle growth/cycling *in vivo*. Despite the great potential, no cell-based product is clinically available for hair regeneration therapy to date. This is mainly due to demanding issues that still hinder the functionality of cultured human hair cells. In this chapter we comprehensively review the hair follicle cyclic regulation, as well as the emergent strategies using different cell sources and tissue engineering approaches aiming to successfully achieve a clinical cure for hair loss. The hurdles of these strategies are discussed, as well as the future directions to overcome the obstacles and fulfill the promise of a "hairy" feat.

Introduction

Hair loss (Alopecia) is a disease that affects a growing number of people worldwide and impacts individuals' physical, psychological and social well-being (Marks et al., 2018). Patients with hair disorders suffer from emotional stress, embarrassment and depression that severely compromise their life quality (Hadshiew, Foitzik, Arck, & Paus, 2004). The most common type of hair loss is androgenetic alopecia (AGA) which affects up to 50% of men by the age 50 and 50% of women throughout their lifetime (Ellis, Sinclair, & Harrap, 2002; Rhodes et al., 1998). It has an increased incidence among Caucasian and American population, and affecting in a lesser extent Chinese, Japanese, and African populations (Otberg, Finner, & Shapiro, 2007). Also, although very prevalent among young individuals, the AGA outcome increases with age (Severi et al., 2003).

AGA is a genetically predispose disorder characterized by nonscarring progressive miniaturization (reduction in the diameter, length and pigmentation) of scalp hair follicles (HF). In men, AGA is commonly known as male pattern baldness where the progressive HF miniaturization replaces terminal hair (thick and pigmented) by fine vellus-like hair on the scalp, exhibiting a very specific

pattern scaled by Norwood-Hamilton classification (Otberg et al., 2007). Female pattern baldness is quite different, being characterized by diffuse thinning of the crown region with maintenance of the frontal hairline (Ludwig classification) (Piraccini & Alessandrini, 2014).

Up to date, treatments include pharmacological and surgical (autologous hair transplant) interventions. Although hair restoration surgery is nowadays the most effective method for AGA, donor HFs scarcity is often its major limitation (Rose, 2015). Besides, pharmacological treatments still not fully satisfy the patient's needs and entail drastic side effects (Talavera-Adame, Newman, & Newman, 2017). Thus, the limited efficacy and possible side effects of the current treatments has fostered the search for alternative therapeutic solutions, capable of generating unlimited number of HFs *de novo*. Noteworthy, stem cell-based tissue engineering is emerging as the most thriving approach, aiming to reconstruct HFs *in vitro* to replace lost or damaged HFs as a consequence of disease, injury or aging. HF bioengineering approaches are based on the accumulated knowledge on reciprocal epithelial-mesenchymal (EM) interactions controlling embryonic organogenesis and postnatal HF cyclic growth. However, despite recent progress in the field, clinical applications of tissue engineering strategies for hair loss are still missing. Neogenesis of human follicles derived from cultured HF dermal cells has not been successfully achieved yet.

This chapter focus on the research approaches being developed to tackle the major limitations of human HF bioengineering, namely the loss of cellular function following *in vitro* HF cells expansion, the loss of *in vivo* tissue context/architecture, and the reconstruction of autologous functional HF germs for clinical procedures. Additionally, it provides an overview of the major limitations of the mainstream mouse models for human hair loss research.

HF morphogenesis and cycling

HF is a mini organ that forms during embryonic skin development. Its functional and cycling activities rely on a coordinated communication between the different cell populations from epithelial, mesenchymal and neural crest stem cell origin (Hardy, 1992), which additionally regulates adult skin homeostasis and wound repair (Mayumi Ito et al., 2005; Panteleimon Rompolas & Greco, 2014). Therefore, understanding the HF anatomy, as well as the stem-cell populations operating during postnatal cyclic regeneration, is crucial for tissue engineering-based solutions.

Morphologically, HF morphogenesis can be organized into three different stages: induction, organogenesis and cytodifferentiation (Schmidt-Ullrich & Paus, 2005). Induction initiates with the formation of dermal condensations, stimulated by diffusible signals from the epithelial compartment (also known as “crowding” of epidermal keratinocytes) (Stark, Andl, & Millar, 2007). Epithelial placode signals then recruit fibroblasts from the underlying dermis to form dermal condensates, the follicular dermal papilla precursors. These clusters of dermal cells then signal back to overlying unspecified epithelial cells, triggering them to proliferation and differentiation to form

the HF and its appendages (Hardy, 1992; Millar, 2002). It is this crosstalk signaling between epithelial-placode and mesenchymal-condensates that drives a massive proliferation of epithelial cells, leading to further engulfed of dermal condensates and HF downgrowth into the deep skin (organogenesis). Finally, a wide range of different signaling factors, as well as the topologic arrangement of different cell populations in distinct HF compartments characterize the cytodifferentiation phase (Schmidt-Ullrich & Paus, 2005) (**Fig 1.1**).

Many signaling pathways have been associated with HF development and growth, tightly regulated by specific waves of regulatory signals between epidermis and dermis. Insight into the molecular signals controlling HF development point out Wnt/wingless family as the most upstream signaling pathway orchestrating epithelial-dermal interaction during HF induction (Millar, 2002; Rachel Sennett & Rendl, 2012; Sick, Reinker, Timmer, & Schlake, 2006). Canonical WNT/ β -catenin signaling is the master regulator of hair follicle fate decision (Y. Zhang et al., 2008). Beyond WNT, some major players are involved in signaling cascades controlling in HF development, as FGF (fibroblast growth factor), SHH (Sonic Hedgehog), TNF (tumor necrosis factor) and TGF β /BMP (transforming growth factor- β /bone morphogenetic protein) families (Fuchs, 2007; Schmidt-Ullrich & Paus, 2005; X. Wang, Tredget, & Wu, 2012). After placode formation, different growth factors - *Fgfs*, *Tgfb2*, *Delta1*, *Noggin*, Follistatin (*Fs*), *Grem1*, *Msx1*, and *Msx2* - are expressed to promote HF development. On the other hand, Bmp family molecules expressed both in placode and dermal condensates counteract Wnt growth signaling (Millar, 2002; X. Wang et al., 2012). Therefore, it is a complex balance between WNT stimulation and BMP restraining signaling that dictates HF development. Once the placode is formed, WNTs/BMP's signal downstream for HF downgrowth and maturation. Early *Shh* expression is crucial for the maturation of dermal condensates and DP formation (Hardy, 1992; Oro & Higgins, 2003).

Remarkably, after birth, no further follicles are formed, but rather the cycling portion of follicle apparatus undergoes consecutive cycles of remodeling controlled by similar molecular pathways involved in embryogenesis. Interestingly, Humans are born with a predefined number of follicles on the scalp (roughly 100,000), that repeatedly cycle throughout life.

Stem cell populations

Follicular dermal stem cells exist in the dermis (skin-derived precursors, SKP) able to regenerate dermal sheath (DS) and populate the dermal papilla (DP) at every growth cycle (Rahmani et al., 2014). Both, DS and DP comprise mesenchymal cells with multi-lineage differentiation capacity (Jahoda, Whitehouse, Reynolds, & Hole, 2003). In the mature HF, the DP is adjoined to connective tissue sheath (DS), together forming the dermal component of the mature HF (Millar, 2002) (**Fig. 1.1**). The DP is thought to be a master regulator of HF cycling, which consists in serial phases of

growth (anagen), apoptotic-driven regression (catagen), and rest (telogen) (G. Cotsarelis, 2006a). On the human scalp, anagen lasts 1-6 years and it involves the complete regeneration of the cycling portion of the HF (Fig 1.1). At the telogen-to-anagen transition, DP stimulates epithelial hair follicle stem cells (HFSC) from the bulge region, which are adult multipotent cells holding self-renewal capability and kept quiescent in their niche surrounded by the sebaceous gland in the outer root sheath (ORS) (Fuchs, 2009; P. Rompolas et al., 2012). When DP stimulatory signaling overcomes the threshold imposed by the inhibitory bulge microenvironment (Woo, Zhen, & Oro, 2012), HFSCs divide generating a new pool of progenitors at the bulge base called the secondary germ cells (M. Ito, Kizawa, Hamada, & Cotsarelis, 2004), which survive catagen-driven apoptosis (A. A. Panteleyev, Jahoda, & Christiano, 2001). These primed hair germ cells migrate to the bulb, while expanding and differentiating into transit-amplifying cells (HF-TACs) that attach to the basement membrane surrounding the DP lower half. HF-TACs likely sit in place throughout much of anagen to fuel HF growth by differentiating into eight distinct epithelial lineages (e.g. shaft, inner root sheath and companion layer cells) and sebaceous glands (Greco et al., 2009; Hsu, Pasolli, & Fuchs, 2011; H. Yang, Adam, Ge, Hua, & Fuchs, 2017) (Fig 1.1).

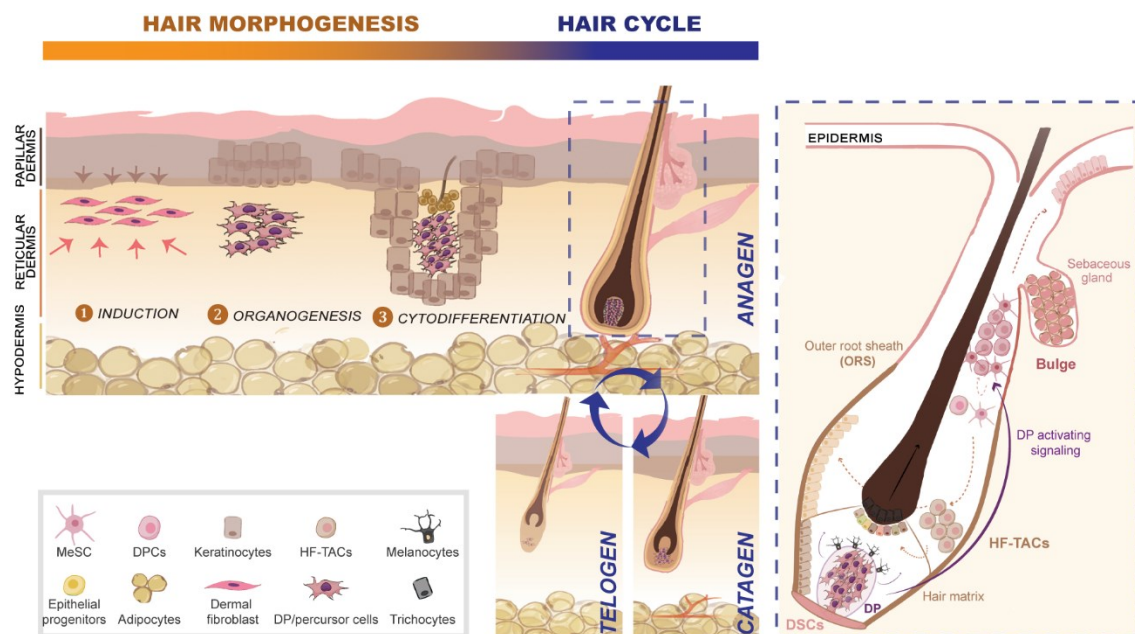


Figure 1.1. Cell population dynamics during HF morphogenesis and cycling stages. Interactions between epithelial and mesenchymal cell populations in the skin determine the embryonic morphogenetic stages of induction, organogenesis and cytodifferentiation, as well as the postnatal hair cycling stages of anagen, catagen and telogen. Inset on the right depicts the complex cell population dynamics operating during anagen. Bulge quiescent HFSC are activated by DP stimulatory signals to proliferate and generate HF-TACs. HF-TACs migrate out of the bulge and differentiate either in ORS or epidermal progenitors. HF-TACs that migrate into the hair matrix give rise to several differentiated epithelial cell lineages (in different colors) that directly contribute to hair growth. Besides HFSCs, melanocyte stem cells (MeSC) in the bulge give rise to

differentiated melanocytes that fuel pigment granules to the adjacent differentiating cells. Dashed lines represent cell trajectories and solid lines molecular signaling.

In addition to HFSCs, melanocyte stem cells (MeSCs) also reside in the bulge. During anagen, they are coordinately activated with HFSCs to generate mature melanocytes, that produce and distribute pigment granules to the adjacent differentiating cells to form pigmented hair fibers (Slominski et al., 2005). Catagen phase can last between 4-6 weeks, where keratinocytes and melanocytes undergo apoptotic processes. This apoptotic-driven regression causes DP to move upwards, bringing it closer to the epithelial bulge (George Cotsarelis, Sun, & Lavker, 1990). Following complete regression, HF enters a quiescent phase (telogen), which can last several months. The replacement of the old hair shaft fiber by the forming club fiber at the end of telogen is called “exogen” (C. A. Higgins, Westgate, & Jahoda, 2009). Finally, HF macroenvironment also encompasses adipocyte tissue containing adipose-derived stem cells (ASCs) in close commitment with hair growth regulation, as patented by increased adipose tissue thickness in anagen (Panteleimon Rompolas & Greco, 2014).

Altogether, the DP, differentiated epithelial cells and the hair matrix constitute the cycling portion of the postnatal HF, being actively renewed each HF cycle. Conversely, the upper portion of the follicle (including bulge, sebaceous gland appendage and the infundibular epidermis) constitute the permanent portion of the postnatal HF, being formed during embryonic development and kept throughout life (R. Paus & Foitzik, 2004; Rachel Sennett & Rendl, 2012). Any aberrant signaling affecting the communication between mesenchymal DP and the surrounding epithelial cells will disrupt the hair cyclic regeneration during postnatal life.

HF cycle regulation

The molecular mechanisms regulating human hair cycle progression are still obscure. However, a multitude of molecular players have for long been documented through mouse model studies (Alonso & Fuchs, 2006; R. Paus & Foitzik, 2004; Ralf Paus, Müller-Röver, & Botchkarev, 1999; Stenn & Paus, 2001). In fact, similar signaling molecules governing hair induction during morphogenesis are thought to trigger *de novo* HF regeneration. WNT/beta-catenin, BMP antagonists (e.g., *Noggin*) and SHH signaling have been demonstrated to act as anagenic growth stimulators. WNT activation results in the stabilization and accumulation of β -catenin in the cytoplasm. When translocated into the nucleus, β -catenin transcriptionally activates members of the *Tcf/Lef1* family of transcription factors (Cadigan & Waterman, 2012). Additionally, *Wnt/beta-catenin* transcriptional targets include Wnt molecules itself, but also *Fgfs*, *Foxn1*, *Msx2*, *Fs*, *Tgfb2* and several cell cycle related genes (Clevers & Nusse, 2012). WNT downstream players act as instructive signals for HF stem cells (HFSC) activation and HF regeneration (Enshell-Seijffers,

Lindon, Kashiwagi, & Morgan, 2010; Greco et al., 2009; Millar, 2002). Some growth factors, as insulin-like growth factor-1 (IGF-1), hepatic growth factor (HGF), and vascular endothelial growth factor (VEGF) were suggested to be important for anagen maintenance (R. Paus & Foitzik, 2004). Conversely, FGF5, TGF β 1, Interleukin-1 β and TNF- α are key catagen inducers (Hebert, Rosenquist, Gotz, & Martin, 1994; R. Paus & Foitzik, 2004).

HF bioengineering: cell sources and challenges

Stem cell-based regenerative medicine is emerging as the most thriving approach for hair loss treatment by holding the potential of HF cloning, i.e., the production of bioengineered instructive germs from human HF cells expanded *in vitro* to generating fully functional HFs upon transplant into the patient's bald scalp. Rationally, such a regenerative therapy may only be possible if combining receptive-epithelial and inductive-mesenchymal populations to mimic the well-orchestrated interactions controlling lifelong HF cycles, which are deeply affected during hair loss (Garza et al., 2011). Ideally, a cell-based regenerative medicine therapy would be autologous, i.e., resort to patients' cells derived from small amounts of tissue biopsies (e.g., HF punch). Thus, the researchers in the field have been mainly focused on developing therapeutic bioengineering solutions using dissociated HFSCs and DP cells (DPCs) isolated from HF biopsies. HFSCs and DPCs retrieved from nonbalding scalp follicles should first be expanded in culture to produce bioengineered structures *in vitro* with hair regenerative potential. Still, an allogeneic cell source could be alternatively used for HF regenerative therapy. Two decades ago, transgender transplantation of microdissected DP and DS was shown to successfully induce HFs (Reynolds, Lawrence, Cserhalmi-Friedman, Christiano, & Jahoda, 1999). This study not only pointed to the need of an inductive dermal component for HF regeneration, but it also disclosed the possibility of using an allogeneic cell source for therapy. Indeed, HF proved to be an immune-privileged site, as it does not express MHC (major histocompatibility complex) class I antigens (Christoph et al., 2000; Westgate, Craggs, & Gibson, 1991). Regardless of autologous vs. allogeneic therapy, the relevance of HFSCs, and mainly DPCs, on tissue-engineering approaches for treating alopecia has been the focus of intensive research over the last decade.

The relevance of the dermal papilla and strategies tackling its hair-inductive potential

Seminal studies demonstrated that papillae isolated from rat, guinea-pig vibrissa, but also human, could induce HF formation when implanted into a recipient non-hairy skin (Horne, Jahoda, & Oliver, 1986; Jahoda, Oliver, Reynolds, Forrester, & Horne, 1996). These experiments

demonstrated that DP, unlike dermal fibroblasts, can reprogram non-hairy epidermis to a follicular fate. Later on, several groups demonstrated hair regeneration in immunodeficient mice skin when combining different populations of dissociated cells (Lichti et al., 1993), including DP and DS (McElwee, Kissling, Wenzel, Huth, & Hoffmann, 2003), but also freshly isolated epithelial bulge cells (Morris et al., 2004). More recently, ablation of DPCs unequivocally demonstrated its pivotal role on hair growth but not on epithelial HF maintenance (P. Rompolas et al., 2012). Moreover, the generation of rodent pelage by transplantation of dissociated DPCs has been possible in a variety of different approaches, as revised in Ohyama et al. (Ohyama, Zheng, Paus, & Stenn, 2010). However, inducing human HF-like structures in nude mice only proved possible when human DPCs were combined with epidermal component (Abaci et al., 2018; J. Qiao et al., 2009; Toyoshima et al., 2012), clearly indicating the need for EM interactions. Thus, a successful bioengineering solution will require an available amount of competent epithelial and inductive DPCs.

DP *in vitro* cell expansion is inevitable towards the development of a clinically relevant tissue engineering-based solution for HF regeneration. However, two major technical burdens have limited the attainment of human trichogenic DPCs. Isolation of human DPCs requires DP microdissection from HF grafts, as enzymatic digestion with trypsin and collagenase inefficiently works to generate single cells for FACS-sorting, and robust cell surface markers (**Fig. 1.2**) remain to be defined (Ohyama et al., 2010). Moreover, enzymatic digestion would deprive DP of its natural and distinctive extracellular matrix microenvironment, which is essential for hair inductive properties. This is also a caveat of *in vitro* expansion, particularly under regular culture conditions lacking key environmental cues (Ohyama, Kobayashi, Sasaki, Shimizu, & Amagai, 2012; Rendl, Polak, & Fuchs, 2008). Considering the relevance of DP for HF cycle, massive effort has been made on finding out an *in vitro* expansion procedure for human DPCs that preserves, or alternatively rescues, their hair inductive ability. One approach consists in co-culturing DPCs with keratinocytes or keratinocyte-conditioned medium, shown to provide DPCs with molecular cues involved in EM crosstalk during hair growth (Havlickova, Biro, Mescalchin, Arenberger, & Paus, 2004; J. Qiao et al., 2009). Also, distinct players in this signaling crosstalk (including Wnt, BMP and FGF) were reported to prolong the hair-inducing ability of cultured DPCs (Kalabusheva, Terskikh, & Vorotelyak, 2017; Kishimoto, Burgeson, & Morgan, 2000; Ohyama et al., 2012; Osada, Iwabuchi, Kishimoto, Hamazaki, & Okochi, 2007; Rendl et al., 2008). More recently, pharmacological modulation of JAK-STAT signaling pathway was shown to improve cultured human DPCs' inductivity (Harel et al., 2015). Another strategy to reproduce native DP trichogenicity is the establishment of three-dimensional (3D) sphere cultures, which has been pointed out as one of the most effective means to restore intact anagenic DP transcriptional signature (C. A. Higgins, Chen, Cerise, Jahoda, & Christiano, 2013). The 3D-spheroid culture help DPCs to aggregate, thereby

reestablishing cell-cell contacts, a crucial feature of *in vivo* DP (C. A. Higgins, Richardson, Ferdinando, Westgate, & Jahoda, 2010). In fact, sphere formation was shown to increase hair-inductive ability in both, cultured human DPCs and multipotent dermal progenitors (SKPs) (B. M. Kang, Kwack, Kim, Kim, & Sung, 2012; Kishi & Hayashi, 2016; Shimizu et al., 2011).

Considering the undoubted contribution of 3D culture for DPCs' fitness, numerous studies have explored the contribution of biomaterial-based strategies to support DP hair inductive behavior *in vitro* (Kageyama et al., 2018; Kalabusheva et al., 2017). Biomaterials have been employed either *i*) as supportive scaffold with known stiffness for 3D cell culture (Y. C. Huang et al., 2013; Tan et al., 2018); or *ii*) as supportive matrix for the encapsulation of dissociated cells (Nakao et al., 2007; Toyoshima et al., 2012). Recently, human placenta extracellular matrix hydrogel was used for DP sphere culture and shown to restore hair-inductive potential of high-passage DPCs (X. Zhang, Xiao, Liu, Miao, & Hu, 2019). Moreover, 3D culture has been combined with different top-up modulations to boost cultured DPCs' inductivity. Importantly, by combining WNT signaling pharmacological activation of DPCs with 3D spheroid culture was recently shown to enhance *de novo* HF formation in a reconstituted human skin grafted into nude mice model (Yoshida, Soma, Matsuzaki, & Kishimoto, 2019).

The requirement of the HFSC epithelial component

Bulge HFSCs are the most prominent stem cell population actively contributing to HF regeneration *in vivo* by replenishing different epithelial lineages (Alonso & Fuchs, 2006; Mayumi Ito et al., 2005). Although their depletion convey in hair follicle loss (Mayumi Ito et al., 2005), in the most common pathological hair loss conditions, i.e. alopecia areata (AA) and AGA, HFSCs are preserved (but not DP cells) (Morgan, 2014). In contrast, aging-driven loss of hair regenerative capacity is associated with terminal differentiation and transepidermal clearance of HFSCs (Matsumura et al., 2016). Thus, from an *in vitro* tissue engineering perspective, mimicking *in vivo* contribution of both HFSC and DP seems critical to establish a successful hair follicle regeneration strategy. Accordingly, several studies evidenced a boost of HF induction when combining the epithelial component (HFSCs $\pm$ keratinocytes) with the inductive DPCs within engineered germs (A. C. Gupta et al., 2018; M. Nilforoushzadeh et al., 2017). Even though bulge-specific cell molecular markers allow direct HFSC isolation from mouse and human HF (Morris et al., 2004; Ohyama, 2007) – as opposed to human DPCs - HFSCs have poor proliferative ability and tend to differentiate during culture passaging (Rhee, Polak, & Fuchs, 2006). Therefore, committed progenitors or keratinocyte precursors are being alternatively used as an epithelial component for HF tissue engineering (Ohyama & Veraitch, 2013). Actually, different EM combinations have been tried to successfully reconstruct hair folliculogenesis *in vitro*. To date, fully functional HF reconstruction

in vitro was only achieved using mouse embryonic skin cells (Asakawa et al., 2012; Toyoshima et al., 2012). Several studies have reported improved HF neogenic potential either by using cells with higher differentiation potential, namely adult HFCS, neonatal keratinocytes and embryonic cells (Kageyama, Yan, Shimizu, Maruo, & Fukuda, 2019; Toyoshima et al., 2012), or by coupling human and mouse cells to form chimeric HFs (e.g. human mesenchymal inductive cells and rodent epithelial keratinocytes) (Ehama et al., 2007; L. Zhang et al., 2019). As expected, such cellular constructs evidence greater HF inductive potential when implanted into a mouse skin. Although interesting from a mechanistic perspective, those studies are far from reproducing a postnatal human HF regeneration. Conversely, they may bewilder the layman community by suggesting successful fully functional HF regeneration (i.e., HF with cycling activity, hair shaft grow and stem cell renewal) by non-translational approaches. Importantly, as most studies have used rodent cell-based assays (**Table 1.1**), the actual contribution HFSCs for human HF bioengineering needs to be further validated. Although challenging, some researchers are now committed to establish *in vitro* human post-natal cell-based HF germs (Kalabusheva et al., 2017; Yoshida et al., 2019).

Non-follicular cell sources for HF bioengineering

While isolation of HFSCs and DPCs seems a promising autologous cell source for human HF regenerative therapy, non-follicular cell populations have been also considered, especially when donor hair scarcity is an issue. Due to their similarity with DPCs, SKPs have been alleged as an autologous multipotent cell source for HF regeneration (Fernandes et al., 2004). They have proved clinically relevant for stem cell-replacement strategies, particularly in central nervous system and spinal cord injury (Fernandes, Toma, & Miller, 2008). Also, SKPs have been extensively studied for its trichogenic potential in mouse skin (Guo et al., 2019; X. Wang et al., 2016). Reconstruction of functional HF and sebaceous glands (SG) in nude mice excisional wound has been achieved following implant of spheroid structures containing epidermal stem cells and SKPs in a hydrogel (X. Wang et al., 2016). However, hair inductive potential of this precursor population in the human remains unclear, in addition to its challenging isolation from human dermis.

To overcome the constraints of cell population scarcity and/or technical isolation, non-autologous reprogramed pluripotent stem cells have been considered as these represent an inexhaustible source of cells with multi-lineage differentiation ability (Takahashi et al., 2007). To date, several groups have already attempted HF regeneration using human-induced pluripotent stem cells (hiPSCs) (Gnedeva et al., 2015; Takagi et al., 2016; R. Yang et al., 2014). Differentiation of hiPSCs obtained from non-hair cell sources can be used to generate both DPCs and HFSCs, or simply an inductive dermal and receptive epidermal cell population. Indeed, hiPSCs may enable HF bioengineering through a 3D integumentary organ system *in vitro*. In these systems, iPSCs-based organ germs were created to stimulate bioengineered organ development *in vivo* through reciprocal EM interactions

(Takagi et al., 2016). Nevertheless, the use of iPSCs for human therapeutic purposes is still controversial due to safety, namely viral integration into the genome and risk of teratoma formation (Hirschi, Li, & Roy, 2014). Stringent safety requirements will need to be satisfied towards cGMP manufacturing and commercialization of hiPSC-derived therapeutic products prior to their clinical application (Abbasalizadeh & Baharvand, 2013). Also, human embryonic stem cells (hESCs) have been tested for differentiation into HF cell lineages. Recently, a two-step procedure reported the derivation of hESCs into neural crest cells and then hair inductive DP-like cells (Gnedeva et al., 2015). Lastly, recent studies described the transformation of human dermal fibroblasts into hair-inducing cells with HF regenerative competence (Fan et al., 2018; L. Zhang et al., 2019).

In sum, several cellular sources are currently being investigated for human HF bioengineering (**Fig. 1.3**) that will deliver distinct HF equivalents *in vitro* with attractive potential to specific research/clinical applications (e.g. preclinical models for drug efficacy testing and reconstructed skin models (Lindner, Horland, Wagner, Atac, & Lauster, 2011; Madaan, Verma, Singh, & Jaggi, 2018)), even if do not answer the demand for functional/cycling/oriented bioengineered HF required for hair cloning.

Table 1.1. Summary of tissue engineering approaches tested with HF regeneration outcome.

Origin	Mesenchymal cell type	Epithelial cell type	<i>In vitro</i> approach	<i>In vivo</i> APPROACH	Outcome	Ref
MOUSE	SKPs	Neonatal epidermal KTs	TSA treatment in SKP aggregates	Transplantation in nude mice excisional wounds	HF neogenesis	(Guo et al., 2019)
	Adult dermal fibroblasts	Dorsal skin epidermal KTs	Treatment with embryonic skin extract	Patch assay in nude mice full thickness wounds	HF neogenesis	(Fan et al., 2018)
	Adult vibrissa DPCs	Adult vibrissa HFSCs	3D culture	Intracutaneous transplantation of HF germs in nude mice	HF formation	(Tezuka, Toyoshima, & Tsuji, 2016)
		Neonatal foreskin epidermal KTs	PRP-based bioactive 3D scaffolds	Mini chamber assay in nude mice	HF formation	(Xiao et al., 2017)
	Neonatal dermal progenitors	Neonatal epidermal progenitors	3D organoid	Transplantation into nude mice	HF formation	(M. Lei et al., 2017)
	Neonatal dermal cells	Neonatal epidermal cells	3D co-culture in collagen scaffold		HF formation	(Kageyama et al., 2018)
	iPSCs-derived MSCs	iPSCs-derived epithelial stem cells	Bioengineered 3D integumentary organ system	Embryonic body transplantation into nude mice	Functional HF regeneration	(Takagi et al., 2016)
	Embryonic back skin follicle-derived mesenchymal cells	Embryonic back skin follicle-	Bioengineered HF germ	Intradermal transplantation into nude mice	Functional HF regeneration	(Toyoshima et al., 2012)

		derived epithelial cells	using organ germ method			
MOUSE AND HUMAN	Mouse neonatal dermal cells	hiPSC-derived bulge stem cells	-	Chamber assay in nude mice	HF formation	(R. Yang et al., 2014)
	Mouse DP-enriched cells	Human neonatal foreskin epidermal KT	Mixed cell suspension	Chamber assay in nude mice	HF formation	(Ehama et al., 2007)
	DP-like cells derived from neural crest hESCs	Mouse neonatal epidermal KT		Subcutaneous injection in nude mice	HF formation	(Gnedeva et al., 2015)
	Mouse whisker DPCs	Human epidermal KT	Cell-matrix composites	Graft skin composite in SCID mice	HF neogenesis in regenerated skin tissues	(L. Zhang et al., 2019)
	Human DPCs	Mouse embryonic epithelial cells	DP encapsulation in collagen-enriched microgel	Patch assay-transplanted slab of nude mouse skin	Microtissue fabrication with increased hair regeneration	(Kageyama et al., 2019)
HUMAN	Intact DP	Bulge derived epithelial cells	Bioengineered HF germ using organ germ method	Intradermal transplantation in nude mice	Human bioengineered hair follicle	(Toyoshima et al., 2012)
		Neonatal epidermal KT	DP aggregates in collagen I 3D bioprinting molds	Engraftment into SCID mice	Generation of Human HF within human skin constructs	(Abaci et al., 2018)
	Scalp HF DPCs	Adult epidermal KT	Hanging drop culture for 3D spheroids	-	Spontaneous cell sorting within mixed aggregates	(Kalabusheva et al., 2017)
		HF KT and HFSCs	3D DP organoid within a gelatin hydrogel	-	Relevance of EMI in HF	(A. C. Gupta et al., 2018)
		Melanocytes and ORS KT	Engineered microfollicles	-	HF fibers similar to vellus hair	(Lindner et al., 2011)
	Scalp HF DPCs and dermal fibroblasts	Fetal epidermal KT	Wnt signaling activation in DP spheroid culture	Reconstructed human skin assay grafted into nude-SCID mouse	Functional HF formation	(Yoshida et al., 2019)
	Adult SKPs	Adult epidermal SCs	Cells mixed in a hydrogel	Transplantation in nude mice excision wound	HF and SG formation	(X. Wang et al., 2016)

SKPs – Skin derived progenitors; **KTs** – keratinocytes; **DPCs** – Dermal papilla cells; **HFSCs** – Hair follicle stem cells; **SCs**- Stem cells; **ORS** – outer root sheath; **PRP** – platelet-rich plasma; **EMI** – Epithelial-mesenchymal interaction; **SG**- Sweat gland; **TSA**- Trichostatin A.

Mouse models for hair research

Different animal models have been used for hair loss and regrowth studies *in vivo*, including mice (reviewed in (Porter, 2003)), rats (Johnson & Ebling, 1964), goats (Su et al., 2018), and monkeys (Uno, 1991). Despite more divergent from humans than other models, mice have been predominantly used in hair research due to their easier handling, well-established protocols and ethical approvals. Several mouse strains are currently used for hair growth studies (John P. Sundberg, Beamer, Uno, Van Neste, & King, 1999), the most popular being *C57BL/6* and *C3H*, whose pelage (but not ear and tail) skin pigmentation is merely dependent on their follicular melanocytes (Tobin, 2011). This way, anagen cycles of hair growth are easily detected by the darkening of shaved skin (Stenn & Paus, 2001). Up to date, foremost knowledge on HF biology and cycling has arisen from studies using spontaneous and/or genetically engineered mouse mutants with hair loss outcome (Schneider, Schmidt-Ullrich, & Paus, 2009). Some relevant examples of spontaneous mutants exhibiting HF defects are listed in **Table 1.2**. These spontaneous mouse mutants significantly contributed to the identification of new genes involved in hair loss, as well as triggered further research on transgenic mouse models (Porter, 2003). Accordingly, a continuous growing number of genetically engineered mutants unveiled the mechanisms controlling hair morphogenesis, cycle and pigmentation (reviewed in (Nakamura, Schneider, Schmidt-Ullrich, & Paus, 2013)). Although these models provided valuable knowledge to conceive hair loss therapeutic interventions, inconsistent outcomes have often arisen when conveyed into a human background. Attention should be given to species-specific differences.

Table 1.2. Mouse models with hair loss.

Mutant strain	Mutant gene / origin	Phenotype	Reference
<i>ragged/opossum</i> (<i>Raop</i>)	Mutation in the Sox18 transcription factor gene	Reduced number of follicles	(James, Hosking, Gardner, Muscat, & Koopman, 2003)
<i>waved 2</i> (<i>Egfrwa2</i>)	Mutation in the EGF receptor tyrosine kinase	Abnormal hair morphogenesis	(Luetke et al., 1994)
<i>hairless</i> (<i>Hr hr</i>)	Hypomorphic mutation in the hairless gene	Severe abnormalities during the first catagen and total alopecia	(A. A. Panteleyev, Paus, & Christiano, 2000)
<i>Nude</i> (<i>Foxn1^{nu}</i>)	Nude allele <i>Foxn1^{nu}</i> in <i>Foxn1</i> transcription factor gene	complete hair loss	(Mecklenburg, Nakamura, Sundberg, & Paus, 2001)
<i>balding</i> (<i>Dsg3^{bal}</i>)	Mutation in the Desmoglein-3 gene	abnormal hair shaft structure	(Montagutelli, Lalouette, Boulouis, Guenet, & Sundberg, 1997)
B6CBAF1	Hybrid cross between C57BL/6 female and CBA male	testosterone inducible model of alopecia	(Matias, Malloy, & Orentreich, 1989)

Mouse vs. human hair

Major differences exist between mouse and human hair, namely in the HF morphogenesis, cycling, structure, and microenvironment (**Fig. 1.2**; e.g., immune and hormonal regulation). First, HF function in humans is vastly different from its role in other mammals. Whereas the fur primarily controls thermoregulation in animals, the human HFs mainly protect the scalp skin from ultraviolet radiation rather than acting as heat isolators (Iyengar, 1998). Second, hair cycle behavior and growth are noticeable different in mice and humans: *i*) anagen growth phase takes 2 weeks in mice compared to 3-5 years in human (Garza et al., 2012; Oh et al., 2016); *ii*) hair shaft shedding (exogen) is a well-controlled process in mice, in which old hair shafts are kept for several HF cycles (Milner et al., 2002); *iii*) contrarily to rodents, human hair cycle occurs asynchronously in the scalp (Halloy, Bernard, Loussouarn, & Goldbeter, 2000; Oh et al., 2016). In addition, the hair types are also different between human and mice. Adult humans have two major hair types visible on the scalp, terminal hairs (pigmented) and vellus hairs (thin, non-pigmented) (Ralf Paus & Cotsarelis, 1999). Melanin is transferred to the hair fiber cortex in humans, whereas primarily to the hair fiber medulla in mice (Tobin, 2011). Mice have many different hair types, namely truncal (pelage) hairs, vibrissae, muzzle hairs, tail, cilia and perianal hair (J. P. Sundberg, 1994). These hair types should be deeply investigated in hair studies since distinct molecular mechanisms orchestrate different HF populations (Schmidt-Ullrich & Paus, 2005). Besides, the mouse hair does not transit from a vellus to terminal hair. Regarding the hair structure during cycling, mouse and human do not differ substantially, with human scalp follicles showing larger and longer hair shafts than mouse pelage follicles. However, in vibrissae follicles the DP never reaches the telogenic bulge, making it hard to explain HFSC migration from the bulge to the follicular base during anagen onset (Oshima, Rochat, Kedzia, Kobayashi, & Barrandon, 2001). HFSCs are a pool of quiescent multipotent stem cells in the outermost layer of the HF which are activated by the DP to proliferate and promote the hair growth.

Moreover, androgenic effects in mice HF proved quite distinct from that observed in human scalp HF. The most common form of human hair loss is AGA, which is mainly caused by an androgen action on the DP. Androgens alter the production of regulatory factors by the DP cells, causing anagen shortening and follicle miniaturization. Strong evidence has pointed out the human scalp HF sensitivity to androgens as the main reason for hair miniaturization, as opposed to androgens' effect on hair from other body regions (e.g. beard) (Miranda, Charlesworth, Tobin, Sharpe, & Randall, 2018). Also, human scalp sensitivity to androgens is distinctive in comparison to other animals, which lack androgens-driven phenotypes (e.g. prostate disorders or alopecia) (Miranda et al., 2018). In fact, mice do not suffer from AGA and key mechanisms controlling androgen-dependent HF miniaturization in human scalp are not recapitulated in mice (Nakamura et al., 2013; Oh et al., 2016). Human and murine HF respond differently to distinct androgen and hormone

stimulation. To circumvent this issue, an androgen-dependent mouse model expressing a human androgen receptor transgene, *K5-hAR*, was generated. Still, and noteworthy, in this model the human AR is expressed in basal epidermis and outer root sheath (ORS) mouse cells, and not in the DP (Crabtree et al., 2010; V. A. Randall et al., 2001), perhaps explaining why these mice do not exhibit a phenotype resembling human AGA (Fu et al., 2021). Therefore, caution is needed when resorting to the *K5-hAR* mice to validate drug therapies. In human AGA, two main factors determine terminal hair miniaturization to vellus: anagen growth shortening and decreased size of the DP and hair matrix (Courtois, Loussouarn, Hourseau, & Grollier, 1994; Elliott, Stephenson, & Messenger, 1999). Studies using human DP spheroid cultures suggested that spheroid size is essential for HF inductivity, although not directly translating into thicker regenerated hair (Y. C. Huang et al., 2013). In mice, selective ablation of DP cells *in vivo* showed that DP cell number determines the thickness and type of hair produced (Chi, Wu, & Morgan, 2013; Tobin, Magerl, Gunin, Handijski, & Paus, 2003). The follicular papilla cell number and total papilla size are maximal by anagen VI, then decreasing by fibroblast migration out of the late anagen/early catagen papilla into the proximal connective tissue sheath (Tobin et al., 2003). Thus, hair cycle-associated plasticity of the HF mesenchyme is likely clinically relevant.

Furthermore, not only hair cycle regulation differs between human and mouse, but also the stem cell niches (organization, markers, features) within the HF. Different HF stem and progenitor cells have been intensively investigated for their ability to generate tissue engineering applications to treat hair loss. However, the identification of specific biomarkers is still missing for their effective isolation and expansion. Even though mouse studies have significantly elucidated stem cell activation mechanisms during hair cycle, major constraints still remain when translating these findings into tissue engineering strategies. Whereas the mouse anagenic bulge compartment (a reservoir of multipotent stem cells in the adult HF ORS) can be easily identified, within the human ORS there is a keratinocyte pool without apparent distinctive morphologies (Akiyama, Dale, Sun, & Holbrook, 1995). Moreover, different biomarkers were found in mouse and human HF. For instance, CD34 and K15 markers, identified as murine bulge stem cell-specific markers and used for HFSC isolation (Liu, Lyle, Yang, & Cotsarelis, 2003; Trempus et al., 2003), are not expressed in the human bulge region (CD34 is alternatively detected in the ORS) (Poblet, Jimenez, Godinez, Pascual-Martin, & Izeta, 2006). On the other hand, CD200, follistatin and DIO2 appear to be human-specific markers of the bulge stem cells (G. Cotsarelis, 2006b; Molina, Giansante, & Finol, 2018; Ohyama et al., 2006). Regarding the mesenchymal DP cells, integrin alpha 9 (Tsai et al., 2011) and CD133 (Y. Ito et al., 2007) are established cell surface markers for mouse but not human DP. Importantly, the development of stem cell-based therapies for hair loss has been limited by the lack of robust human DP cell surface markers, which precludes their isolation by cell sorting (Ohyama et al., 2010). Therefore, another mesenchymal multipotent pool in the skin, the SKPs, has

gained alternative attention in the hair research field. Mouse SKPs express neural crest biomarkers (e.g., Slug, Snail, Twist, Pax3, Sox9, p75) and their role in DP renewal during the hair cycling has been demonstrated (Biernaskie et al., 2009; Fernandes et al., 2004; Rahmani et al., 2014), as well as their dermal stem cell properties explored in bioengineering approaches (Biernaskie et al., 2009). However, the study of human SKPs (hSKPs) has remained technically challenging and barely reported. The nestin, fibronectin and vimentin markers expressed by hSKPs are also present in other skin mesenchymal populations (Toma et al., 2001; Toma, McKenzie, Bagli, & Miller, 2005), thus constraining the isolation, expansion, and validation of hSKPs' therapeutical potential.

Finally, despite their similar structural layering and dramatic changes in dimension during the hair growth cycle, human and mouse skins differ in overall thickness. In mice, epidermis is loose comprising only three cell layers (<25µm), whereas human epidermis is firm comprising many layers (>50µm). Also, human dermis is substantially thicker than mouse dermis and contains fewer HFs (Gudjonsson, Johnston, Dyson, Valdimarsson, & Elder, 2007). Therefore, their percutaneous absorption capacity is likely distinct, which should be considered in translational drug studies. Furthermore, immunological specificities during wound healing might dictate paradoxical outcomes in mice and human. For example, mouse epidermal dendritic T cells secrete FGF9 during wound healing, further triggering FGF9 secretion by dermal fibroblasts (Gay et al., 2013). This mechanism explains the presence of HFs in mice but not in human scars (Zomer & Trentin, 2018). Such immunological specificities of the mouse skin are also disadvantageous to study AA, an autoimmune disease that is the second most common cause of hair loss (A. Gilhar, Etzioni, & Paus, 2012). Still, mouse models have provided invaluable means for studying the factors underlying immune regulation of this autoimmune skin disease (Yu & Petersen, 2018). For example, the therapeutic effect of JAK inhibitors (Ruxolitinib and Tofacitinib) on AA was validated in the C3H/HeJ mouse model (Xing et al., 2014). However, C3H/HeJ mice develop spontaneous AA at very low frequency, with histologic features that do not resemble those in human AA (Amos Gilhar, Laufer Britva, Keren, & Paus, 2020). Human AA is characterized by intra- and pre-follicular infiltration of CD4⁺ and CD8⁺ T lymphocytes. In murine AA, inflammatory cells infiltrate to the distal follicle even reaching the bulge. Key differences between the C3H/HeJ model and human AA pathobiology, genetics and immunobiology have been reviewed (Amos Gilhar et al., 2020; A. Gilhar, Schrum, Etzioni, Waldmann, & Paus, 2016), questioning the suitability of the mouse model to validate the efficacy of therapeutic compounds for human AA. For this reason, a humanized mouse model has been generated for preclinical studies (A. Gilhar, Paus, & Kalish, 2007).

Validation of hair-inducing capacity in mouse models

Animal models have been extensively used to functionally validate the ability of stem cell populations expanded *in vitro* to induce HF formation. Specifically, most hair inductive studies

monitor the formation of “hair-like” structures upon implementation of bioengineered instructive mini-germs in mouse skin. HF bioengineering approaches have resorted primarily to human HFSCs or DPCs co-transplanted with mouse neonatal cells. Yet, successful generation of functional mature HFs exclusively from human adult HFSCs and DPCs has not been achieved (reviewed in (Castro & Logarinho, 2019; Mohammadi, Youssef, Abbasalizadeh, Baharvand, & Aghdami, 2016; M. A. Nilforoushzadeh et al., 2019)). To cope with any constraining differences between mice and humans, human scalp skin xenografts in severe combined immunodeficiency (SCID) mice have been used. Noteworthy, this is currently the single preclinical assay that allows the establishment of a human hair cycle *in vivo* (Oh et al., 2016). Nude and SCID mutant mice have been routinely used in hair bioengineering approaches because: *i*) nude mice constitute a unique model to study *de novo* hair formation, as they lack visible hair fibers, making xenografts easy to follow and study; *ii*) SCID mice are deficient in T and B cells, which prevents the rejection of human scalp skin or human bioengineered transplants (Valerie A. Randall, Sundberg, & Philpott, 2003; John P. Sundberg et al., 1999).

Apart from xenografts, animal models have been also used to explore the efficacy of topic treatments to induce hair growth *in vivo* (Matias et al., 1989). Alternatively, human HF organ cultures (HFOCs) *ex vivo* have become increasingly popular in preclinical studies for hair growth/anti-AGA drugs (Langan, Philpott, Kloepper, & Paus, 2015). One common pitfall of preclinical drug validation in both mice and HFOCs is the fact that only gain/loss of anagen HFs can be monitored, and not the reversion of HF catagen and/or miniaturization (Langan et al., 2015; Pantelireis & Higgins, 2018). In fact, only maximally growing anagen VI HFs are used in HFOCs, which are thus unsuitable to study hair shaft elongation. Moreover, only occipital (not frontotemporal or vertex) HFs are used in HFOCs, which are more resistant to DHT-induced miniaturization (reviewed in (Magerl et al., 2004)).

In sum, although several morphogenic and regenerative signaling pathways are evolutionarily conserved, species-specific differences may dictate if one given therapy will succeed in the human background.

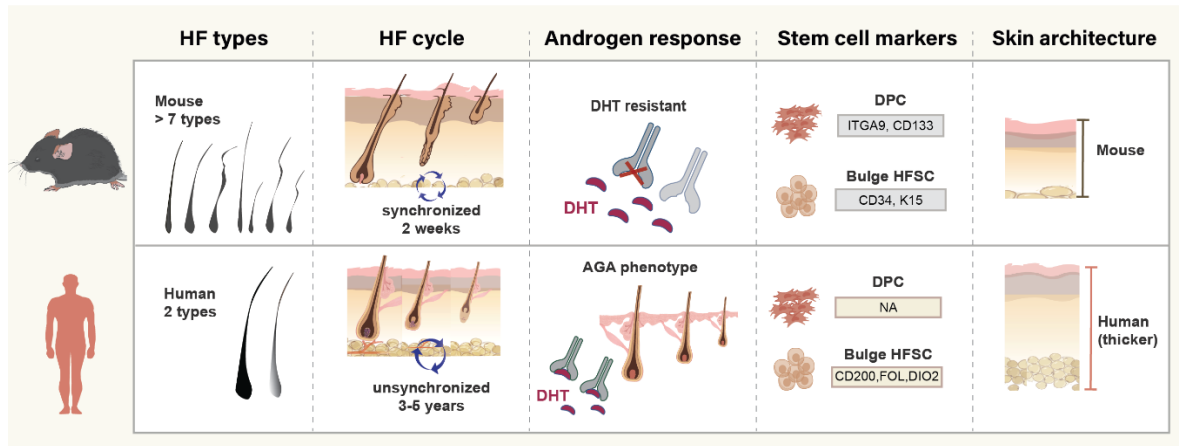


Figure 1.2. Schematic summary of the main differences between mouse and human HFs. Mice have many different types of HFs whereas human only have two (terminal pigmented and vellus non-pigmented). Anagen growth phase takes 2 weeks in mice compared to 3-5 years in human. DP moves upward to contact the stem cell niche during mice catagen, which is not observed during human hair cycle. Mice do not exhibit AGA that in human is due to expression of androgen receptors in the DP. The DP and bulge stem cell niches' biomarkers are distinct between mouse and human. Despite their similar structural layering, human and mouse skins differ in thickness and stiffness.

Regenerative medicine therapy for human hair loss: how far?

Considering that transferring isolated stem cells into a hostile microenvironment may encumber proper hair formation, different tissue engineering strategies have been designed to overcome this gap consisting either in: *i*) biomaterial-based platforms, including organic or inorganic matrices and scaffolds; *ii*) scaffold-free microtissues, through the creation of multicellular spheroids or organoids; or more recently, *iii*) tissue bioprinting techniques (Laschke & Menger, 2017). Using those different approaches, bioengineered instructive mini-germs have been generated *in vitro*, which can be then transplanted into the host to produce HFs (**Fig. 1.3**).

Inspired by HF embryonic development, a 3D organ germ method was proposed for HF bioengineering, consisting in providing a native-like 3D environment and maximizing EM cell interaction to mimic hair organogenesis (Tezuka et al., 2016). Bioengineered follicle germs were shown to develop a correct structure when transplanted into the back skin of nude mice (Tezuka et al., 2016; Toyoshima et al., 2012). Additionally, two recent studies have achieved growth of mouse hair *in vivo* after transplantation of *in vitro* formed structures, either consisting of mouse adult DP and epidermal cells (M. Lei et al., 2017), or of mouse iPSCs (Takagi et al., 2016), encapsulated in hydrogel matrices. Different artificial 3D microenvironments composed of silk-gelatin (A. C. Gupta et al., 2018), hyaluronic acid (Kalabusheva et al., 2017), and collagen (Kageyama et al., 2019) were shown to improve hair germs for hair regenerative medicine. In a similar approach, human HF resembling vellus hair were recreated *in vitro* from a mixture of DP cells, keratinocytes and

melanocytes in a collagen matrix, and named “microfollicles” (Lindner et al., 2011). Presently, researchers are joining efforts to develop more complex structures that can mimic the DP tridimensional morphology while providing a spatiotemporal delivery of molecular cues needed for human hair morphogenesis. Such a combined approach may favor cellular interactions *in vitro*, hopefully guiding the development and differentiation of both epithelial and mesenchymal counterparts to form a mature HF. To this end, recent work disclosed an interesting approach using a bioactive scaffold based on platelet-rich plasma (PRP) that synergistically combines 3D culture environment with natural release of endogenous growth factors (Xiao et al., 2017).

Envisioning the large-scale production of HF germs needed for a clinical set-up, different innovative high-throughput strategies have been conceived, using both mouse and human cells. 3D-printing technology is currently being used in the hair research field to print 3D molds resembling HF microenvironment. Effective 3D-printing of skin substitutes with human HFs has been recently reported (Abaci et al., 2018). Additionally, custom-designed array plates were produced to allow scalable fabrication of inductive DP microtissues (Kageyama et al., 2019; Kageyama et al., 2018). Finally, an emerging trend in HF research is the *in vitro* reconstruction of artificial hair-bearing skin. Relevantly, Zhang et al. were able to generate HFs from cultured mouse dermal papilla cells in *de novo* engineered skin model (L. Zhang et al., 2019). Also, hair-bearing human skin constructs were produced using innovative scaffolds that allow the development of properly oriented HFs (Abaci et al., 2018).

Despite the above-mentioned progress in HF bioengineering, the reconstitution of a fully organized and functional human HF resorting to cultured human cells is still missing. A regenerative medicine therapy for human hair loss will only be successfully achieved when HF are formed *de novo* following implementation of *in vitro* bioengineered structures into the patient’s bald scalp. Importantly, although from a scientific perspective studies have achieved and reported HF regeneration from human cells (Abaci et al., 2018; Lindner et al., 2011), the caveats are whether i) there is any mouse contribution in HF neogenesis from human bioengineered structures transplanted into mouse skin, and ii) human bioengineered structures will generate HF that besides growing/cycling also mimetic natural hair type and are responsive to physiological stimuli.

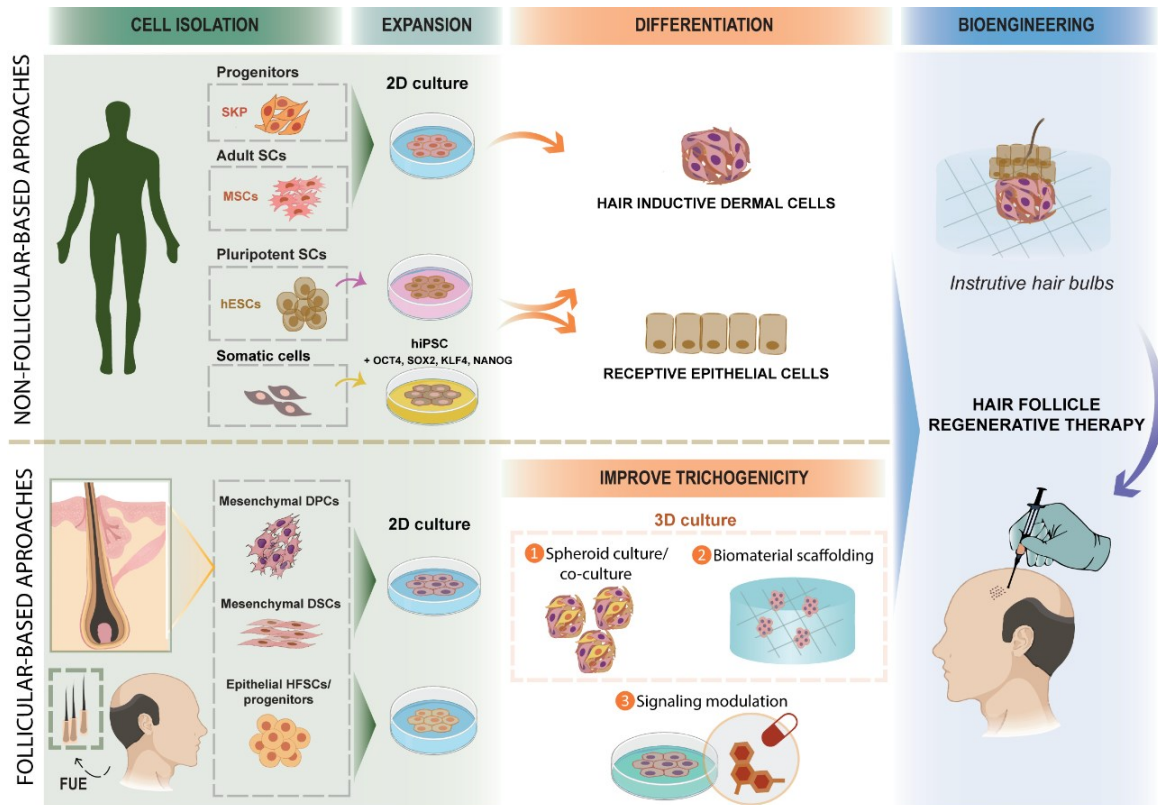


Figure 1.3. Prospective cell-based strategies for HF regenerative therapy. Different cell sources are being explored for HF tissue engineering. Non-follicular cell sources (top left): SKPs from human skin, which are similar to DPCs; adult stem cells (e.g., isolated from bone marrow- MSC); and pluripotent stem cells (embryonic/ESC or induced/iPSC with Yamanaka factors), which may be differentiated into a mesenchymal inductive population (akin to follicular DP) or a receptive epithelial population. Follicular cell sources (bottom left): DPCs and DSCs mesenchymal inductive; and bulge HFSC epithelial receptive. Considering the negative effects of *in vitro* follicular cell culture expansion, signaling modulation, 3D culture, biomaterial-based culture, or a combination of these approaches may be used to restore cell's trichogenicity. By combining epithelial and mesenchymal components, engineered instructive mini-bulbs can be obtained *in vitro* for a successful tissue engineering solution able to generate mature and functional HFs in the bald scalps; FUE - Follicular Unit Excision.

From a clinical perspective, an effective regenerative medicine would provide an autologous cell-based bioengineered product able to cure hair loss without adverse side effects. Although promising, so far only hematopoietic stem cell-based therapies have been implemented in the clinics. Moreover, significant limitations may further hamper an operational clinical solution for hair loss. First, bioengineered hair reconstruction will imply large-scale production of cell-based structures and the development of xeno-free and well-defined culture expansion media for clinical usage. Robust culture systems that allow stem cell multiplication while maintaining their intrinsic properties are still missing. Second, even if generation of functional and cycling hair follicle units is achieved, a huge gap still exists until the conception of a clinically relevant bioengineered product

that responds to physiological stimuli (e.g., neuronal stimuli) and aesthetic context (hair type, density, pigmentation and orientation). For instance, larger bioengineered DPs could be required to generate thicker hair fibers, as DP size has been reported to impact on hair's diameter (Elliott et al., 1999). Third, the low efficiency of organ induction, together with glitches in HF eruption and/or growth direction, may hinder the establishment of the effective therapy. Finally, from an economic perspective, a cost-effective cellular growth and *in vitro* cell-based bioengineering for hair loss will be challenging. The establishment of a patient-customized therapy will necessarily make it highly expensive.

Considering all the above-mentioned pitfalls that the hair-cloning premise has faced over the last decades, it is not surprising why hair rejuvenation (by stimulating existing follicles) has become the goal post for treating hair loss.

Conclusion

Comprehensive knowledge of HF morphogenesis and cyclic regenerative regulation, together with optimized protocols for HF/stem cells isolation and culturing have boosted the creation of a wide range of bioengineering solutions aiming to cure hair loss. However, future efforts are still needed to bridge such knowledge into an effective translational tissue engineering solution. Importantly, the successful development of *in vitro* engineered human HFs will certainly suit major biological applications far beyond hair loss cure. The conception of biologically improved skin replacement therapies (whose usage has been limited by the absence of HF), or even their application as a research model for skin drug development or cosmetic products testing, turn the HF bioengineering a knowhow seeker by several medical and pharmaceutical industries.

II

Introduction and aims of the thesis

Chapter 2

Introduction and aims of the thesis

Presently at the US, 50 million men and 30 million women suffer from the hair loss condition known as androgenic alopecia (AGA). There is an increasing demand for both preventive and therapeutic hair loss options as people are now more concerned about beauty standards, lifestyle and general health. Hair loss is acknowledged as an age-related physiologic process, and much has been described on the evolution of human hairlessness (Schwartz and Rosenblum, 1981; Jablonski, 2010). Considering that people aged over 65 years will more than double over the next three decades, expectedly reaching 1.5 billion in 2050 (United Nations, 2019), the millions of dollars spent every year on hair restoration will continuously increase. Moreover, the world digitalization has magnified the consumers' requirements for aesthetic and youth appearance, but also the mental distress caused by hair loss conditions. In addition, lifestyle changes and anxiety that dominate the millennials and coming generations have contributed to the early onset of AGA (Agaoglu et al., 2021; Cannarella, La Vignera, Condorelli, & Calogero, 2017). To note, the hair loss industry has also benefited from the COVID-19 pandemic, since coronavirus infection triggers telogen effluvium, further pushing for hair regenerative solutions (Monari et al., 2022). Despite this fast-growing market, the expensive cost of hair loss treatments and their limited efficacy fall short over the patients' needs and expectations. Currently, hair transplant is the most popular and effective therapy for AGA. Hair restoration surgery has markedly evolved over the past years prompted by significant technological and clinical advances, both with the use of robotics and follicular unit extraction (FUE) methods, to provide patients excellent results (Rose, 2018). Market analysts have predicted the global hair transplant industry will value more than \$28 billion by 2027 (<https://www.globenewswire.com/en/news-release/2021/06/18/2249450/28124/en/Global-27-9-Billion-Hair-Transplant-Market-Forecast-to-2027.html>). However, this procedure is strongly limited by the available number of HFs in the donor area (Rose, 2015). Besides hair transplant, to date, only Minoxidil (potassium channel agonist) and Finasteride (α 5-reductase inhibitor) have been approved by the US Food and Drug Administration (FDA) as pharmacological treatments for hair loss (Adil and Godwin, 2017). Yet, these drugs have revealed marginally effective at ceasing the rate of hair loss and entail drastic side effects (Madaan et al., 2018; Talavera-Adame et al., 2017). Intriguingly, no other FDA-approved drug has been marketed over more than 40 years of research (Rose, 2015).

Aims of the thesis

The present research aims to improve the understanding of the human hair loss and to develop novel therapeutic strategies for its treatment.

Using a source of fresh human HF, this thesis proposes to address the following specific aims:

Aim 1. Unveil molecular mechanisms responsible for dermal papilla hair inductive potential (Chapter 4).

This unbiased analysis of dermal papilla transcriptional changes will provide us with mechanistic insights governing DP hair induction regulation. A methodological description and analysis of next-generation sequencing was performed to cover the whole genome and more accurately understand the DP transcriptional signature. Unbiased transcriptional analysis of DP cells retrieved from intact papilla vs. expanded 2D vs. aggregated 3D provides us with mechanistic insights governing DP loss of hair inductive properties *in vitro*.

Aim 2. An educated approach to modulate loss of DP cell's properties *in vitro* (Chapter 5).

A major factor hampering functional human HF regeneration is the failure to preserve DP cells trichogenicity during *in vitro* expansion. DP cells gradually lose their inductivity in 2D culture and gain stress markers, which seem to be partially restored when transferred into a 3D spheroid culture (C. A. Higgins et al., 2013). We hypothesize that external mechanical cues are able to trigger the activation of different signaling pathways in DP cells responsible for guiding their differentiation into a committed non-hairy lineage. Therefore, educated approaches targeting TGF β 1-SMAD signalling during DP multiplication will be used to directly assess the role of TGF β 1-SMAD signalling in DP cells physiology.

Aim 3. Therapeutically modulate human hair follicle growth cycle (Chapter 6).

Previous studies have suggested an androgen mediated TGF β regulation in AGA (Inui, Fukuzato, Nakajima, Yoshikawa, & Itami, 2002, 2003). Hence, functionally reestablishing the TGF β signaling pathway in HF could potentiate its therapeutic exploration for hair growth. Validation studies were performed in Human HF organ cultures to assess the efficacy of a potent TGF β receptor I (T β RI) inhibitor against TGF β repressive signaling and validate its potential as a pharmacological intervention to treat AGA.

Aim 4. Compound screening for DP cells “reprogramming” factors (Chapter 7).

Epigenetic modulation of key transcription factors (TFs) controlling DP cells trichogenicity may allow us to “reprogram” the DP signature. Reestablishing the “native” epigenetic state and transcriptional activity of DP cells in culture would allow reprogramming back to their hair

inductive state. A new therapeutic approach for hair loss may be uncovered if an efficient reprogramming of DP cells allows them to reestablish the natural DP trichogenic signature *in vitro*. Thus, this chapter proposes a novel set up to screen for new pharmacological compounds that impact on human hair follicle trichogenicity and we discover novel epigenetic compounds as promising candidates for hair loss treatment.

III

Methods

Chapter 3

Methods

3.1. Dermal papilla cell (DPC) culture

Human DPC cultures can be established from either balding or occipital non-balding hair grafts (**Fig. 3.1**). The experimental set-up for human DPCs isolation is schematically shown in **Fig. 3.2**. We present representative images from successfully established human DP cell cultures. Before initiating a DP cell culture, we highly recommend training the micro-dissection of human dermal papillae and check for the efficient isolation of DPCs.

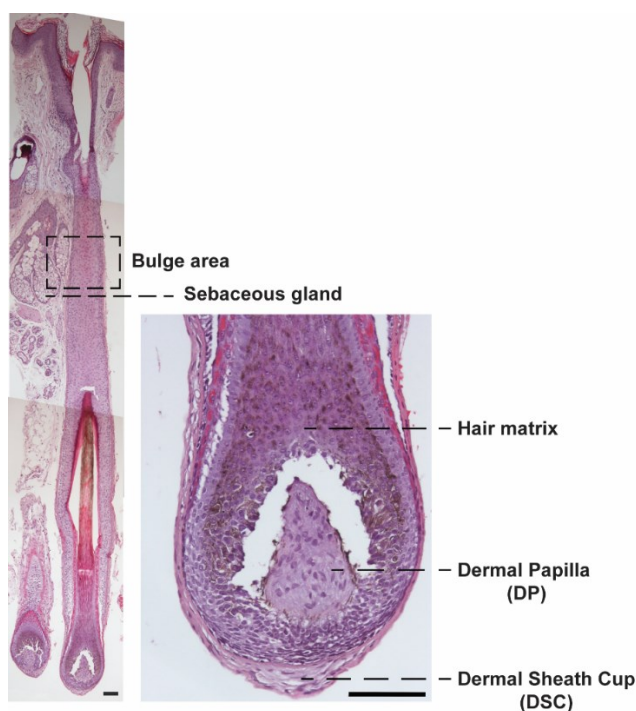


Figure 3.1. Histological analysis of human occipital HF unit. Samples were stained with Hematoxylin and Eosin (H&E). Scale bar, 1mm.

Freshly collected occipital hair grafts were placed in HBSS solution (Thermofisher, Gibco 14025-092) supplemented with 2% antibiotics-antimycotics (Merck Life Science, cat. no. A5955) and dissected in a sterile petri dish within 1-3h after isolation. In a normal scalp, approximately 90% of the extracted HF grafts will be in anagenic growth, and thus can be used for explant culture. Human DP have approximately $530\mu\text{m}^3$ (Elliott et al., 1999), therefore can be easily observed under a

stereomicroscope at 35x magnification (Leica EZ4W, equipped with a digital camera 5.0x mega-pixel CMOS camera). Isolated DPs were aspirated from the Petri dish and incubated in pre-warmed digestion solution [0,2mg/mL collagenase D (Merck Life Science, cat. no. 11088858001) in MEM α (Gibco, cat. no. 41061037)] for 4h at 37°C with shaking. 250uL of this digestion mix were transferred into a 24-well (Corning, cat. No. CLS3526) previously coated with 0.1% gelatin (CAS Number: 9000-70-8). 750uL of prewarmed MEM α supplemented with 15% FBS (Gibco, cat. no. 10270-106), 2.5 mM l-glutamine and 1x antibiotic–antimycotic (Gibco, Thermo Fisher Scientific, CA, USA) were added to each well. The 24-wells were left undisturbed for 1 week so that the DPs collapsed, and DPCs started spreading out. Once a reasonable density of adherent DPCs could be seen in the explant culture, medium was replaced every 3 days, and cells trypsinized (Gibco, cat. no. 12605010) at confluency and diluted 1:4 to establish a new passage, until a maximum of 3 passages (p3) (3-4 weeks).

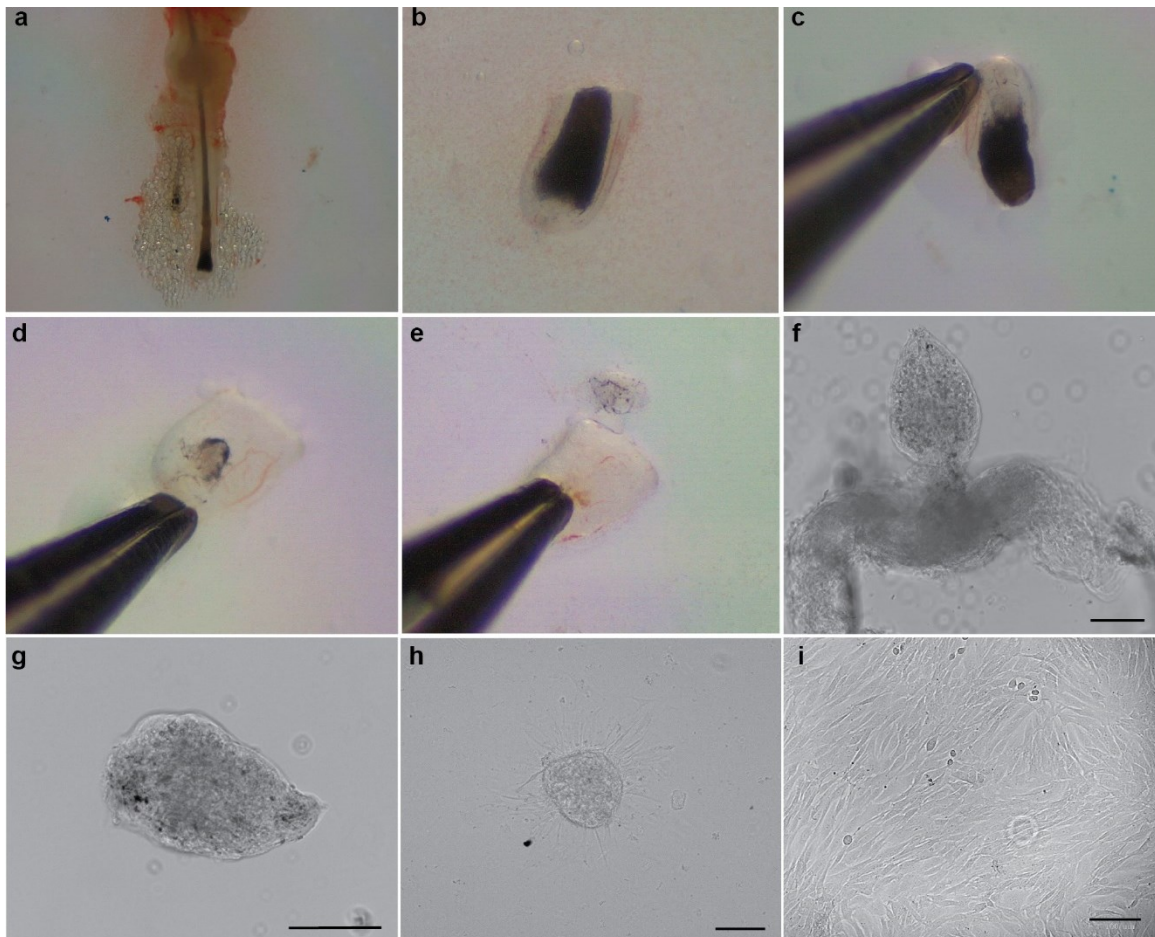


Figure 3.2. Human dermal papilla micro-dissection method. a) Skin biopsy to collect hair follicle units (10x); b) Transect the HF with scalpel blade to isolate the hair bulb (35x); c) Push the hair matrix using sterile Dumont forceps; d-f) Transect the DP by pressing the dermal sheath end bulb; g) Aspirate the isolated DP and transfer to a digestion solution: 0,2mg/mL collagenase D in MEM α ; h) DP adherence in a 24-well plate

coated with 0,1% gelatin and DPCs outgrowth around the DP after 1 week; **i**) Confluent DPC culture (scale bar, 100 μ m).

3.2. 3D culture

A micro-molding non-adhesive agarose hydrogel (UltraPure™ Agarose, Invitrogen cat. no.16500100) was used as a supportive scaffold to obtain 3D self-assembled DPC spheroids. To generate the agarose micro-mold, we used a commercially available 3D polydimethylsiloxane (PDMS) Petri dish (MicroTissues®, Merk cat. no. Z764000-6EA). Agarose was dissolved at 2% w/v in sterile saline solution (B. BRAUN 0.9% NaCl). Molten agarose was then pipetted into a sterile PDMS micro-mold. Upon solidification, agarose hydrogel molds were gently removed from the PDMS. Using the PDMS stamp as a negative mold, 81 microwells with 800 μ m diameter were formed. After casting, agarose micro-molds were transferred to a 12-well plate and equilibrated in MEM α overnight. 1.6×10^5 DPCs in a 190 μ L monodispersed suspension were then seeded per micro-mold. This cell density was calculated so that each spheroid contains approximately 2000 cells to mimic the native DP size *in vivo* (Elliott et al., 1999). 72h after seeding, spheroids were collected for analysis (**Fig. 3.3**). To harvest the microtissues, medium was carefully removed from the well plate. Using a sterile spatula, the micro-mold was placed inverted into a 24-well containing 1 mL of medium. After gentle shaking, spheroids were released. The media containing the spheroids was transferred to a 15mL tube, and centrifuged at 1200xg for 5 min. The spheroids' pellet was used for further analysis.

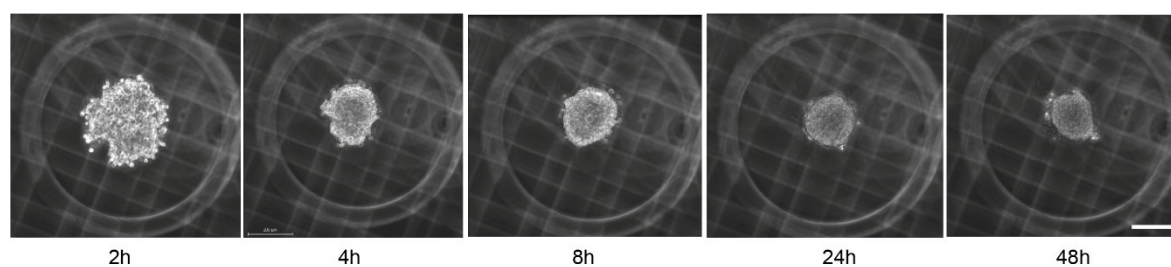


Figure 3.3. Time-lapse imaging of DPCs' self-assembly into 3D spheroids. Top views of the agarose micro-mold under a bright field microscope showing spheroid formation over 48h period. Scale bar, 200 μ m.

The use of agarose hydrogel for micro-molding is a very reproducible high-throughput method which allows the formation of several similar spheroids with a given size. In general, this method enables the fabrication of a large number of spheroids and their use for diverse molecular and cell biology analyses. Histological analysis comparing a freshly isolated DP and a 3D spheroid generated *in vitro* revealed high morphological similarity (**Fig. 3.4**).

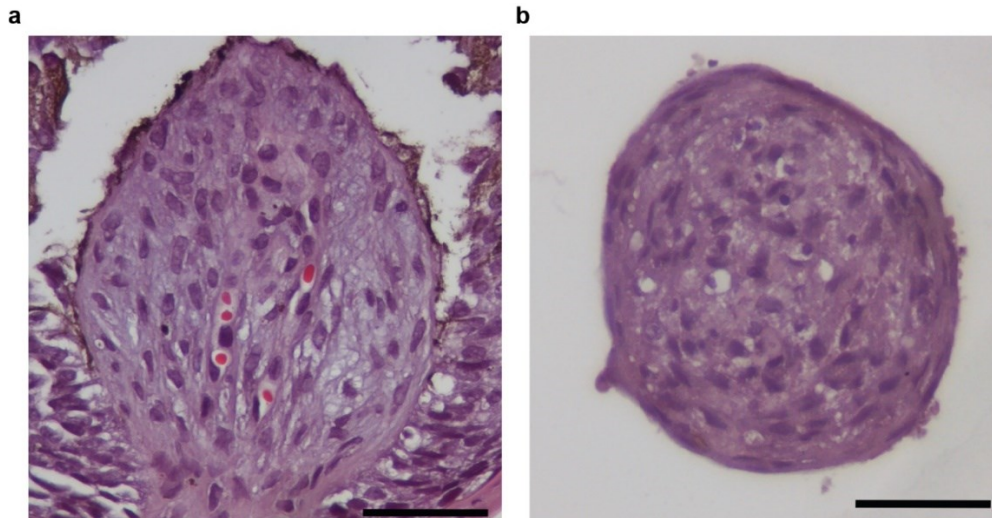


Figure 3.4. Freshly isolated DP vs. DP spheroid. **a)** H&E staining of a fresh DP– a specialized mesenchymal cell population at the base of the HF that is histologically distinct from the adjacent dermis and stimulates HF development and cyclic growth *in vivo*; **b)** H&E staining of a DP spheroid generated *in vitro*. Scale bars, 100 μm.

3.3. RNA extraction

For transcriptome analysis, RNA was extracted from freshly isolated occipital DPs (FDPs), 2D cultured DPCs at passage 3 (p3), and DP 3D spheroids (72h after being established from p3 cells from the same patient). For 2D cultured DPCs and DP 3D spheroids, RNA was extracted using Quick-RNA Microprep kit (cat no. R1050, Zymo Research) accordingly to manufacturer's instructions.

Importantly, given the low amount of HF tissue donated from each patient during hair transplant, whole transcriptome analysis (RNA-seq) from fresh isolated dermal papillae tissue is quite challenging. Furthermore, akin to a skin tissue it contains multiple nucleases and its collagenous nature make it one of the most challenging tissues to homogenize (Troost et al., 2007) turning it a very demanding tissue for gene expression analysis. Indeed, obtaining a meaningful RNA extraction from human dermal papilla is a critical limitation to the study of human hair follicle regulation. Therefore, here we detailed described a technique to successfully extract RNA from human fresh dermal papillae, by improving dermal papillae digestion and overcoming the shortage of RNA. Here, we described an optimized technique for DP digestion and RNA extraction.

HFs were extracted using a 0.95-mm micropunch (Scharf CE, Istanbul, Turkey), and immediately fast frozen in nitrogen and transported to the lab. The working bench was cleaned with RNase AWAY™ Surface Decontaminant (cat no. 7000TS1, Thermofisher). Hair grafts were washed in PBS (pH 7.4, RNase-free). 15 anagenic HFs were selected under a stereomicroscope and dissected to collect the DP. Isolated DPs were immediately transferred into a digestion solution containing

180uL Tissue Lysis Buffer (Buffer ATL, cat. no 939011, Qiagen) and 20uL proteinase K solution (Cat. No: 19131, Qiagen). 200uL Buffer AL (cat. no 19075, Qiagen) were added to the digestion mix, and shaking 10 min at 56°C and 300rpm. RNA extraction was done using Quick-RNA Microprep kit upon adding Lysis buffer 1:1. Considering the low number of HF's donated by each patient, HF's from 2 patients were pooled to obtain enough amount of high-quality RNA for optimal detection of FDP transcripts.

3.4. Galunisertib treatment

Galunisertib (LY2157299) is a potent TGF β receptor I (T β RI) inhibitor (Selleck Chemicals) was diluted to a concentration of 50mM in DMSO. Passage 0 (p0) DPCs (at 60% confluency) were treated with 10 μ M galunisertib in. Media was replaced on every other day until passage 2 (p2). P2 DPCs were collected 48h after treatment and processed for further analysis.

3.5. Real-Time quantitative PCR (RT-qPCR)

600 ng of RNA were reverse transcribed into complementary DNA with iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA), according to the manufacturer's protocol. cDNA was stored at -20 °C. RT-qPCR reactions were performed in a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). 10 μ l reaction mixes were prepared consisting of iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories), forward and reverse primers (125nM each) and cDNA (30 ng). The amplification program used was: 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s, 60 °C for 35 s, and melt curve from 55 °C to 95 °C with 0,5 °C increment for 10 s. All primers were designed to span at least one exon-intron junction (**Table 3.2**). Primer's efficiency was tested by plotting Ct values versus log cDNA dilution for each gene studied using CFX Maestro™ Software (Bio-Rad Laboratories). Transcript levels were quantified by the 2- $\Delta\Delta$ Ct method. Δ Ct values were determined by subtracting the geometrical mean of the Ct values for GAPDH and UBQ from the mean target gene Ct value.

3.6. Western blot

DPCs were collected and treated with RIPA lysis buffer (89900, Thermo fisher Scientific) containing halt protease inhibitor cocktail (78429, Thermo fisher Scientific) and halt phosphatase inhibitor cocktail (78420, Thermo fisher Scientific). Lysates were sonicated and protein content quantified by the lowry method (DC™ Protein Assay, Bio-Rad, CA, USA). Hair bulbs were dissected from HFOCs treated with DMSO, DHT and galunisertib for 72h. Upon rinse in PBS, hair bulbs were lysed in laemmli buffer (without bromophenol blue) with halt protease inhibitor Cocktail

and Halt Phosphatase Inhibitor Cocktail. Sonicated lysates from 10 hair bulbs were used for each condition tested.

Twenty micrograms of total extract were loaded in SDS-polyacrylamide gels and transferred onto nitrocellulose membranes for western blot analysis. Membranes were blocked during 1 h with TBS + 5% BSA. Primary and secondary antibodies were diluted in TBS + 5% BSA accordingly to **Table 3.1**. Signal was detected using Clarity Western ECL Substrate reagent (Bio-Rad Laboratories) according to manufacturer's instructions. A GS-800 calibrated densitometer with Quantity One 1-D Analysis Software 4.6 (Bio-Rad Laboratories) was used for quantitative analysis of protein levels.

3.7. Immunofluorescence

Cells were fixed in freshly prepared 4% paraformaldehyde (PFA) in PBS for 20 min. Following fixation, cells were rinsed in PBS and permeabilized in PBS + 0.3% Triton-X100 for 7 min. Cells were next blocked in 10% FBS in PBS-T (PBS + 0.05% Tween-20) for 1 h, and then incubated overnight at 4 °C with primary antibodies diluted in PBS-T + 5% FBS (**Table 3.1**). For β -catenin staining cells were fixed with 3% PFA for 15 min at RT, and permeabilized in PBS + 0,01% triton + 0,05% SDS for 10 min. Cells were next blocked in 0,2% BSA in PBS-T for 1 h and then incubated overnight at 4 °C with primary antibodies diluted in PBS-T + 5% FBS. DNA was counterstained with DAPI (Sigma-Aldrich, MO, USA).

All images were acquired under a Laser Scanning Confocal Microscope (Leica TCS SP5 II, Leica Microsystems) operated by the Leica Confocal Software -LAS, using a 40x/NA1.1 water immersion objective and an acquisition speed of 400Hz.

3.8. Immunohistochemistry

2-day HFOCs were used for immunohistochemistry analysis. Briefly, HF's were embedded on a drop of OCT (Shandon Cryomatrix, Thermo Fisher Scientific) and stored at -80°C. 8 μ m cryosections were air-dried and fixed in 4% PFA (for TGF β 2, cleaved Caspase 3, p-SMAD2 and KI67 immunostainings) or cold acetone (for LEF1 and β -catenin immunostainings). Following 2x 5min rinse with PBS, microsections were permeabilized 2x 10 min with PBS + 0.3% Triton X-100 (PBS-T) and then blocked with PBS + 10% FBS for 1h at room temperature. Antibodies (**Table 3.1**) were diluted in PBS-T + 1% FBS and incubation done overnight at 4°C. Upon 2x 5min rinse with PBS-T, samples were incubated with the secondary antibodies, goat anti-human Alexa-488 (1 :1000; Life Technologies) and goat anti-human Alexa-568 (1 :1000; Life technologies). Nuclei were counterstained with DAPI (Sigma-Aldrich), and slides were then mounted with 90% glycerol, 0.5% N-propyl-gallate, 20 nM Tris, pH 8 solution. All images were acquired under a Laser Scanning Confocal Microscope (Leica TCS SP5 II, Leica Microsystems) operated by the Leica

Confocal Software - LAS, using 40x/NA1.1 water immersion objective and an acquisition speed of 400Hz.

3.9. Library preparation and RNA-Seq

Bioanalyzer High Sensitivity DNA chip (Agilent technologies) was used to measure total RNA concentration and purity. Only total RNAs that received an RNA integrity number (RIN) of 9 or higher were used for RNA-sequencing. Library prep and sequencing were done at Novogene (Cambridge, United Kingdom). Low quality reads were removed (>10% Ns). The percentage of reads with quality score above 20 (Q20) was > 97% for all samples. Over 80M reads per sample aligned to the hg38 version of the human genome using HISAT2 (v2.0.5) (D. Kim, Paggi, Park, Bennett, & Salzberg, 2019), with a rate of unique mappings >93%. Gene expression based on reads mapped to genes was quantified by FeatureCounts (v1.5.0-p3). Principal component analysis was done on the $\log_2(\text{FPKM}+1)$ counts using the PCAtools package (Lun, 2021). Differential gene expression analysis was done with the DESeq2 (v1.20) (Love, Huber, & Anders, 2014) package on the raw counts.

Differentially expressed genes (DEGs) in galunisertib-treated vs. untreated samples were obtained with $\text{abs}(\log_2(\text{FC})) \geq 1$ and $p\text{-value} \leq 0.05$, represented in a volcano plot using the R package ggplot2 and in a heatmap upon Z-scoring and hierarchically clustering with the Euclidean distance, using the ComplexHeatmap package (Gu, Eils, & Schlesner, 2016). GO term and KEGG pathway enrichment on DEGs were done with the ClusterProfiler package (Wu et al., 2021) as well as KEGG pathway gene set enrichment analysis for all genes. Pearson correlation was calculated between samples and plotted using the R package corrplot (v0.9).

Gene over representation analysis (ORA) were performed with the ClusterProfiler R/Bioconductor package (v4.4) using mSigDB collections (<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>). A minimum of 10 genes per set was required as well as an enrichment $p\text{-value} \leq 0.05$.

3.10. Hair grafts [*ex vivo* studies]

A total of 35 Caucasian male patients aged between 18-55 years, who had given their informed consent for this study, were included in our analysis. 10-20 micrografts were harvested from the occipital scalp of patients diagnosed with androgenic alopecia and undergoing autologous hair transplant by the routine FUE technique. All micrografts used were intact, with visible amount of fat and connective tissue. In addition, only anagen HFs were used in this study.

3.11. *Ex vivo* human scalp hair follicle organ culture (HFOC)

HF units from human adult scalp tissue were cultured in Williams E medium supplemented with 10 ng/mL hydrocortisone, 10 g/mL insulin, 2 mM l-glutamine and 1x antibiotic–antimycotic (all from Gibco, Thermo Fisher Scientific). HFs were maintained at 37°C in 5% CO₂ atmosphere. Minoxidil (M4145, Merck Life Science) was used at 1μM, galunisertib (S2230, Selleck Chemicals CO) at 10μM optimal concentration, and DHT (D-073, Merck Life Science) at 1μM. Culture medium was replaced every 2 days, and images of individual follicles were acquired under a Leica M80 stereomicroscope (Leica Microsystems). HFs elongation was measured from images acquired over 10 days using the opensource ImageJ software. Hair growth percentages were normalized to day 0.

3.12. Statistical analysis

Sample sizes and statistical tests used in each experiment are indicated in figure legends. Statistical analyses were performed with Prism GraphPad v6.00 (Prism, CA, USA). Data were tested for parametric or non-parametric distribution using D'Agostino-Pearson omnibus normality test. Student's *t*-test was used to check for the significance of qPCR, Western blot and HFOC data. Two-tailed Mann-Whitney or *t*-test were used to determine statistical differences between groups. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. All values are mean $\pm$ SD.

Tables

Table 3.1. Antibodies used in the study.

Name	Reference	Application
Anti-phospho-Smad2 (Ser465/467)	Rabbit monoclonal Cell Signaling Technology, 3108S	1:250, WB
Anti-Smad2/3	Mouse monoclonal BD Bioscience, 610843	1:1000, WB
Anti-LEF1 [EP2030Y]	Rabbit monoclonal Abcam, ab53293	1:200, IHC
Anti-Ki-67 (8D5)	Mouse monoclonal Cell Signaling Technology, 9449S	1:500, IF 1:250, IHC
Anti-cleaved Caspase-3 (Asp175)	Rabbit polyclonal Cell Signaling Technology, 9661S	1:250, IHC
Anti-β-Catenin	Mouse monoclonal BD Bioscience, 610153	1:250, IHC

Anti-TGFβ2 (220ct16.4.3.1)	Mouse Monoclonal Thermo Fisher Scientific, MA5-37505	1:100, IHC
Anti-α-Smooth Muscle Actin	Mouse monoclonal Sigma-aldrich, A5228	1:500, IF 1:250, IHC
Anti-GAPDH	Mouse monoclonal ProteinTech Group, 60004	1:50000, WB
Goat anti-rabbit IgG (H+L)	Rabbit polyclonal HRP Jackson ImmunoResearch Europe, 111-035-003	1:3000, WB
Goat Anti-Mouse IgG (H+L)	Mouse polyclonal HRP Jackson ImmunoResearch Europe, 115-035-003	1:3000, WB
Anti-mouse AlexaFluor-488	Mouse polyclonal Life Technologies	1:1000 IF 1: 250 IHC
Anti-rabbit AlexaFluor-568	Mouse polyclonal Life Technologies	1:1000 IF 1: 250 IHC

Table 3.2. Primers used for Real-time qPCR analysis.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>GAPDH</i>	GAAGGTGAAGGTCGGAGTCAAC	GGTCATTGATGGCAACAATATCCAC
<i>UBQ</i>	TGAAGACTCTGACTGGTAGACC	CATCCAGCAAAGATCAGCCTC
<i>LEF1</i>	CTGACAGTGACCTAATGCACGT	GTACACTCAGCAACGACATTCTG
<i>TGFB1</i>	TGGACACCAACTATTGCTTCAGG	AGAAGTTGGCATGGTAGCCC
<i>TGFB2</i>	AGAGTGCCTGAACAACGGATT	GTCGATGTAGCGCTGGGT
<i>TGFB3</i>	TCTCAACAGCCACTCACGCACA	CTAAGCGGATGAGCAGAGGATC
<i>ACTA2</i>	TGTTCCAGCCATCCTTCATCG	CATTGTAGCATAGAGGTCCTTCCT
<i>SERPINE1</i>	TCATCATCAATGACTGGGTGAAG	GCCGTTGAATAGAGGGCATT

IV

**The native dermal papilla transcriptome: the
holy grail of human hair growth induction?**

Chapter 4

The native dermal papilla transcriptome signature: the holy grail of human hair growth induction?

Despite the recent advances in HF research, a clinical solution for hair loss remains intangible as human follicle neogenesis has not been successfully achieved yet. *In vitro* multiplication of DP cells is a key step for tissue engineering, but DP cells lose their hair inductive properties over-time *in vitro* (Ohyama et al., 2012; Rendl et al., 2008; C. C. Yang & Cotsarelis, 2010).

Whereas the DP and primordial dermal condensates from mice have been comprehensively characterized (Ge et al., 2020; K. Gupta et al., 2019; Hagner et al., 2020; R. Sennett et al., 2015), our knowledge of the human native DP and the molecular mechanisms controlling human hair growth is still limited. So far, only two recent studies have profiled the transcriptome of human DP samples in order to i) study androgen-related changes in human DP (Z. Deng et al., 2022) and ii) identify similarities with the onychodermis of nail units (Shim et al., 2022). In addition, microarray comparative analysis was done for native DP vs. DPC cultures (C. A. Higgins et al., 2013). Although these studies have contributed to the understanding of human HF regulation, one limitation is that native DPs and *in vitro* cell cultures were from different patients and were not followed-up during culturing steps. Hence, fundamental research on the human DP transcriptome is still needed to unequivocally identify the human specific traits of a hairy DP and their deregulation upon *in vitro* culturing.

In this chapter, we performed a comparative analysis of freshly isolated DPs vs. DPCs in 2D cultures vs. DPCs in 3D spheroids. We foresee that this analysis will bring valuable insight to human hair regulation and modulation.

Introduction

The current challenge on a hair loss definitive therapy is promoting the HF's neogenesis using the emergent knowledge in the field of regenerative medicine and tissue engineering. The limitation to

this therapeutic approach has been the inability to understand the molecular and cellular mechanisms that determine the stemness and inductive properties of human follicle cells, and to sustain these mechanisms during *in vitro* cell expansion, an indispensable step for tissue engineering solutions.

Transcriptome profiling portrays the biological pathways and processes operating under different experimental conditions. RNA-sequencing is a powerful tool to measure transcript levels and to determine changes in gene expression levels between distinct conditions.

It has been suggested that two-dimensional (2D) culturing of DP cells triggers the loss of hair follicle fate determinants, and that three-dimensional (3D) culturing can partially restore the native DP signature (C. A. Higgins et al., 2013). However, a comparative transcriptome analysis of DP cells under the three conditions (native, 2D and 3D) has not been reported yet. This is likely due to the challenging isolation procedure and significant number of HFs needed to attain high RNA quality and amount for sequencing.

Here, and upon optimizing an RNA isolation protocol from native DP (see **Chapter III, section 3.3**), we set out to study the impact of both 2D and 3D culturing in the native DP transcriptional signature. DPs were isolated from human scalp HF biopsies, then used for DP cell expansion in 2D cultures, and subsequently for 3D spheroids assembly. An unbiased transcriptional analysis of DP cells retrieved from intact papilla vs. expanded 2D vs. aggregated 3D is unprecedentedly provided along sequential culturing stages. We expect this approach to provide mechanistic insights governing DP loss of hair inductive properties *in vitro* and to disclose: i) new molecular targets that can be modulated during *in vitro* expansion to preserve DP hair inductive potential, which may prompt the development of bioengineering constructs for hair loss treatment; and ii) new therapeutic targets with hair inductive capacity that instruct hair growth.

Experimental design

Nine Caucasian male patients undergoing FUE at the Insparya Science and Clinical Institute were enrolled in this study (aged 20–40 years) upon giving their written informed consent. 15 micrografts harvested from the occipital scalp of each patient were used.

For transcriptional analysis, RNA was extracted from i) freshly isolated DP from occipital follicle units, ii) DP cells in 2D standard culture at passage 3 (p3), and iii) DP spheroids assembled during 48h from p3 DP cells from the same patient (**Fig. 4.1**) (see **Chapter III, section 3.2**).

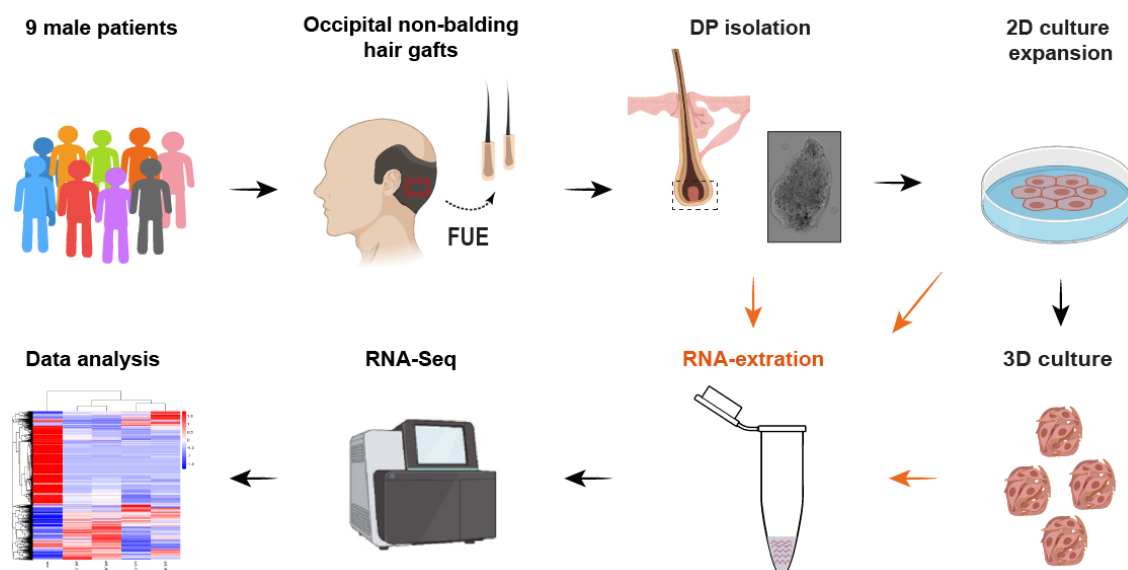


Figure 4.1. Experimental workflow. 15 micrografts were collected from each patient (9 in total). DP was isolated by manual microdissection and either used to implement DP cell 2D cultures, or to extract RNA for transcriptomic analysis. Comparative transcriptomic analysis was performed for fresh native DP (FDP) and for DP cells upon 2D culture expansion and subsequent assembly in 3D spheroids.

Results

RNA-seq analysis was performed to uncover transcriptional differences between FDP and cultured DP cells *in vitro*. We initially set out to determine baseline differences between native DP and cultured DP cells, which likely account for the loss of hair induction capacity upon *in vitro* culture. Principal component analysis (PCA) indicated that only 8,75% of variation is due to donor's genetic heterogeneity in a total of $n=3$ biological replicates (PC2), whereas 76,56% of variation is due to culture conditions (PC1) (**Fig. 4.2a**). This supports cell culturing as the main determinant of large transcriptome differences between samples.

We started by cross comparing our RNA-seq datasets. 11967 genes were common to all datasets of the FDP, 2D and 3D samples from $n=3$ donors. Within this group of the 'DP transcriptome', there are both unchanged and differentially expressed genes upon *in vitro* DP culturing. 437 genes were specifically expressed *in vitro* (either 2D or 3D), 110 genes were exclusively expressed in DP 2D cultures, and 297 exclusively transcribed in DP 3D cultures. We considered the sum of all these genes (a total of 844 genes) to represent an '*in vitro* DP signature' (absent in native DP). Moreover, from this comparative analysis we observed that the expression of 3519 genes of the native DP is lost upon *in vitro* expansion of DP cells ('lost DP transcriptome') (**Fig. 4.2b**). Top GO terms obtained from MSigDB databases revealed 'Epidermis development' 'Skin development' and 'Intermediate filament organization' as primarily represented biological processes lost during *in vitro* culture (**Fig. 4.2b**).

Canonical WNT signaling has been shown to be critical for HF morphogenesis and cycling (Andl, Reddy, Gaddapara, & Millar, 2002; M.-X. Lei, Chuong, & Widelitz, 2013), with its repression leading to the loss of DP inductive potential. Consistent with previous studies (Kishimoto et al., 2000; Ohyama et al., 2012), our whole-transcriptome profiling of DP cells revealed that key WNT signaling players (*WNT2B*, *WNT10B*, *WNT10A*, *WNT6*) are lost upon 2D culture. Likewise, previously reported genes regulating the WNT pathway, *LEF1*, *TCF7* and *AXIN2*, and also *WLS* and *WNT3* ligands, are highly repressed in DP cells *in vitro*. On the contrary, *WNT5A* and *WNT5B* that together with *DKK1* antagonize the canonical WNT signaling pathway (Kwack et al., 2008), are upregulated in *in vitro* cultures. *WNT5A* and *WNT5B* are described to act through the β -catenin-independent signaling pathway to induce cytoskeleton rearrangements (Suthon, Perkins, Bryja, Miranda-Carboni, & Krum, 2021), which suggests that *in vitro* DP culturing may activate the non-canonical WNT signaling (**Fig. 4.2b**). Interestingly, although *TGF β 2* is a *WNT* transcriptional target (Clevers & Nusse, 2012), it was consistently overexpressed in *in vitro* cultures. The TGF β pathway is involved in several cellular processes, as cell proliferation, differentiation, motility and adhesion (Massagué, 2012), and TGF β pathway players are expressed in intact FDP samples. However, our data suggests that both *in vitro* 2D and 3D cultures triggers an activation of this pathway (**Fig. 4.3**). Gene Set Enrichment Analysis (GSEA) for GO terms of TGF β signaling reveal that *in vitro* culturing significantly upregulates this pathway. Accordingly, *THBS1*, an activator of TGF β signaling, was found highly upregulated (**Fig. 4.3a**), suggesting *THBS* as putative *in vitro* DP cell marker. Interestingly, *THBS* has been reported as an hair growth-modulating protein upregulated in catagen, indicating TGF β signaling as a potential therapeutic target for hair follicle induction (Yano, Brown, Lawler, Miyakawa, & Detmar, 2003).

The BMP signaling players (*BMP 3*, *5*, *7*, *8a* and *8b*) were also found significantly downregulated in *in vitro* cultures. Interestingly, *GREM1* and *GREM2* (BMP inhibitors) are specifically expressed during *in vitro* culture, emphasizing BMP signaling pathway inhibition. Similarly, *SHH* is only expressed in intact anagenic DP, and *SUFU* (a negative regulator of Hedgehog signaling) is highly expressed in *in vitro* culture, in contrast to its low expression observed in mouse pelage DP cells (Kishimoto et al., 2000).

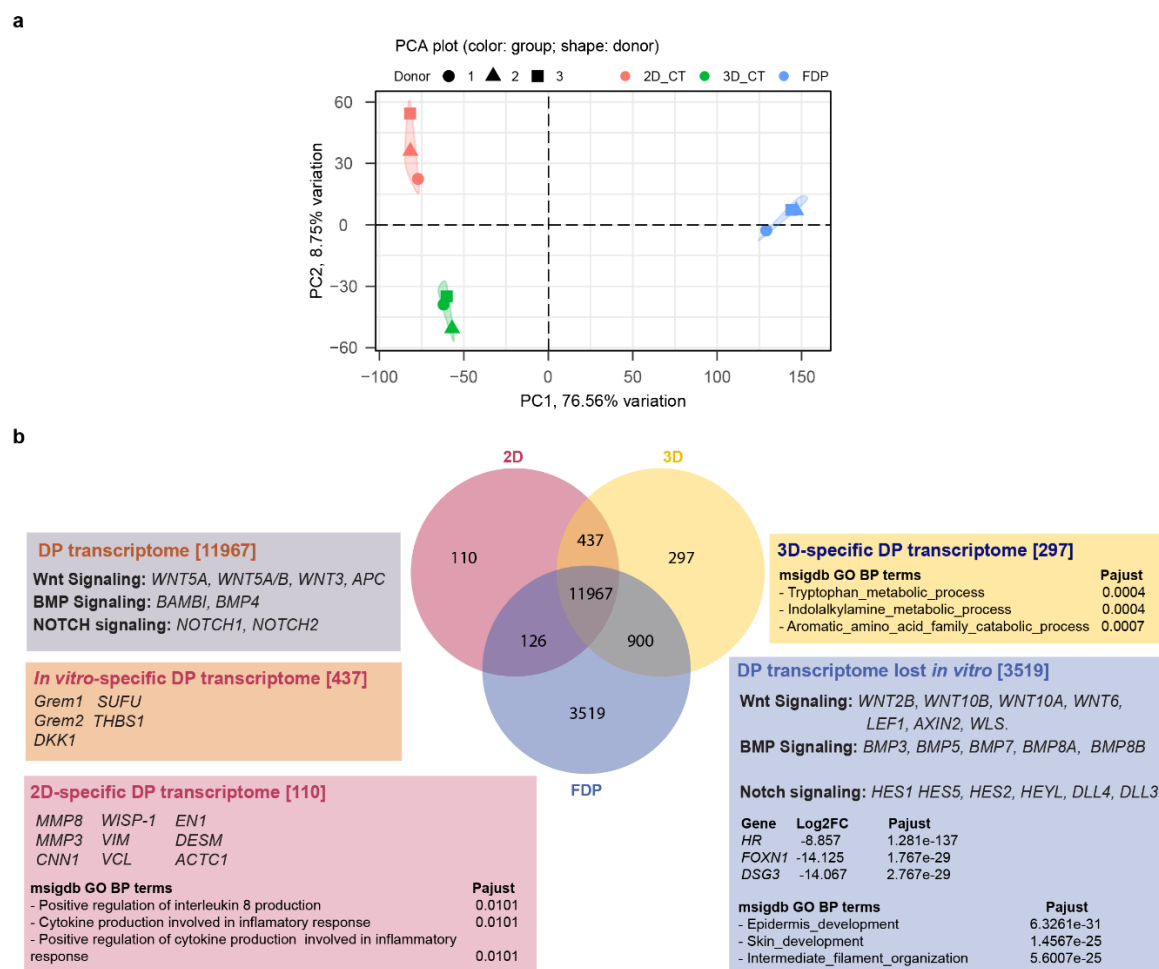


Figure 4.2. Overall correlation between gene expression profiles in DP cells in native (FDP) and 2D and 3D culture conditions. DPs were isolated from $n=15$ donors and used both for RNA extraction (FDP samples) and for the establishment of 2D cultures (2D_CT samples). 2D DP cells were subsequently used (at passage 3) for 3D spheroids assembly (3D_CT samples). **a)** Bidimensional principal component analysis (PCA) of the variance between samples; **b)** Venn diagram displaying genes found expressed in FDP vs. 2D vs 3D, as well as gene ontology (GO) biological processes (BP) found enriched on Molecular Signatures Database (MSigDB) analysis.

To further understand the upstream regulation of such a marked change in gene expression, we examined the expression of transcription regulators. A total of 431 transcription factors were downregulated in *in vitro* cultures compared to FDP. Among the top downregulated transcription factors in both 2D and 3D cultures were the *HES1*, *HES5*, *HES2* and *HEY1* involved in the Notch signaling (**Fig. 4.2b**). Hes1, a major transcriptional repressor downstream of NOTCH, regulates anagen initiation and hair follicle regeneration through modulation of Hedgehog signaling (W. J. Suen, S. T. Li, & L. T. Yang, 2020). It is also described as an effector of Notch signaling and a regulator of cell fate decisions (W.-J. Suen, S.-T. Li, & L.-T. Yang, 2020). GO terms obtained from

MSigDB databases disclosed inflammation and metabolism as primarily biological processes altered during *in vitro* culture (**Fig. 4.2b**).

Taken together, our data revealed that the transcriptional signatures of *SHH*, *WNT*, *BMP* and *NOTCH* signaling pathways are profoundly compromised in *in vitro* vs. *in vivo* DP cells. Considering that these signaling pathways are critical for hair induction and cyclic growth, their perturbation expectedly accounts for the loss of inductive potential in cultured DP cells.

Remarkably, we also observed, in the *in vitro* conditions a total transcriptional shutdown of key genes involved in hair growth, which when mutated lead to alopecia: Hairless (*HR*) (that encodes an intrinsic histone H3K9 lysine demethylase) (Andrei A. Panteleyev, Botchkareva, Sundberg, Christiano, & Paus, 1999), Forkhead box protein N1 (*FOXN1*) (associated with Nude/SCID syndrome) and Desmoglein 3 (*DSG3*) (Koch et al., 1998) (**Fig. 4.2b**). Of note, these transcription regulators play critical roles on HF cycling by impacting HFSC activation, self-renewal, differentiation, as well as by modulating the crucial *SHH*, *WNT*, *BMP* and *NOTCH* signaling pathways (Rendl, Lewis, & Fuchs, 2005).

In agreement with the reported resistance of occipital HFs to androgens, androgen receptor (*AR*) transcripts were barely detected in native DP samples (obtained from non-bald region HFs). But interestingly, we observed *AR* overexpression induced by the *in vitro* culturing.

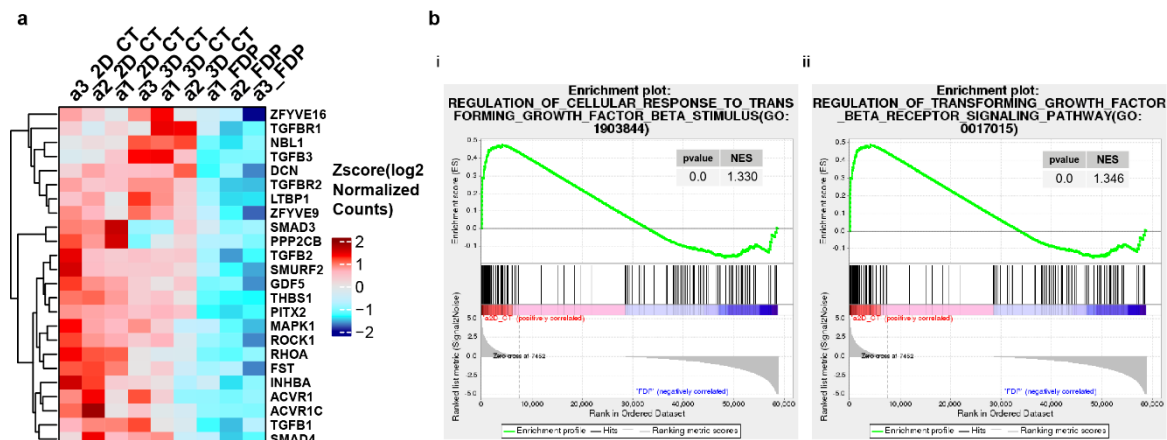


Figure 4.3. Upregulation of the *TGFβ* pathway during *in vitro* DP culturing. **a)** Heatmap of genes of the KEGG *TGFβ* signaling pathway found differentially expressed in 2D, 3D and FDP experimental conditions of each donor (a1-a3). Z-score column color intensities indicate higher (red) to lower (blue) expression; **b)** Gene Set Enrichment Analysis (GSEA) for the GO terms i) ‘Regulation of cellular response to transforming growth factor beta stimulus’ and ii) ‘Regulation of transforming growth factor beta receptor signaling pathway’ in 2D vs. FDP; NES, negative normalized enrichment score.

Given that DP controls HF induction and cycling (Houschyar et al., 2020; Morgan, 2014), we examined enriched DEGs in native vs. *in vitro* conditions using DESeq2 (*adj p value* <0.05). More

than 12000 differentially expressed genes (DEGs) were found in intact DP papilla (FDP) vs. *in vitro* DP cultures (2D and 3D) ($\text{abs}(\log_2\text{FC}) \geq 1$, $p \leq 0.05$) (**Fig. 4.4a**).

4978 genes were found upregulated, and 7422 genes downregulated in 2D vs. FDP samples ($p \leq 0.05$, false discovery rate (FDR) ≤ 0.01 ; **Fig. 4.4a**). Gene Ontology (GO) analysis revealed downregulation of the ‘hair follicle development’, ‘hair cycle’ and ‘hair cycle process’ GO terms, supporting the loss of hair inductive potential in 2D cultured DP cells (**Fig. 4.4b**). Upregulated GO terms included ‘actin cytoskeleton’, ‘extracellular matrix organization’ and ‘regulation of actin filament-based process’ (**Fig. 4.4c**). Within these upregulated GO terms, we found genes encoding metalloproteinases (*MMP3*, *MMP8*) and cytoskeleton proteins (*DESM*/desmin, *ACTC1*/actin alpha cardiac muscle 1, *CNN1*/calponin 1, *VIM*/vimentin, *VCL*/vinculin) that were exclusively expressed in 2D cultures (see **Fig. 4.2b** above). In addition, we observed specific expression in 2D vs. FDP of fibrosis-associated genes such as engrailed-1 (*EN1*) (Mascharak et al., 2021) and WNT1-inducible signaling pathway protein 1 (*WISP-1*), which drive the transition of fibroblasts into myofibroblasts (Sun, Ma, Yin, Zhang, & Liu, 2021) (**Fig. 4.2b**). Thus, our data indicate that mechanical stress imposed by 2D culturing triggers the transcription of genes associated with ECM and actin cytoskeleton remodeling.

IV

The native dermal papilla transcriptome: the holy grail of human hair growth induction?

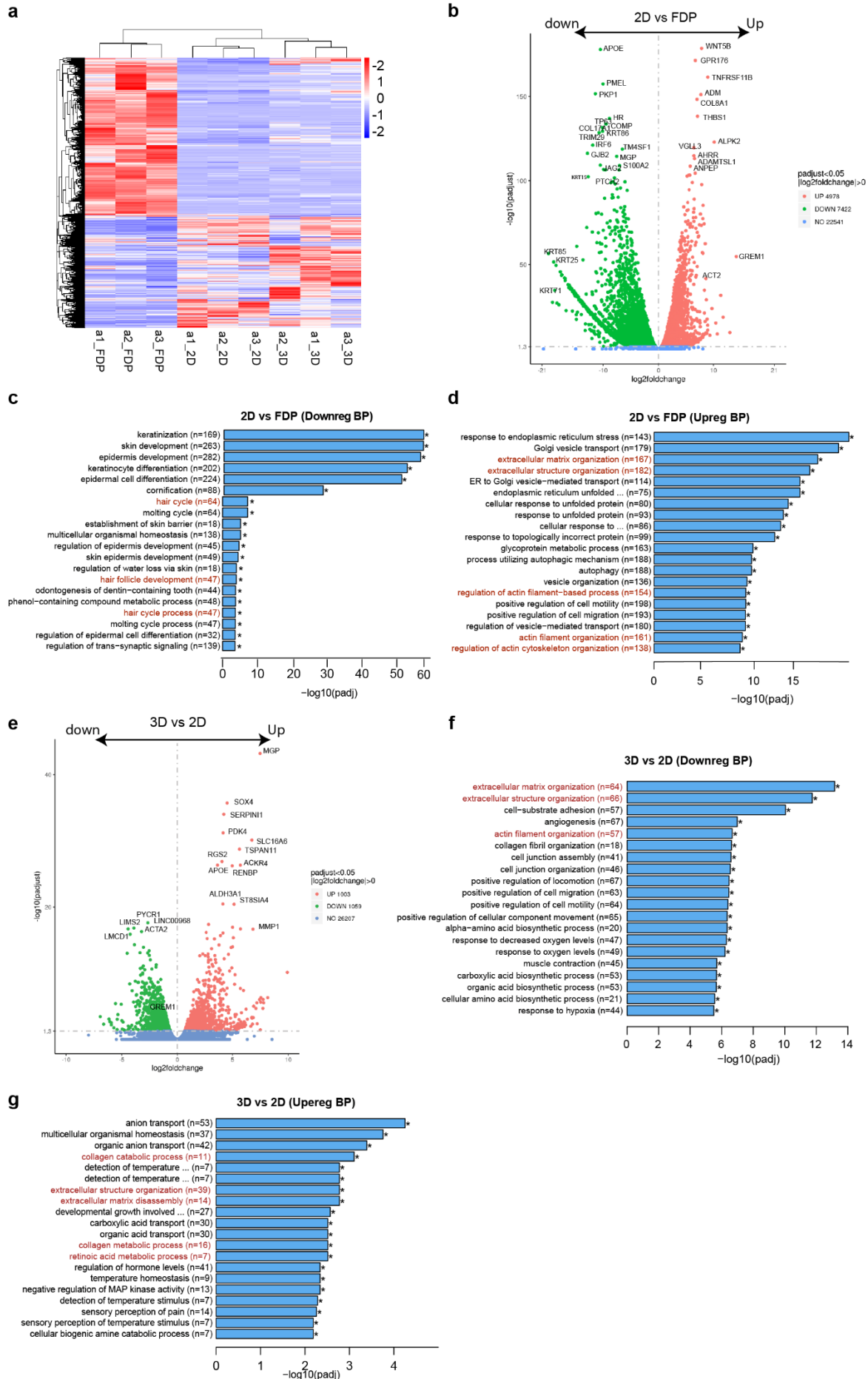


Figure 4.4. Comparative analyses of DP cells transcriptional signatures in native (FDP), 2D culture and 3D spheroid conditions. **a)** Heatmap of the total DEGs in FDP vs. 2D vs 3D. Z-score column color intensities indicate higher (red) to lower (blue) expression; **b)** Volcano Plot of the total DEGs ($\text{abs}(\log_2\text{FC}) \geq 1$, $p \leq 0.05$) found in 2D vs. FDP samples; **c)** Top 20 downregulated biological processes (BP) found in the Gene Ontology (GO) analysis; **d)** Top 20 upregulated biological processes (BP) found in the Gene Ontology (GO) analysis; **e)** Volcano Plot of the total DEGs ($\text{abs}(\log_2\text{FC}) \geq 1$, $p \leq 0.05$) found in 3D spheroid vs 2D culture samples. **f)** Top 20 upregulated BP found in the GO analysis of 3D vs. 2D cultured DP cells; **g)** Top 20 downregulated BP found in the GO analysis of 3D vs. 2D cultured DP cells.

Lastly, we examined which biological processes are modulated when DP cells transit from bidimensional culturing to 3D spheroid format, considering that 3D culturing has been proposed to mimic tissue architecture (Schmeichel & Bissell, 2003) and to partially restore the DP gene signature (Abaci et al., 2018; C. A. Higgins et al., 2013). By intersecting the two datasets, we found 2063 DEGs, 1003 upregulated and 1059 downregulated in 3D vs. 2D samples (**Fig. 4.4e**). GO enrichment analysis for all DEGs revealed ECM and actin filament organization as major biological processes (BP) downregulated in 3D (**Fig. 4.4e, f**). Thus, the assembly of 3D spheroids following 2D culturing seems to partially rescue transcriptional changes driven by 2D mechanical stress. As depicted in **Fig. 4.2b**, 3D samples express 774 (900-126) more genes, vs. only 16 (126-110) in 2D, in common to native samples. Noteworthy, *MGP* (matrix Gla protein) and *APOE* transcripts lost in 2D cultures, were recovered in 3D spheroids, in agreement with previous observations (C. A. Higgins et al., 2013). Additionally, 3D spheroids ameliorate 2D-driven mechanical stress by partially downregulating actin cytoskeleton activation (e.g., repression of *ACT2*/smooth muscle actin). Interestingly, the expression of key players in the *Wnt*/ β -*Catenin*/*LEF1* pathway, such as *TCF7* and *WNT11*, was restored in 3D spheroids. Still, although 3D DP spheroids exhibit a transcriptional signature which is closer to native DP than 2D DP cells, 2.7-fold more non-signature genes are triggered in comparison to 2D condition. These 3D-specific transcripts are associated with tissue remodeling and metabolic processes (**Fig. 4.2b and 4.4g**).

Conclusions

Although transcriptional signatures for mouse DP and DP cells have been described (Z. Deng et al., 2022; R. Sennett et al., 2015; Shim et al., 2022), a comprehensive comparative analysis between the transcriptional profiles of human adult anagenic DP and cultured DP cells was still missing. To further define the transcriptional programs associated with inductive DP signature and to identify key DP markers lost upon culture expansion, we isolated RNA from fresh human DP tissue and compared its transcriptional profiling with that in DP cells expanded in 2D conventional culture and subsequently used in the assembly of 3D spheroids. The advantages of our approach were to

compare the transcriptomes of DP cells from the same donor in native, 2D and 3D conditions, thus avoiding excessive interference from distinct genetic backgrounds, as well as optimizing the RNA isolation from native DPs and using low passage DP cultures (p3).

Our results showed that DP cells *in vitro* lose key signaling pathways controlling epithelial stem cell regulation and hair cycling. Although we observed that 3D culturing into spheroids seems to partially recover genes of the DP native signature that were lost in the 2D culturing, this procedure is still far from reestablishing the intact hair inductive capacity (**Fig.4.4a**). Indeed, we found that both *in vitro* culture systems share many molecular similarities, characterized by the repression of *NOTCH*, *WNT*, *BMP* and *SHH* signaling pathways and the activation of the *TGF β* signaling pathway. Altogether, the alterations in these key pathways controlling hair cycle, certainly account for the loss of DP hair inductive potential upon DP cell expansion *in vitro*.

One caveat of our approach is that, due to the manual microdissection of HF DPs, we cannot exclude the presence of melanocytes and/or hair matrix epithelial cells in the native DP samples. Indeed, epithelial keratin transcripts (such as *KRT85*, *KRT84* *KRT82*) and the *PMEL* melanocyte marker (Hellström et al., 2011) were detected in the FDP samples. Therefore, DEG analysis could be refined to exclude transcripts from contaminant cell types.

Overall, our work identified several transcription factors and fundamental pathways regulating human hair trichogenicity that could be targeted to prevent or revert hair loss. However, deeper analysis of our transcriptional data is still needed to identify upstream master regulators controlling hair induction, critical for the discovery of new therapeutic targets. Our next step will be to build up the regulatory network of differentially expressed transcription factors and their targets.

What drives the major transcriptional changes in DP cells *in vitro*? Mechanical stress has been shown to induce epigenetic modifications on DNA (CpG methylation) or histones (acetylation, methylation and phosphorylation) that alter chromatin structure and gene expression ((Shiu, Aires, Lin, & Vogel, 2018; Stowers & Chaudhuri, 2021)). Fibroblasts cultured *in vitro* exhibit alterations both in methylation profile of DNA cytosine residues and in lysine deacetylation profile of histones (Albregues et al., 2015). Thus, epigenetic alterations driven by the bidimensional mechanical stress most likely trigger the loss of DP transcriptional signature genes and the DP cell identity and function. Accordingly, epigenetic modulation of key transcription factors (TFs) controlling DP cells' trichogenicity could work to reprogram the DP signature *in vitro*. Although the assembly of 2D expanded DP cells into 3D spheroids was found to positively impact on the transcriptome signature, DP cell multipotency appears irreversibly lost. Therefore, only by reprogramming their "native" epigenetic state and transcriptional activity, expanded DP cells will get back their hair inductive potential. Interestingly, upregulation of the BMP pathway and restored hair inductive capacity was observed upon histone acetylation reinstatement in mouse SKPs (Guo et al., 2019).

Also recently, inhibition of class I HDACs was proposed to preserve HF inductivity in postnatal dermal cells (M. Park, Jang, Chung, Kwon, & Jo, 2021).

V

Targeting TGF β to improve human dermal papilla therapeutic potential

Chapter 5

Targeting TGF β to improve human dermal papilla therapeutic potential

Here, we set out to investigate TGF β signaling activation in the human HF DP, as well as to validate the efficacy of a potent TGF β receptor I (T β RI) inhibitor, galunisertib (LY2157299), against TGF β repressive signaling during DP cell 2D culturing. We found that inhibition of 2D culture driven TGF β signaling improves DP cell molecular signatures by inhibiting pro-fibrotic and inflammatory gene expression and preserving DP trichogenicity. Thus, we disclose galunisertib as a critical pharmacological modulation during DP cell therapy for hair rejuvenation, and future in bioengineering approaches that depend on DP culture expansion.

Introduction

Typically, HF growth and regeneration involve a huge set of hormones and growth factors that act synergically on stem cell niches of the dermal compartment, the DP and the dermal sheath (DS). DP is at the basis of the HF and is surrounded by epithelial matrix cells. DPCs provide instructive signals required to activate epithelial progenitors and regulate the HF cycle, which consists in phases of growth (anagen), apoptotic-driven regression (catagen), and rest (telogen) (Cotsarelis, 2006). Importantly, AGA (also known as baldness) is the phenotypic outcome of dysfunctional dermal trichogenic signaling caused by depletion of DPCs. The balding process is associated with shorter anagen, longer telogen phases, as well as with a gradual reduction in the HF size that positively correlates with DPC number (Pantelireis & Higgins, 2018).

Up to date, pharmacological treatments to AGA are still limited to the FDA approved Minoxidil (potassium channel agonist) and Finasteride (α 5-reductase inhibitor) (Adil & Godwin, 2017). Although these drugs prove effective for some patients, they entail significant side effects such as erectile dysfunction and skin itching (Madaan, Verma, Singh, & Jaggi, 2018; Talavera-Adame, Newman, & Newman, 2017). Another mainstream treatment, hair transplant, is limited by the number of hair grafts in the donor region. Consequently, there is a high demand for more effective therapeutic solutions targeting AGA, ideally improving DP fitness for DP cell-based therapy.

DPCs represent an abundant source of autologous cells of mesenchymal lineage with differentiation capacity to exploit for cell-based therapy. However, their expansion *in vitro* severely compromises

their trichogenic function due to changes in the expression of determinant genes of the DP signature and function, such as the transcription factor *LEF1* (Abaci et al., 2018). Pharmacological activation of WNT signaling and inhibition of JAK/STAT signaling have been shown to improve the inductive properties of cultured DPCs (Harel et al., 2015; Yoshida et al., 2019). The establishment of 3D sphere cultures and the use of biomaterial supportive scaffolds have been also reported to partially restore DPCs signature (Claire A. Higgins, Chen, Cerise, Jahoda, & Christiano, 2013; C. A. Higgins, Richardson, Ferdinando, Westgate, & Jahoda, 2010). Still, pharmacological inhibition of molecular pathways activated earlier on by 2D cell culture could prove advantageous.

Besides its reported role on hair cycle regulation, TGF β is a major signaling pathway involved in several biological processes, namely regeneration and wound healing (Akhurst & Hata, 2012). Given that *i*) DP cells are fibroblast progenitors with ability to differentiate into osteogenic lineage (Logan, Camman, Williams, & Higgins, 2018), and that *ii*) stiffness of the extracellular matrix regulates fibroblast function and differentiation (Engler, Sen, Sweeney, & Discher, 2006), we reasoned that TGF β activation during 2D cell culture may trigger DP cell differentiation into non-trichogenic lineages.

Mesenchymal cells, such as fibroblasts, have the ability to sense biomechanical inputs from the extracellular environment. Mechanical stress, for instance induced by substrate stiffness during 2D culturing, can trigger fibroblast differentiation to a profibrotic myofibroblast lineage (X. Huang et al., 2012). Contractile myofibroblasts secrete mediators of inflammatory and fibrotic response, such as the TGF β 1 cytokine and collagen type I, which further aggravate the stress phenotype by a positive feedback loop (Darby, Laverdet, Bonté, & Desmoulière, 2014). The use of biomaterial scaffolds to counteract 2D mechanical stress or the use of drugs targeting profibrotic pathways have been explored (Meli et al., 2020; W. Zhang, Huang, & Xu, 2020). Considering the reported role of TGF β in hair cycle regulation, we reasoned whether TGF β inhibition during 2D cell culture may prevent DP cell differentiation into non-trichogenic lineages, thus far undetermined.

Here, we set out to investigate the effect of pharmacological inhibition of TGF β signaling in human DPC 2D cultures. We used galunisertib (LY2157299), which is a specific TGF β receptor type I tyrosine kinase inhibitor. We found that galunisertib treatment restores transcriptional signatures associated with improved trichogenic potencial in DPC 2D cultures, namely downregulation of profibrotic signaling pathways and upregulation of cell adhesion molecules and antioxidant enzymes associated with hair induction. Overall, we disclose galunisertib-treated dermal papilla cells as a promising cell therapy for hair regeneration and rejuvenation.

Results

Galunisertib rescues TGF β signaling activation in 2D DP cell cultures

TGF β is a major signaling pathway involved in several biological processes, namely regeneration and wound healing (Akhurst & Hata, 2012). Given that i) DP cells are fibroblast progenitors with ability to differentiate into osteogenic lineage (Logan, Camman, Williams, & Higgins, 2018), and that ii) stiffness of the extracellular matrix regulates fibroblast function and differentiation (Engler, Sen, Sweeney, & Discher, 2006), we reasoned that TGF β activation during 2D cell culture may trigger DP cell differentiation into non-trichogenic lineages. To test this hypothesis, we started by evaluating if TGF β signaling is activated in cultured DP cells at early p0 and p2 passages. TGF β signaling is composed of TGF β 1, TGF β 2, and TGF β 3 ligands that, when bond to TGF β type II receptor (T β RII), phosphorylate and activate the TGF β type I receptor (T β RI/ALK-5) (Vander Ark, Cao, & Li, 2018). We indeed found increased in transcription levels of TGF β downstream target (SERPINE1/PAI-1) in 2D cultured DP cells (**Fig. 5.1a**).

Activated T β RI phosphorylates SMAD2 and SMAD3, which then bind to SMAD4. The phospho-SMAD2/3-SMAD4 complex translocate into the nucleus to regulate canonical gene transcription (Vander Ark et al., 2018). Besides, noncanonical TGF β signaling (SMAD-independent) can be activated by the recruitment of other proteins to the ligand–receptor complex (Mu, Gudey, & Landström, 2012). In agreement with TGF β signaling activation and DP cell differentiation towards a profibrotic lineage, the direct gene target plasminogen activator inhibitor-1 (*SERPINE1/PAI-1*), and the indirect target actin α 2 smooth muscle (*ACTA2/ α SMA*), were upregulated in DP cell culture over time (**Fig. 5.1b, c**).

To investigate if galunisertib, a potent small molecule inhibitor of TGF β receptor I (T β RI), can suppress TGF β signaling activation in DP cell 2D cultures, we treated cells accordingly to the experimental setup shown in **Fig. 5.1d**, using a 10 μ M optimal concentration as determined by efficient reduction of SMAD2 canonical target phosphorylated protein levels (**Fig. 5.2a**). We found that galunisertib was able to inhibit SMAD2 phosphorylation along cell passaging, and even more efficiently than upon aggregation of p2 DP cells into 3D spheroids (**Fig. 5.1e**). Moreover, galunisertib prevented the upregulation of *SERPINE1* profibrotic player during 2D cell culturing (**Fig. 5.1g**). Interestingly, whereas aggregation of untreated p2 DP cells into 3D spheroids was unable to rescue *TGFB1* expression driven by early 2D cell passaging, it efficiently worked in *SERPINE1* repression (**Fig. 5.1f, g**). Overall, our data showed that mechanical stiffness introduced by 2D culturing, activates TGF β signaling in DP cells which can be rescued by galunisertib under a working dose that does not impact DP cell proliferation nor viability (**Fig. 5.2b, c**).

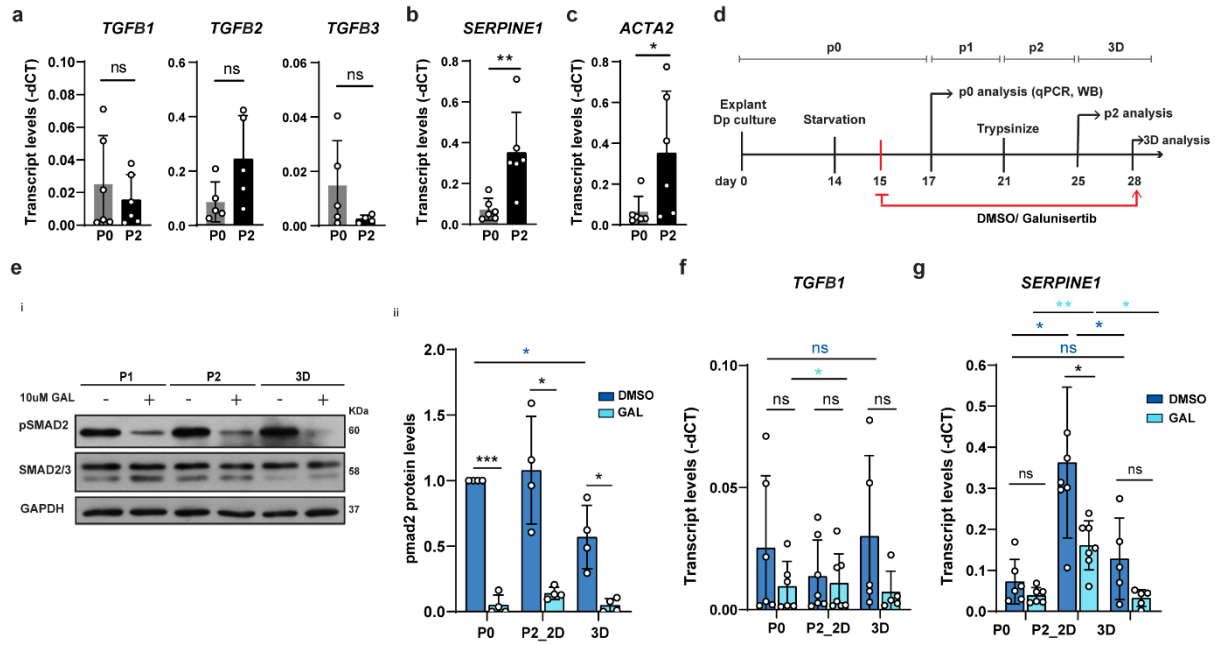


Figure 5.1. Galunisertib rescues TGFβ activation in human dermal papilla cells (DPCs). **a)** RT-qPCR analysis for *TGFB1*, *TGFB2*, *TGFB3* in early passage DPCs cultures (P0 and P2). *GAPDH* and *UBQ* were used as reference genes; **b-c)** Transcript levels of TGFβ signaling downstream players *SERPINE1* and *ACTA2*. **d)** Experimental step up for vehicle (DMSO) and drug (galunisertib) treatments of DPCs' cultures established from dissected DPs from the same patient. 1 day after starvation, DMSO or galunisertib were added to p0 cultures. 2 days after treatment, cells were collected for gene expression analysis (RT-qPCR and Western blot/WB) and subsequent cell passaging (p1 and p2). Experimental procedure was repeated at p2. **e)** Western blot and quantitative analysis of SMAD2/3 and phospho-SMAD2 protein levels as readout for canonical TGFβ signaling activation in DPCs untreated and treated with galunisertib at p1, p2 and upon 3D spheroid assembly from p2 cells (n=4). *GAPDH* was used as loading control and for normalization. **f-g)** RT-qPCR analysis of *TGFB1* and *SERPINE1* target transcript levels in DPCs untreated and treated with galunisertib at p1, p2 and upon 3D spheroid assembly from p2 cells. Values are mean ± SD of three biological samples representing different donors. ns not significant, *p < 0.05, **p < 0.01, ***p < 0.001, using paired t-test (a-c and g) or Wilcoxon (e and f).

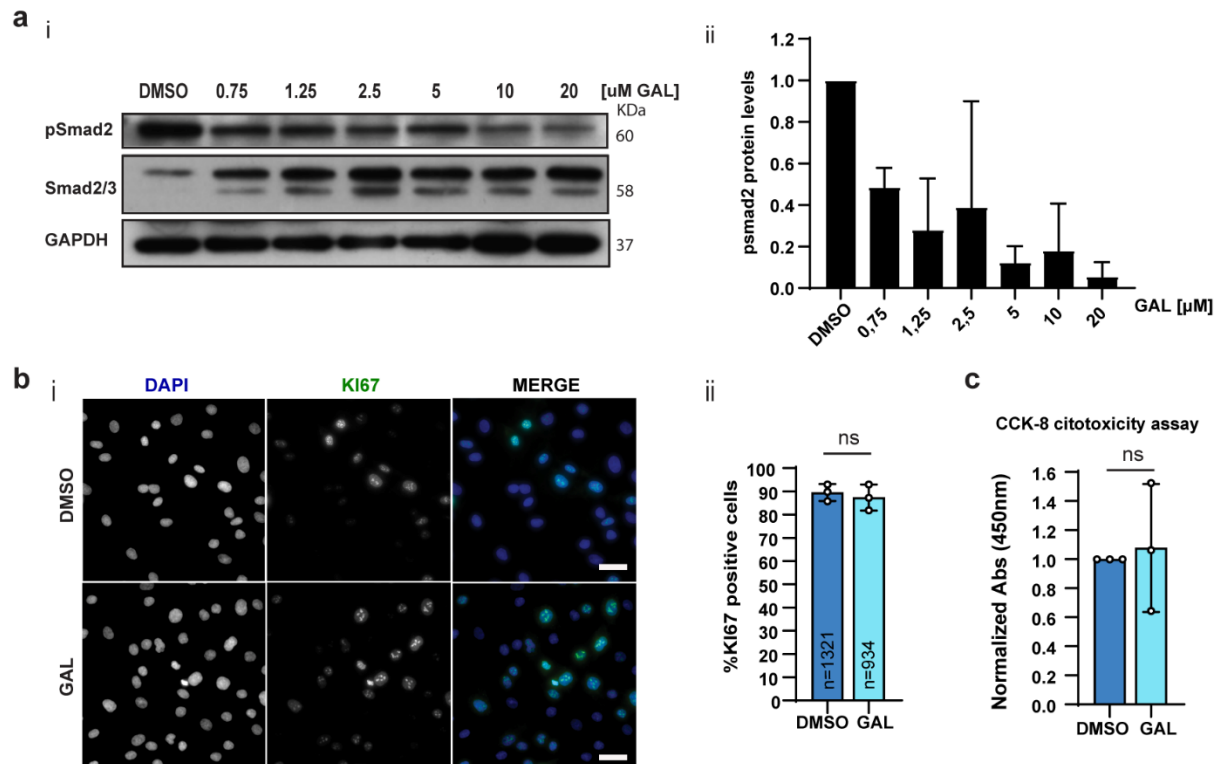


Figure 5.2. Titration of Galunisertib optimal working concentration *in vitro*. **a)** Western blot and quantitative analysis of SMAD2/3 and pSMAD2 protein levels as readout for canonical TGF β signaling inhibition in DPCs treated with galunisertib at p2; GAPDH was used as loading control and for levels normalization. **b)** Immunofluorescence analysis of Ki67 proliferation marker during *in vitro* DPCs culture until p2. Scale bar, 25 μ m. **c)** Cell Counting Kit-8 colorimetric assay for cytotoxicity assessment in DPCs untreated (DMSO) and treated (galunisertib) until p2. Ns not significant by χ^2 (b, c) statistical tests.

TGF β signaling inhibition counteracts DP cell transition into profibrotic lineage during 2D culturing

We observed that DP cells cultured *in vitro* acquire a profibrotic gene signature characterized by *SERPINE1* and *ACTA2*/ α SMA upregulation. We asked if galunisertib was able to prevent the accumulation of contractile stress fibers (α SMA-positive) in 2D cultured DP cells. Indeed, we found that the percentage of α SMA-positive DP cells was significantly reduced at both p0 and p2 cell passages under galunisertib treatment (**Fig. 5.3a, b**). The percentage of α SMA-positive DP cells at p2 under drug treatment was similar to that found at p0 untreated control (**Fig. 5.3b**). α SMA protein levels were also quantified by western blot analysis, showing that galunisertib prevents stress fiber accrual along cell passaging as efficiently as 3D spheroid assembly (**Fig. 5.3c, d**). To determine if DP cell biomechanical improvement by galunisertib preserves the Wnt/ β -catenin inductive signaling, we measured the *LEF1* transcription factor and β -catenin staining. *LEF1* transcript levels were not improve in DP cell cultures treated with galunisertib (**Fig. 5.3e**). Additionally, B-

CATENIN protein staining suggests that galunisertib induce a nuclear shift in β -catenin staining (Fig. 5.3f)

Overall, our results show that Galunisertib counteracts the accrual of stress fibers in DP cells *in vitro*, thereby ameliorating mechano-transduction signaling towards DP hair inductive signature.

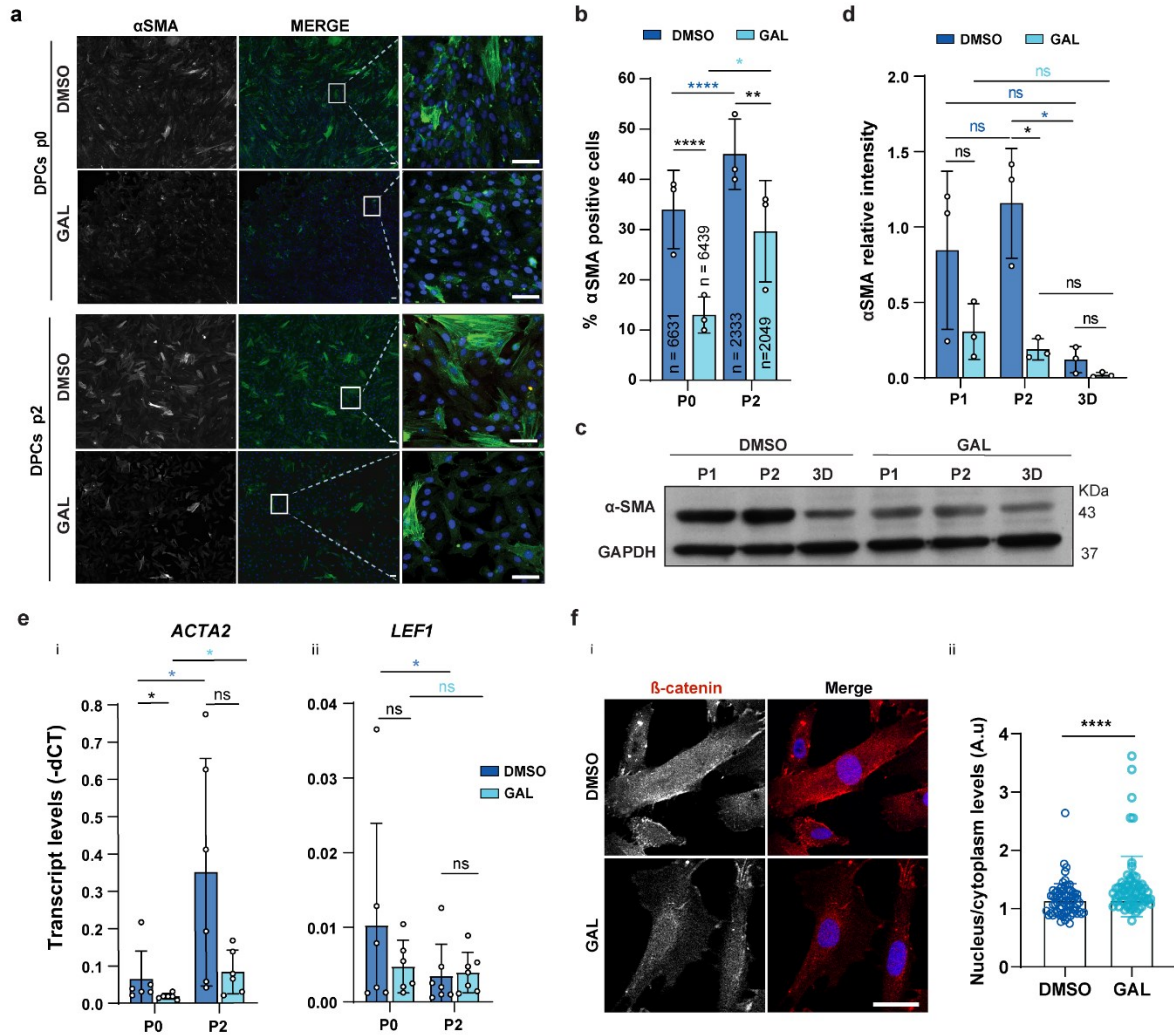


Figure 5.3. Galunisertib counteracts DP cell transition into profibrotic lineage during 2D culture. **a)** Representative images of α SMA immunostaining (green) in early passage (p0 and p2) DPCs' cultures. **b)** Percentage of α SMA-positive DP cells at p0 and p2 cultures treated with DMSO or galunisertib (Gal). **c-d)** Western blot analysis and quantification of total α SMA protein levels in DMSO- or Gal-treated DPCs at p1, p2 and upon p2 3D spheroid assembly. GAPDH was used as loading control. **e)** RT-qPCR analysis of *ACTA2* and *LEF1* transcript levels in DMSO- and Gal-treated DPCs at p1, p2 and upon p2 3D spheroid assembly. **f)** β -catenin protein intensity levels by immunofluorescence analysis of $n \geq 60$ human DP p2 cells treated with DMSO or galunisertib. Values are mean $\pm$ SD of biological samples from three independent donors. Ns not significant, $*p < 0.05$, $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ by χ^2 (b) or paired t-test (d, e, f) statistical tests.

Galunisertib improves the inductive DP cell signature in 2D cultures

To portray the transcriptional programs modulated by specific inhibition of TGF β signalling in DP cells, we performed RNA-sequencing profiling of early passage (p2) galunisertib-treated (2D_GL) vs. untreated (2D_CT) DP from 3 donors. Principal component analysis (PCA) indicated 66.92% of variation covered by PC1 and PC2, with PC1 correlating with variation between donors (biological replicates) and PC2 correlating highest with GL treatment (**Fig. 5.4a**). A total of 327 differentially expressed genes (DEGs), 142 downregulated and 185 upregulated, were found in 2D_GL vs. 2D_CT samples ($\text{abs}(\log_2\text{FC}) \geq 1$, $p \leq 0.05$) (**Fig. 5.4b, c**). PCA analysis focusing on the 327 DEGs revealed PC1 to account for 78.72% of the variation and to correlate with GL treatment (**Fig. 5.5a**), which supports galunisertib as main determinant of large transcriptome differences between samples.

Amongst the most downregulated genes, we found the *COMP* (cartilage oligomeric matrix protein), *POSTN* (periostin), *CDH2* (cadherin-2) and *FOXS1* (Forkhead Box S1), all previously associated with fibrosis (**Fig. 5.4b**) (C.-C. Deng et al., 2021; Noizet et al., 2016; Wan et al., 2021). Gene ontology (GO) analysis of the DEGs based on their biological processes revealed downregulation of the ‘extracellular matrix organization’ and ‘extracellular structure organization’ GO terms, which are typically upregulated under profibrotic conditions (**Fig. 5.4d**). Indeed, we observed a downregulation for ITGB5 and ITGAV integrins (**Fig. 5.5b**), cell surface mechanosensors whose upregulation is associated with matrix contraction and osteogenic differentiation (Olivares-Navarrete et al., 2011). Additionally, core matrisome components were also downregulated by TGF β inhibition, including several collagens (*COL7A1*, *COL8A2*, *COL5A1*) and matrix metalloproteinase-2 (*MMP2*), suggesting decreased collagen deposition and remodeling, normally upregulated in fibrotic programs (**Fig. 5.5b**). In agreement, downregulation of genes related to ‘connective tissue development’, ‘ossification’, ‘muscle tissue development’ and ‘cartilage development’ further supported the inhibitory effect of galunisertib on the transition of DP cells to profibrotic myofibroblast or osteoblast lineages. On the other hand, GO analysis revealed upregulation of the ‘cell-substrate adhesion’, ‘regulation of peptide secretion’ and ‘regulation of protein kinase activity’ GOs, suggesting that galunisertib counteracts changes in migration/invasion and ECM remodelling that are associated with fibrotic transition (Wynn, 2008) (**Fig. 5.4d**).

To gain mechanistic insight into biological pathways associated with the DEGs, we did an enrichment analysis for KEGG pathways (**Fig. 5.4e**, **Fig. 5.5c** which highlights the DEGs found within significantly enriched KEGG pathways). Interestingly, enrichment analysis (ClusterProfiler (Wu et al., 2021)) indicated the ‘PI3K-AKT signalling among the top downregulated KEGG pathways ($\text{padj} \leq 0.05$, **Fig. 5.4e**) and gene set enrichment analysis (GSEA) further revealed extensive pathway modulation with the majority of genes being either upregulated or downregulated (**Fig. 5.5d**). PI3K/AKT pathway is a downstream target of the non-canonical

SMAD-independent TGFβ signaling, thus supporting the efficacy of galunisertib treatment in the DPC 2D cultures, further upheld by the fact that TGFβ signaling pathway was also downregulated (**Fig. 5.4f** for GSEA). The *MAPK* pathway, which is another target of the non-canonical TGFβ signaling pathway, was also downregulated (**Fig. 5.4e**). GSEA analysis for the *JAK/STAT* signaling pathway whose inhibition was previously reported as beneficial for the maintenance of DPC signature during 2D culturing (Harel et al., 2015), revealed galunisertib treatment to significantly downregulate this pathway (**Fig. 5.4fii**). Consistently, the ‘cytokine-cytokine receptor interaction’ inflammation-related pathway was significantly downregulated in GSEA analysis (**Fig. 5.5diii**).

Upregulated KEGG pathways comprised ‘Cell adhesion molecules (CAMs)’ ‘*RAP1* signaling pathway’ and ‘Axon guidance’ (**Fig. 5.4e**). Cell adhesion molecules (CAM) have for long been associated with hair morphogenesis and DP aggregative behavior (Müller-Röver et al., 1999; A. A. Panteleyev, Paus, Ahmad, Sundberg, & Christiano, 1998). For example, neural cell adhesion molecules NCAM1/2 and Myelin Protein Zero (MPZ) were correlated with hair induction potential (Combates, Chuong, Stenn, & Prouty, 1997; Leiva et al., 2014). GSEA analysis further confirmed significant upregulation of the ‘Cell adhesion molecules’ pathway (**Fig. 5.4fiii**). The Rap1 pathway comprises several regulatory proteins involved in diverse cellular processes, such as cell differentiation and integrin-mediated cell adhesion (Bos, de Rooij, & Reedquist, 2001). Within this pathway, upregulation of *ANGPT2* suggests that galunisertib might induce angiogenesis (Endo et al., 2018). The KEGG pathway ‘axon guidance’ includes genes reported to be involved in HF formation (Sennett et al., 2015). Semaphorin (SEMA3B/SEMA6D), Neurotrophin (NDNF), Netrin (NTN4), Ephrin (EFNA5) were also identified as dermal condensate signature genes (Sennett et al., 2015) (**Fig. 5.5c**). Additionally, galunisertib seems to impact DPCs’ metabolism, as observed by upregulation of the ‘Histidine metabolism’ KEGG pathway (**Fig. 3E**). Aldehyde dehydrogenase 3 family members, *ALDH3A1* and *ALDH3A2*, were reported to play critical role in cell stemness and homeostasis by maintaining redox balance (Duan, Cai, Guo, Bian, & Yu, 2016). Moreover, ‘pathways regulating pluripotency of stem cells’ were enriched in KEGG functional enrichment analysis, which suggests that galunisertib might improve DPCs’ stemness. In addition, the WNT-signalling pathway is also highly modulated, namely *WNT2B* shared with other relevant pathways and upregulated (**Fig. 5.5d**), and a large proportion of genes being up- or down-regulated (**Fig. 5.5dii**, GSEA).

Overall, the data suggest that galunisertib acts by preventing profibrotic and inflammatory pathways driven by 2D culturing, while preserving DPC function.

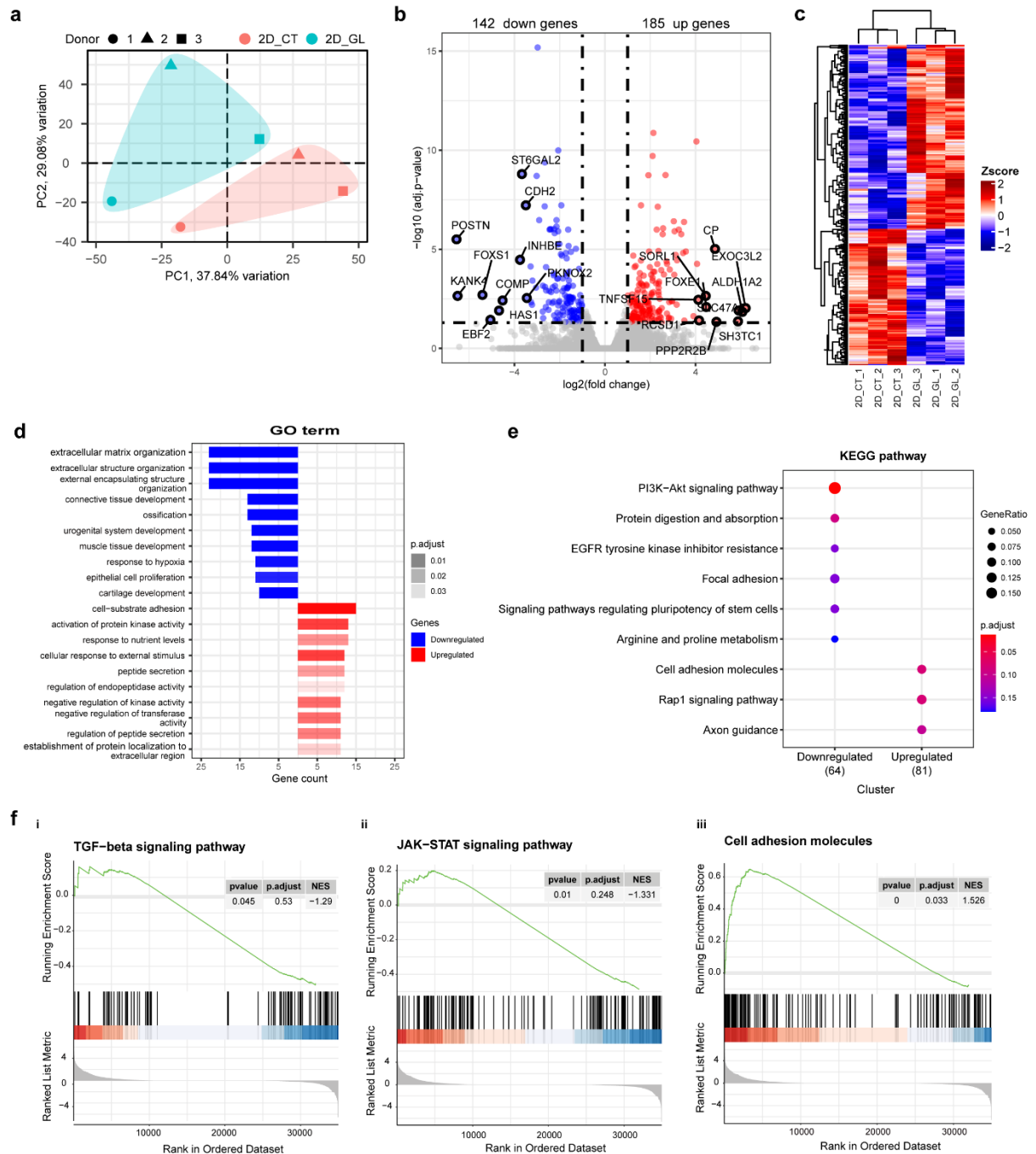


Figure 5.4. Galunisertib counteracts profibrotic inflammatory signaling pathways driven by DPC 2D culturing. **a**) Bidimensional principal component analysis (PCA) of the variance between RNA-seq samples. **b**) Volcano Plot of the total DEGs ($|\text{abs}(\log_2\text{FC}) \geq 1$, $p \leq 0.05$) found in 2D_GL vs. 2D_CT comparative analysis. Top 10 upregulated (red) and downregulated (blue) genes are indicated. **c**) Heatmap of the total DEGs in 2d_GL vs. 2D_CT. Z-score column color intensities indicate higher (red) to lower (blue) expression. **d**) Top 10 downregulated (blue bars) and upregulated (red bars) biological processes found in the Gene Ontology (GO) analysis. **e**) Top KEGG pathways for the DEGs ($p_{\text{adj}} \leq 0.20$). **f**) Gene Set Enrichment Analysis (GSEA) for the KEGG pathways 'TGFβ signaling' (i), 'JAK/STAT signaling' (ii) and 'Cell adhesion molecules' (iii). NES, negative normalized enrichment score.

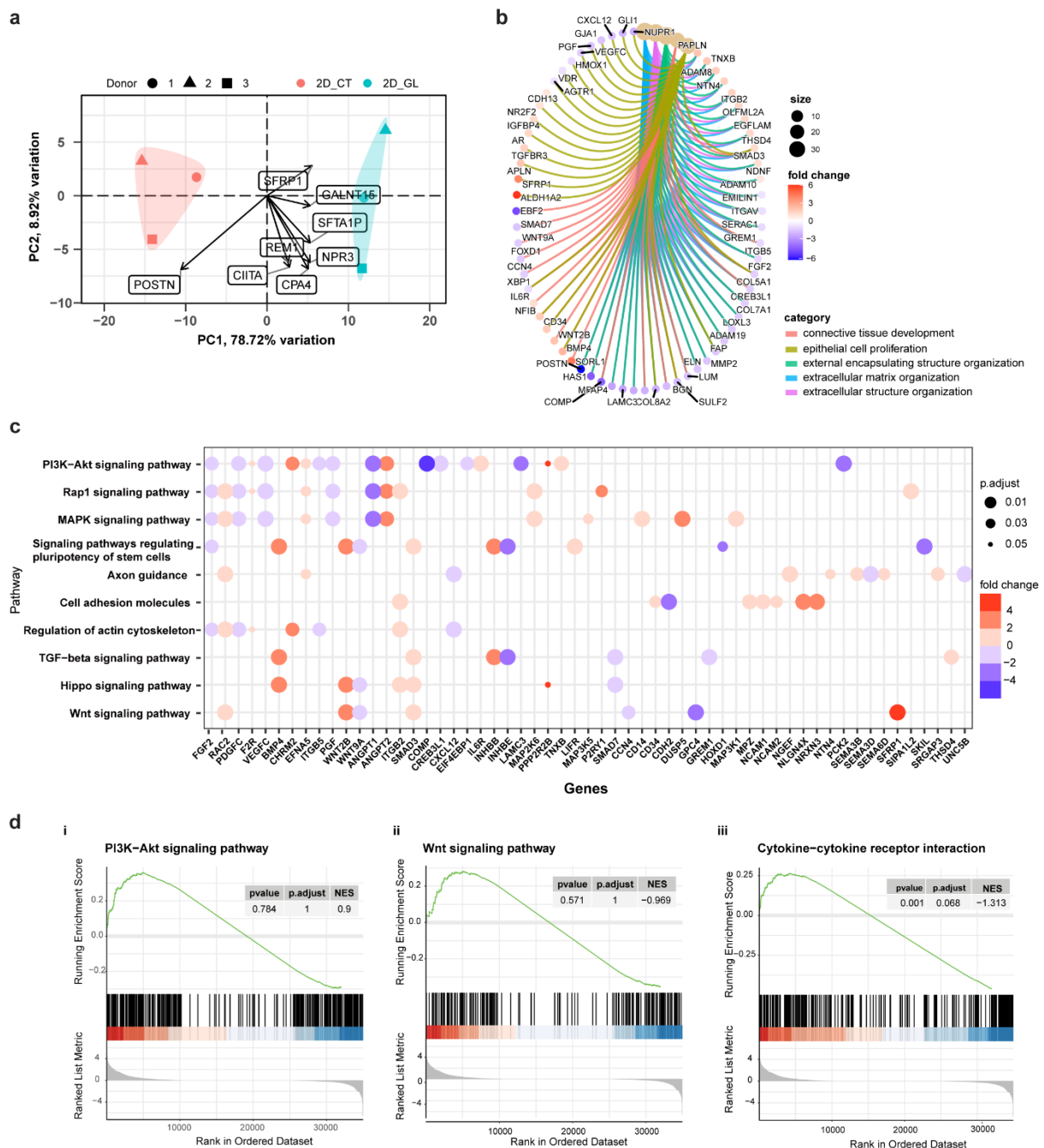


Figure 5.5. **a)** Bidimensional principal component analysis (PCA) of the total DEGs in 2D-GL vs. 2D_CT. **b)** Network plot of enriched GO terms for BP:biological processes and key DEGs. **c)** DEGs (color intensities are fold change values) associated with specific KEGG pathways found altered in 2D_GL vs. 2D_CT. **d)** Gene Set Enrichment Analysis (GSEA) for the KEGG pathways ‘PI3K/AKT signaling’ (i), ‘Wnt signaling’ (ii), and ‘Cytokine-cytokine receptor interaction’ (iii). NES, negative normalized enrichment score.

Discussion

Given that TGF β is a multifunctional pathway involved in the hair follicle cycle, skin healing process and regeneration, we carefully examined the impact of galunisertib on DPCs.

During the skin healing process, dermal and hair follicle fibroblasts are known to transit into a myofibroblastic repair state, where they activated contractile function and upregulate the production of matrisome proteins. Although it is an essential mechanism during *in vivo* tissue wound healing, it can trigger a non-regenerative tissue repair. Hypertrophic scars that replace normal skin tissue during wound healing are characterized by a thicker dermis due to excessive collagens production, increased mechanical stiffness and it's unable to regenerate hair follicle (Plikus et al., 2021).

TGF- β 1 is a master regulator of the healing process, regulating myofibroblast differentiation and maturation via canonical TGF β 1/SMAD2/3 signaling (Phan et al., 2020; Plikus et al., 2021). Here we proposed that biomechanical properties (e.g., substrate rigidity) underlying *in vitro* DP cell culture may trigger their activation into a more fibrotic lineage, which deeply impact DPCs molecular regulation, phenotype, and function. We also proposed that this fibrogenic state may hamper functional human HF regeneration as it fails to preserve DP cell trichogenicity during *in vitro* expansion. Consistently, we have shown that TGF β is responsible for inducing the expression of actin-associated proteins (including α SMA) through a non-SMAD dependent signaling, which accounts for the prominent contractile behavior of DPCs *in vitro* (Malmstrom et al., 2004). Here, we have shown that galunisertib modulates both canonical and non-canonical TGF β signaling. We proved that canonical TGF β signaling is blocked as observed by the remarkable decrease in psmad2 phosphorylation, blocking its translocation to the nucleus where it regulates the transcription of downstream players. Additionally, we demonstrated that galunisertib can modulate the non-canonical signaling by regulating major players of the PI3K/AKT, Rho-like GTPase and MAPKs signaling pathway. RNA-seq data supports that galunisertib shifts DPCs into a regenerative healing state by showing the recovery of biomechanical properties and collagen synthesis. Overall, our work provides evidence that galunisertib acts directly on DPCs potentiating their trichogenicity and reducing stress.

To the best of our knowledge, this is the first study to provide evidence of a small molecule inhibitor targeting TGF β signaling alone to revert fibrotic dedifferentiation of human DPC *in vitro*. The ability to revert DPCs back to a trichogenic-primed state in opposition to a differentiated and osteogenic-committed lineage can be explored for future cell and tissue engineering solutions for hair loss.

A cell therapy solution may comprise the following steps: 1) obtaining occipital androgen resistant cells from hair follicle tissue; 2) culturing the androgen resistant cells in 2D cell culture under galunisertib treatment during the expansion process; 3) implanting the expanded DP trichogenic population near to miniaturised hair follicles in the balding scalp. The application of

DP trichogenic cells is expected to rejuvenate miniaturised hair follicles and delaying alopecia progression.

A tissue engineering solution may comprise the following steps: 1) obtaining occipital androgen resistant cells from hair follicle tissue; 2) culturing the androgen resistant cells in 2D cell culture under galunisertib treatment during the expansion process population; 3) Engineering hair follicle germs containing DP trichogenic cells; 4) implanting the hair follicle germs into the balding scalp.

VI

Pharmacological inhibition of TGF β signaling improves human hair follicle growth cycle

Text extracts of the following patent were included in this chapter:

Castro & Logarinho (2022). Galunisertib for use in the treatment of androgenic alopecia; INPI n°20221000000803, filed on February 16th, 2022. Patent pending.

Chapter 6

Pharmacological inhibition of TGF β signaling improves human hair follicle growth cycle

The sustained binding of dihydrotestosterone (DHT) to androgen receptors (AR) in the dermal papilla (DP) of balding scalp hair follicles (HF) is the leading cause of HF miniaturization in androgenic alopecia (AGA) patients. DHT-AR transcriptionally activates the TGF β signaling pathway that is a key pathological mediator in AGA. Here, we set out to investigate TGF β signaling activation in human non-balding HF organ cultures (HFOCs), as well as to validate the efficacy of a potent TGF β receptor I (T β RI) inhibitor, galunisertib (LY2157299), against TGF β repressive signaling during *ex vivo* HF growth cycle. We found TGF β signaling activation in the DP of non-balding scalp HFOCs, which inhibition by galunisertib translated into improved proliferation and β -catenin levels in the hair bulb epithelial compartment and delayed catagen onset. Thus, we disclose galunisertib as a potential pharmacological intervention to treat AGA.

Introduction

Typically, HF growth and regeneration involve a huge set of hormones and growth factors that act synergically on stem cell niches of the dermal compartment, the DP and the dermal sheath (DS). DP is at the basis of the HF and is surrounded by epithelial matrix cells. DP cells (DPCs) provide instructive signals required to activate epithelial progenitors and regulate the HF cycle, which consists in phases of growth (anagen), apoptotic-driven regression (catagen), and rest (telogen) (Cotsarelis, 2006). Importantly, hair loss (also known as alopecia or baldness) is the phenotypic outcome of dysfunctional dermal trichogenic signaling caused by depletion of DPCs. The balding process is associated with shorter anagen, longer telogen phases, as well as with a gradual reduction in the HF size that positively correlates with DPC number (Pantelireis & Higgins, 2018).

AGA is characterized by HF miniaturization (reduction in diameter, length and pigmentation) during repeated hair cycles with shortened anagen (Otberg, Finner, & Shapiro, 2007). Androgens, in particular DHT generated by α 5-reductase metabolization of testosterone, act via the DP in inducing hair loss (Martinez-Jacobo, Villarreal-Villarreal, Ortiz-López, Ocampo-Candiani, &

Rojas-Martínez, 2018). The higher levels of type II $\alpha 5$ -reductase and of AR in DPCs from balding vs. non-balding scalp HFs, drive a gene expression program that negatively impacts hair growth (Itami, Sonoda, Kurata, & Takayasu, 1994; Winiarska et al., 2006). The DHT-AR complex alters the expression of key hair regulatory pathways, for example inhibiting the Wnt/ β -catenin signaling pathway.

Androgens induce TGF β 1, which has been suggested as key player in AGA pathogenesis (Inui & Itami, 2011). Injection of TGF β 1 into the back skin of mice induces premature catagen and keratinocyte apoptosis, while *tgfb1* deficient mice exhibit delayed HF regression (Foitzik et al., 2000). DHT stimulates the expression of *TGF β 2*, interleukin 1 alpha (*IL1A*) and 6 (*IL6*), that act paracrinally on matrix epithelial cells, inhibiting their proliferation and hair growth (Fu et al., 2021; Hibino & Nishiyama, 2004; Kwack, Ahn, Kim, Kim, & Sung, 2012). *TGF β 2* also induces pro-apoptotic proteins in human HF DP, such as tumor necrosis factor alpha (TNF α) and caspases, leading to premature catagen and progressive miniaturization (Soma, Tsuji, & Hibino, 2002; Tsuji et al., 2003). Besides, TGF β activation at the mesenchymal compartment leads to epithelial cell death and reduced HFSC compartment (Mesa et al., 2015).

Here, we set out to investigate TGF β signaling activation in the DP of human HFOCs established from non-balding regions of AGA patients, as well as the effect of pharmacological inhibition of TGF β signaling in the *ex vivo* HF growth. We found galunisertib (LY2157299), a specific TGF β receptor type I tyrosine kinase inhibitor, to inhibit TGF β signaling in the DP, translating into improved cell proliferation and β -catenin expression in the hair bulb epithelial compartment, and importantly, delaying catagen onset. Overall, we disclose galunisertib as a promising pharmacological therapy for hair loss treatment.

Results

TGF β signaling activation in human HF organ cultures from AGA patients' non-balding scalp

Previous studies have suggested that DHT action in DP cells expressing androgen receptors, i.e., from balding region HFs, drives TGF β signaling activation and accelerates HF regression, causing miniaturization in AGA (Inui, Fukuzato, Nakajima, Yoshikawa, & Itami, 2002). We asked whether TGF β signaling is activated in the DP of human HFs collected from non-balding scalp regions and cultured *ex vivo* (HF organ cultures - HFOCs). Despite being supposedly non-responsive to DHT, comparative analysis was nevertheless done between HFOCs untreated and treated with DHT. TGF β signaling is composed of TGF β 1, TGF β 2, and TGF β 3 that, when bond to TGF β type II receptor (T β RII), phosphorylate and activate the TGF β type I receptor (T β RI/ALK-5) (Vander Ark, Cao, & Li, 2018). We thus started by quantifying the transcript levels of *TGFB1*, *TGFB2*, *TGFB3* in DP isolated from human HFs cultured *ex vivo* for 48h (**Fig. 6.1a**). qRT-PCR analysis revealed

that only *TGFB2* is slightly increased in DHT-treated vs. untreated HFOCs, with *TGFB1* and *TGFB3* levels remaining unchanged (**Fig. 6.1b**).

Activated T β RI phosphorylates SMAD2 and SMAD3, which then bind to SMAD4. The phospho-SMAD2/3-SMAD4 complex translocate into the nucleus to regulate canonical gene transcription (Vander Ark et al., 2018). To determine if TGF β signaling pathway is indeed activated in HFOCs $\pm$ DHT, we measured the protein levels of phosphorylated SMAD2 (p-SMAD2) in the hair bulbs. We found that TGF β signaling appears activated in both conditions, as inferred by the detection of p-SMAD2 (**Fig. 6.1c**). p-SMAD2 levels were equivalent in untreated and DHT-treated HFOCs, confirming that TGF β signaling activation in human non-balding HF organ cultures occurs independently of DHT-AR triggering.

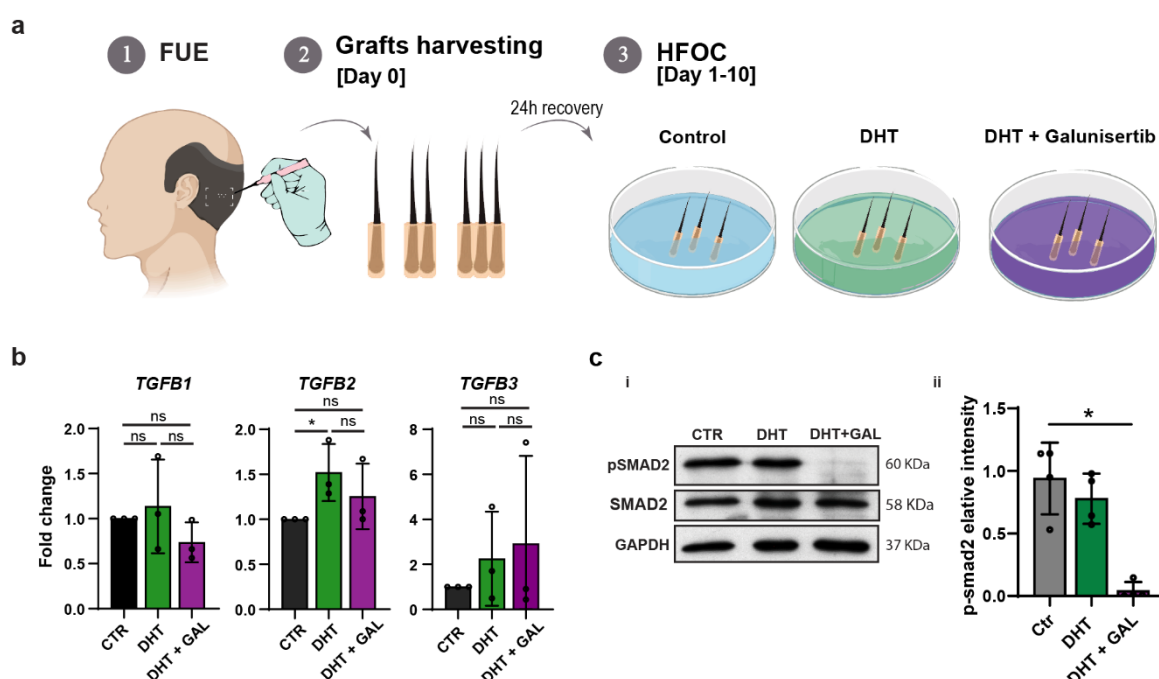


Figure 6.1. Galunisertib blocks TGF β activation in human hair follicle organ cultures (HFOCs). **a)** Schematic representation of DHT and galunisertib treatments in human HFOCs; **b)** RT-qPCR analysis of *TGFB* transcripts in dermal papilla isolated from HFOCs treated with DHT, galunisertib or DHT+galunisertib, for 48h. Values are mean $\pm$ SD of samples from 3 independent donors; **c)** Western blot and quantitative analysis of SMAD2/3 and phospho-SMAD2 protein levels as readout for canonical TGF β signaling activation in HFOCs treated with DHT or DHT+galunisertib, for 72h. *GAPDH* was used as loading control and for normalization. Ns not significant, * $p \leq 0.05$ by unpaired t-test (b) and paired t-test (c) statistical tests.

T β RI inhibition restrains TGF β signaling activation in HF organ cultures

To ascertain the potential role of TGF β signaling on HF regression, we asked if galunisertib (LY2157299), counteracts TGF β signaling and its possible detrimental effect on DP fitness and HF

growth cycle. We first titrated the optimal concentration of this compound using as readout the mean time (days) that HFOCs took to enter catagen (**Fig. 6.2a-c**). A working dose of 10 μ M revealed capable of delaying catagen entry in comparison to untreated controls, although without any impact in the hair shaft growth rate (**Fig. 6.2a-c**). DHT treatment did not significantly accelerate catagen onset in non-balding HFOCs, again in line with neglectable AR expression in their DP (**Fig. 6.3a-c**). Accordingly, galunisertib exhibited equivalent beneficial effect in both the presence and absence of DHT (**Fig. 6.3a-c**). Nevertheless, we found that galunisertib suppressed DHT-driven TGF β 2 upregulation in the DP during HF organ culturing (DHT+GAL condition in **Fig. 6.1b**), as well as inhibited SMAD2 phosphorylation (**Fig. 6.1c**), which confirms the small-molecule effect in the inhibition of TGF β signaling. We also assessed for a potential synergic effect of galunisertib with minoxidil (**Fig. 6.4a-c**). Combination treatment did not potentiate the effect of each drug alone in catagen delay.

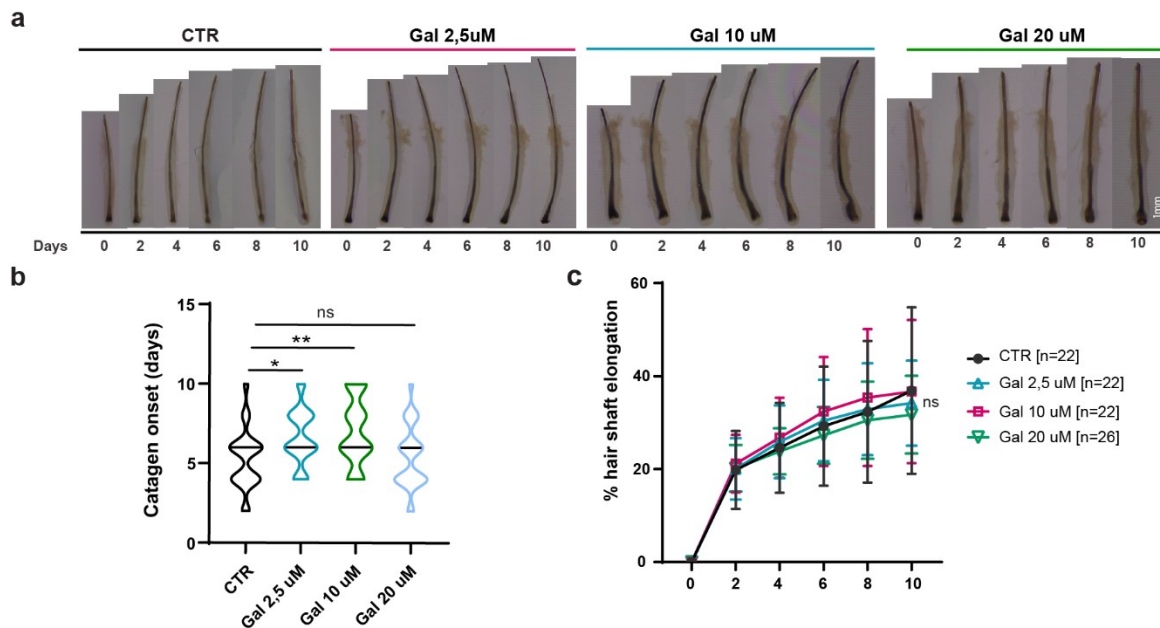


Figure 6.2 Titration of galunisertib optimal working dose in HFOCs. a) HFOCs treatment with increasing doses of galunisertib for 10 days. A concentration of 10 μ M was established as optimal based on the observation of catagen onset delay (DP detachment as readout); **b)** Catagen onset timing in HFOCs treated with increasing galunisertib concentrations as indicated; **c)** Percentage of hair shaft elongation in comparison to day 0. In b) and c), values are mean $\pm$ SD of HFOCs established from $n=5$ independent donors ($n \geq 22$ HF per condition). *ns* not significant, * $p \leq 0.05$ by Mann-Whitney statistical test.

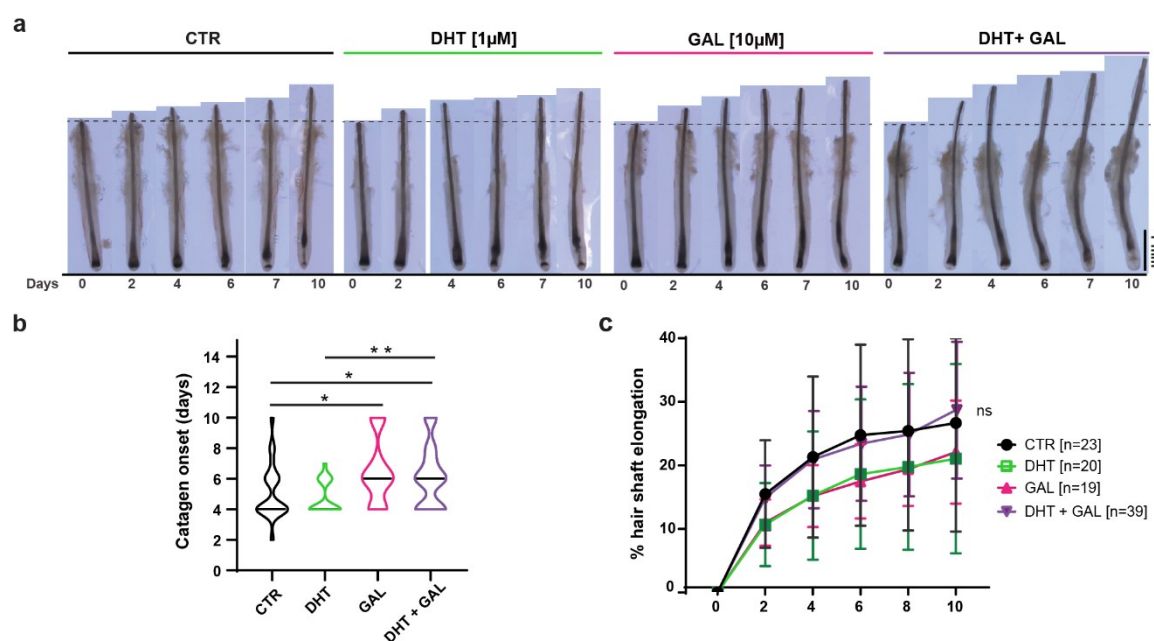


Figure 6.3. Galunisertib delays catagen entry in HFOCs. **a)** Hair growth *ex vivo* (HFOCs) under different treatment conditions for 10 days. Representative HF photos for each condition over time are shown in the panels. DP detachment was used as readout for catagen onset. Scale bar, 1 mm; **b)** Catagen onset timing in HFOCs under different treatment conditions as indicated; **c)** Percentage of hair shaft elongation in comparison to day 0. In **b)** and **c)**, values are mean $\pm$ SD of HFOCs established from $n=5$ independent donors ($n \geq 20$ HFs per condition). *ns* not significant, * $p \leq 0.05$, ** $p \leq 0.01$ by Mann-Whitney (**b,c**).

Galunisertib decreases TGF β 2 protein levels in human dermal papilla of HFOCs

Although DHT induces *TGFB2* transcription in the DP, TGFB2 protein has been suggested to paracrinally act on the adjacent epithelial compartment (Fu et al., 2021; Hibino & Nishiyama, 2004; Kwack et al., 2012). Thus, to directly visualize TGFB2 protein localization pattern in HFOCs $\pm$ galunisertib for 48h, HFs were cryo-sectioned for immunohistochemistry analysis (**Fig. 6.5a**). We found a significant change in the DP levels of TGFB2 upon galunisertib treatment (**Fig. 6.5a,b**), as expected from our transcript analysis (**Fig. 6.1b**) and previous studies in mouse DP (Hibino & Nishiyama, 2004). Also, the p-SMAD2 canonical TGF β target was significantly reduced in DP (**Fig. 6.5a, c**). However, we noticed that TGFB2 protein was predominantly located in the adjacent epithelial hair matrix, pre-cortex, and inner root sheath of human scalp HFs (**Fig. 6.5a**). Quantitative analysis of TGFB2 and p-SMAD2 fluorescence intensity levels at the bulb epithelial compartment surrounding the DP revealed decreased levels in galunisertib-treated HFOCs although not significant. Thus, our data support that induced *TGFB2* transcription and transduction in the DP of

HFOCs, is followed by TGFB2 protein accumulation on the adjacent epithelial compartment. Importantly, galunisertib was found to inhibit catagen induced TGFB2 deposition in DP.

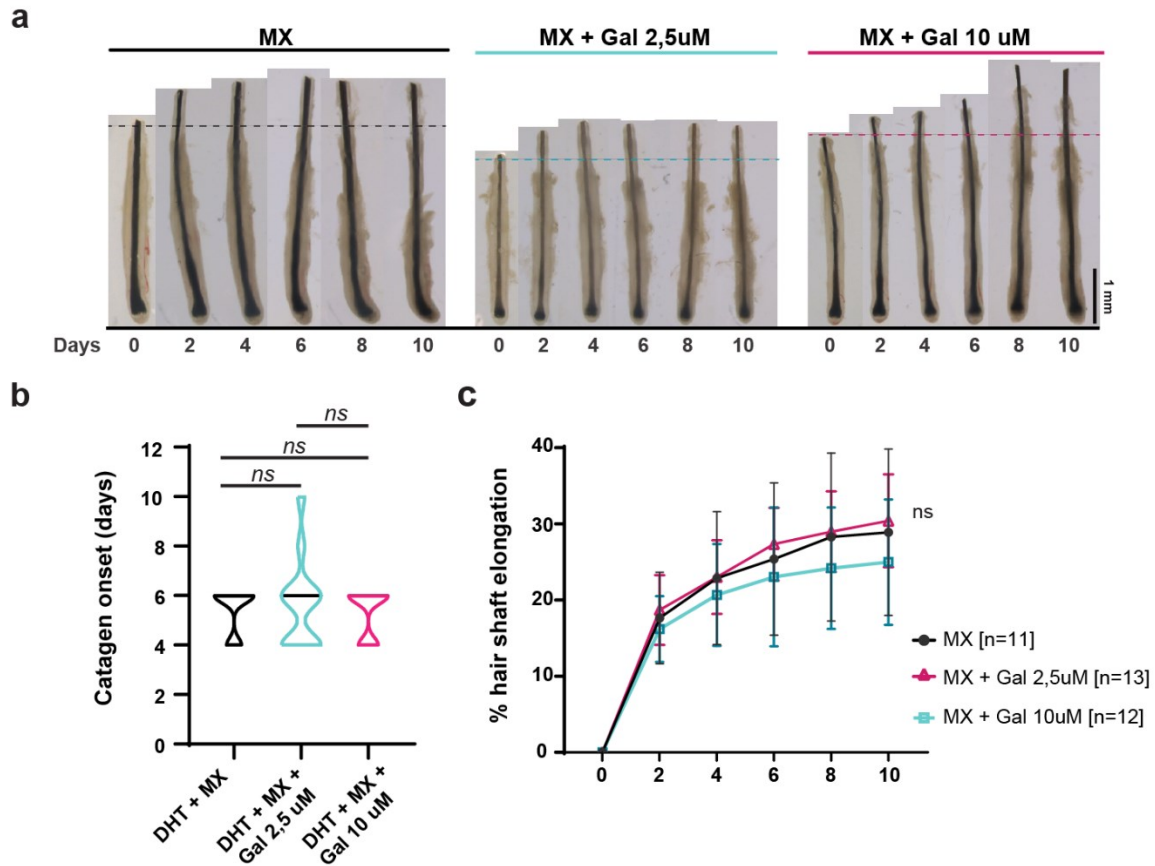


Figure 6.4. Evaluation of combination treatment with galunisertib and minoxidil in HFOCs. **a)** HFOCs treatment with minoxidil (MX) and galunisertib (Gal) for 10 days to evaluate drug synergic effect. **b)** Catagen onset timing in HFOCs treated as indicated. **c)** Percentage of hair shaft elongation in comparison to day 0. In **b)** and **c)**, values are mean $\pm$ SD of HFOCs established from $n=2$ independent donors ($n \geq 10$ HF per condition). *ns* not significant, * $p \leq 0.05$ by Mann-Whitney statistical test.

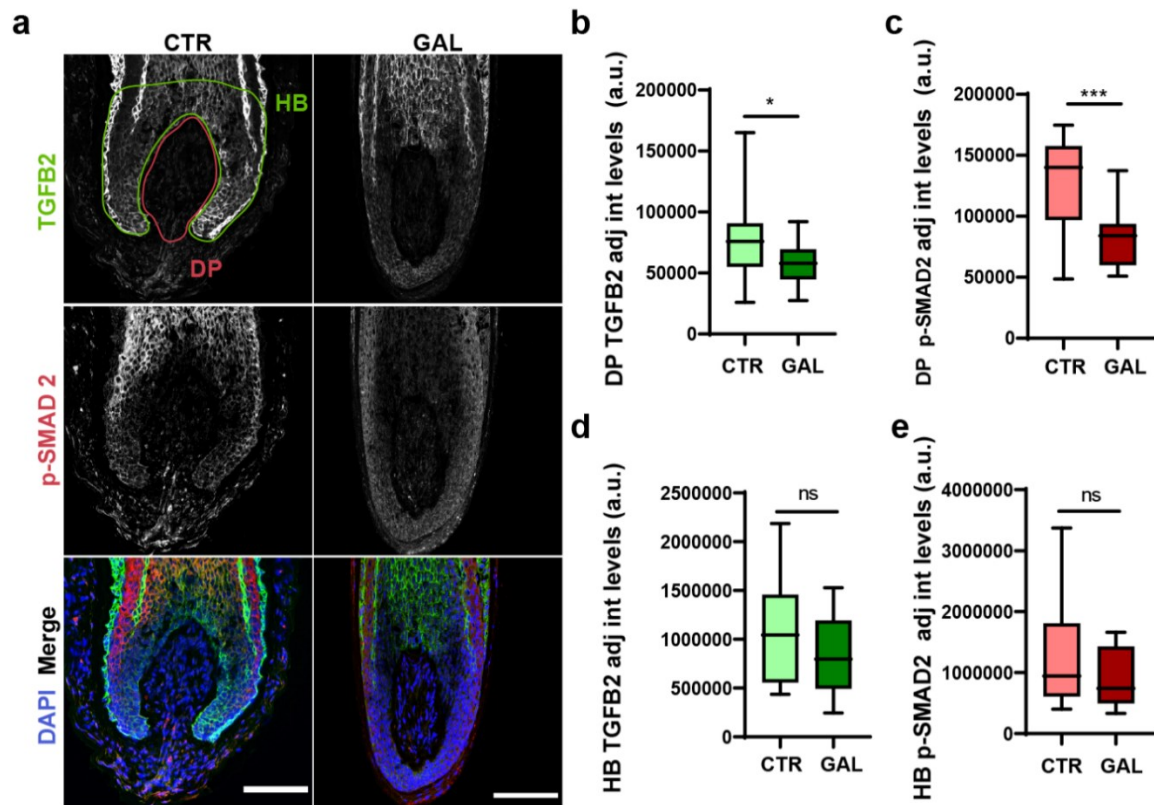


Figure 6.5. Galunisertib inhibits TGF β 2 paracrine signaling in the bulb epithelial compartment. a) Representative IHC images of tissue sections of HFOCs untreated and treated with galunisertib as indicated and stained for TGF β 2 (green) and p-SMAD2 (red). In the upper left frame is exemplified how the dermal papilla (DP) and adjacent bulb epithelial compartment (HB) regions were delineated for quantitative analyses shown in B-D. Nuclei were counterstained with DAPI (blue). Scale bar, 100 μ m; **b-e**) Adjusted intensity levels of TGF β 2 and p-SMAD2 per DP and HB areas as measured in tissue sections from untreated and galunisertib-treated HFOCs. Values are mean $\pm$ SD of HFOCs established from $n=8$ donors ($n \geq 13$ HF per condition). ns $p \geq 0.05$, * $p \leq 0.05$ by unpaired t-test.

Galunisertib improves cell proliferation and β -catenin expression in the bulb epithelial compartment of HFOCs

To further dissect the role of TGF β 2 signaling in the HF cycle, we evaluated the effect of galunisertib treatment in HFOCs' cell proliferation and apoptosis. Immunohistochemistry analysis in cryosections of HFOCs treated with galunisertib for 48h, revealed an increased number of bulb epithelial cells staining positive for the Ki67 proliferation marker, as well as higher Ki67 fluorescence intensity levels, in comparison to untreated HFOCs (**Fig. 6.6a-c**). Fluorescence intensity levels of the apoptosis marker cleaved Caspase 3, mainly detected in the DP, did not show significant changes, suggesting that galunisertib acts by stimulating cell proliferation instead of inhibiting apoptosis (**Fig. 6.6b, c**).

Moreover, we additionally found that galunisertib significantly enhances β -catenin intensity levels in the DP adjacent epithelial compartment, suggesting that WNT/ β -catenin signaling activation (**Fig. 6.7a-e**). Interestingly, the transcription factor LEF1, a marker of DP cell trichogenicity, although not significantly upregulated in the DP, displayed increased levels in the bulb epithelium upon galunisertib treatment (**Fig. 6.7b, d**). Noteworthy, we observed LEF1 to also localize at the epidermal basement membrane surrounding the DP, which we consider as distinctive feature of human HF (**Fig. 6.7a**).

Overall, the data suggest that galunisertib counteracts TGF β 2 signaling activation from the DP to the epithelial compartment, thereby improving WNT/ β -catenin and Lef1 trichogenic signaling and delaying catagen in human HFOCs.

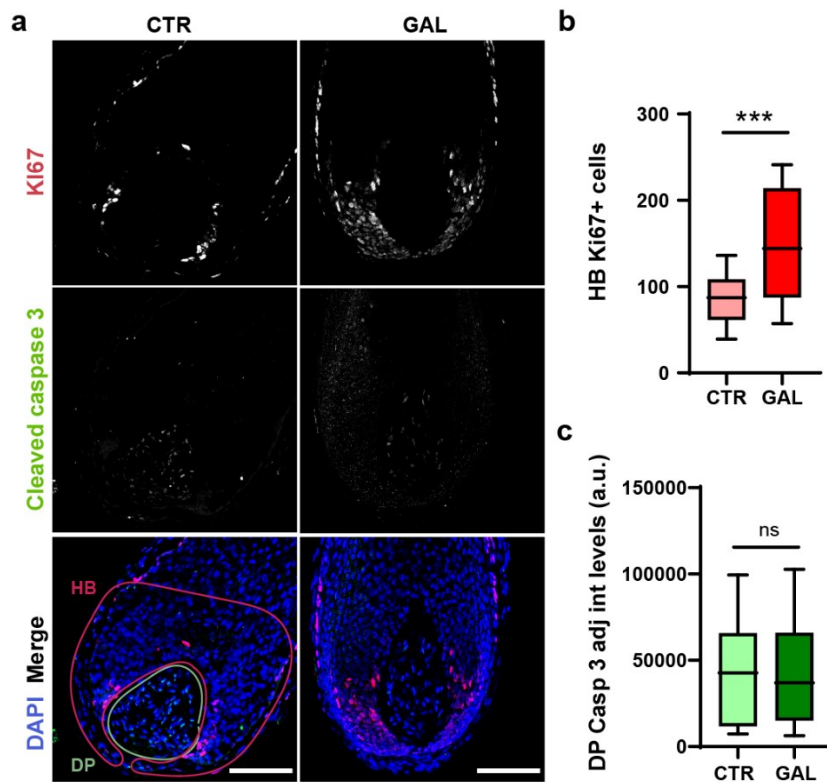


Figure 6.6. Galunisertib improves cell proliferation in the bulb epithelial compartment. a) Representative IHC images of tissue sections of HFOCs untreated and treated with galunisertib and stained for KI67 (red) and cleaved caspase 3 (green). Nuclei were counterstained with DAPI (blue). Scale bar, 100 μ m; b, c) Adjusted intensity levels of KI67 and cleaved caspase 3 per DP and HB (bulb epithelium) areas, respectively, as measured in tissue sections from HFOCs $\pm$ galunisertib; All values are mean $\pm$ SD of HFOCs established from $n=8$ donors ($n \geq 16$ HF per condition). ns $p \geq 0.05$, * $p \leq 0.05$ by unpaired t-test.

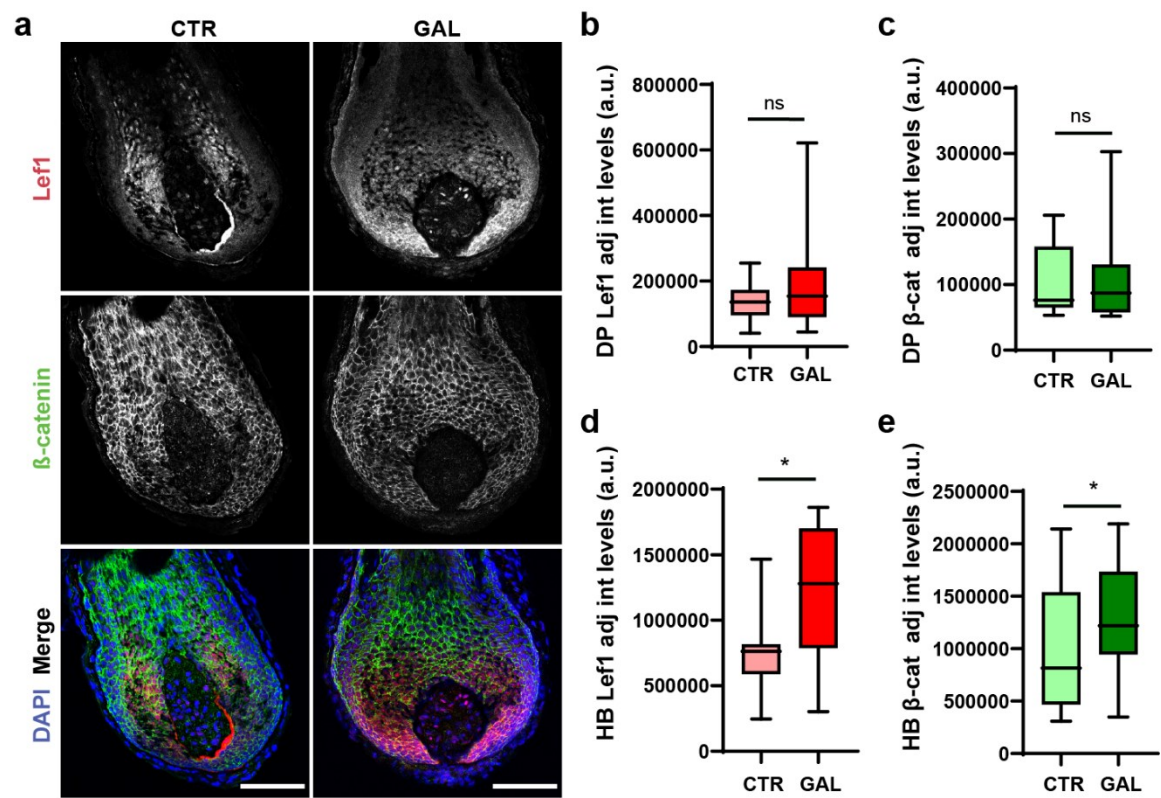


Figure 6.7. Galunisertib improves WNT signaling in the bulb epithelial compartment. **a)** Representative IHC images of tissue sections of HFOCs $\pm$ galunisertib and stained for LEF1 (red) and β -catenin (green). Nuclei were counterstained with DAPI (blue). Scale bar, 100 μ m; **b- e)** Adjusted intensity levels of LEF1 and β -catenin per DP or HB areas, as measured in tissue sections from HFOCs $\pm$ galunisertib. All values are mean $\pm$ SD of HFOCs established from $n=8$ donors ($n \geq 16$ HF per condition). ns $p \geq 0.05$, * $p \leq 0.05$ by unpaired t-test.

Discussion

Hair follicle homeostasis is maintained by a strict balance of cell differentiation (anagen growth) and elimination (catagen regression). Androgens are suggested to stimulate TGF β 1/TGF β 2 that act as hair cycle catagen inducers, leading to progressive HF miniaturization (Inui & Itami, 2011). Here, we set out to investigate the effect of the small-molecule T β RI inhibitor, galunisertib, in human HF cycle. We resorted to human HF organ cultures from non-balding scalp due to the limited access to miniaturized HF from AGA patients. However, since non-balding HFs are non-responsive to DHT, this study model could at first prove unfitting to study TGF β signaling activation. Excitingly, we found that notwithstanding the fact that DHT did not accelerate catagen in non-balding HFOCs as expected, TGF β signaling was still activated in the absence of DHT this way rating non-balding HFOCs as suitable model to test the effect of galunisertib. We found that galunisertib inhibits TGF β signaling in HFOCs as inferred by the decreased levels of the phosphorylated SMAD2 canonical target. Immunohistochemistry analysis of the DP and the adjacent epithelial compartment further demonstrated that galunisertib counteracts TGF β 2 accrual in the dermal papilla, which translated into improved hair matrix cell proliferation required to sustain anagen (Oh et al., 2016). Intensity levels of the apoptosis marker cleaved Caspase 3 in the DP remained unchanged in agreement with previous observations in *in vitro* and mouse models (Tsuji et al., 2003; Winiarska et al., 2006). Thus, rather than inhibiting DP cell apoptosis, galunisertib appears to inhibit *TGF β 2* gene and protein expression in the DP and its detrimental paracrine signaling in the adjacent epithelial compartment, as previously reported (Soma et al., 2002).

Notably, galunisertib was able to rescue the levels of the trichogenic markers, transcription factor LEF1 and β -CATENIN, which may validate the increased proliferation of follicular keratinocytes and anagen-inducing properties of the DP (Enshell-Seijffers, Lindon, Kashiwagi, & Morgan, 2010), thereby attesting galunisertib effect in the delay of catagen onset. Interestingly, a combined treatment with galunisertib and minoxidil did not revealed advantageous in delaying catagen onset during HF organ culturing.

To the best of our knowledge, this is the first study disclosing the efficacy of pharmacological inhibition of TGF β signaling to improve human hair follicle growth cycle. Given that TGF β is a multifunctional pathway involved in the hair follicle cycle, skin healing process and regeneration, galunisertib holds the promise for an effective AGA treatment, as well as for scarless hair restoration.

VII

**A high-throughput screening approach
towards the discovery of novel compounds
that restore the dermal papilla inductive
potential**

Chapter 7

A high-throughput screening approach towards the discovery of novel compounds that restore the dermal papilla inductive potential

Human mesenchymal dermal papilla cells (DPCs) modulate hair follicle (HF) development and growth cycle and represent a reservoir of easily accessible adult progenitor cells (P. Rompolas et al., 2012), which has been largely explored for HF tissue engineering therapies against hair loss. However, hair cloning strategies resorting to human DPCs expanded *in vitro* remain intangible due to DPCs' loss of trichogenic properties upon cell culturing (C. A. Higgins et al., 2013). This represents the major bottleneck in HF bioengineering for replacement therapy. Hence, the step forward will require the understanding of mechanistic cues behind DPCs' loss of trichogenic properties in order to come up with counteracting strategies. So far, several approaches have been tested for their capacity to restore the *in vivo* signature of human DPCs (Castro & Logarinho, 2019). Yet, critical differences between mouse and human HFs, such as the signaling pathways controlling hair growth and the DP molecular markers, have hampered the translation of findings into the clinic setup (Castro, Portinha, & Logarinho, 2022). Here, we describe the protocol of a cell-based assay for compounds screening designed to identify putative epigenetic remodelers capable of restoring the inductive signature in cultured DPCs. Detailed instructions are provided for a controlled and reproducible protocol that can be performed in a laboratorial setup for aseptic cell culturing and equipped with a robotic automated workstation. The advantages of this high-throughput screening, as well as its potential extrapolation to other drug libraries, are discussed.

Introduction

DPCs expansion *in vitro* abrogates their trichogenic potential, and this caveat has not been surpassed by any approaches used so far. In order to improve culture conditions, adaptations have

been made on 2D monolayer cultures, including: 3D cultures, co-culturing of DPCs with other cell types, and biomimetic approaches using 3D bioprinting to reconstruct tissue context/architecture (C. A. Higgins et al., 2010; Ohyama et al., 2012; Jizeng Qiao et al., 2009) (Abaci et al., 2018; C. A. Higgins et al., 2013). Recently, cellular reprogramming through the expression of the *OSKM* transcription factors, has emerged as a promising strategy to develop trichogenic cells from autologous iPSCs (Pinto & Tersikh, 2021). This highlights the relevance of the epigenetic control of chromatin structure in the transcriptional changes during cell transitions or fate commitment (Venkatesh & Workman, 2015). Chromatin compaction, for example, inhibits gene transcription by blocking the access of transcription factors and RNA polymerases. Chromatin compaction can result from histone methylation (by polycomb complexes PRC1 and PRC2), histone deacetylation (by HDACs) or DNA methylation (by DNMT and TET family members) (Goldberg, Allis, & Bernstein, 2007).

Very recently, by combining single-cell transcriptomics, assay for transposase-accessible chromatin sequencing (ATAC-seq), and chromatin immunoprecipitation sequencing (ChIP-seq), postnatal papillary fibroblasts were disclosed to lose regenerative ability due to decreased H3K27 acetylation and subsequent reduction in chromatin accessibility (J. Y. Kim et al., 2022). Also, distinct chromatin rearrangements were found in DP vs. papillary fibroblasts in response to *in vitro* stimuli, even though these cells arise from common progenitors during embryonic development (Niall J Logan, 2021). Furthermore, DP mechanical shock was shown to induce changes in the methylation status of gene promoters, leading to altered transcription profiles (e.g., *ITGAV* promoter hypomethylation) (Logan, Camman, Williams, & Higgins, 2018).

DPCs expanded *in vitro* exhibit significant changes at the transcriptome level, with repression of several HF fate determinants (as described in **Chapter IV**). Rationally, the progressive loss of hair inductive properties upon *in vitro* expansion likely arises from epigenetic-driven repression of genes responsible for DP cell maintenance and progenitor cell asymmetric division. Therefore, cellular reprogramming through drugs targeting chromatin remodelers could work to restore the DP lineage trichogenicity.

Here, we set out to develop a cell-based assay compound screening to identify drugs targeting epigenetic remodelers that potentially restore the gene expression signature of DPCs and their hair-inductive potential. This will provide a functional expanded pool of DPCs that when reimplanted back into the scalp, and upon interaction with other microenvironment cellular players, may induce hair follicle regeneration.

Experimental design and layout

HF's collected from patients undergoing FUE were used for DP microdissection and subsequent expansion of DPCs from cultured explants. Patient-derived cells have proven instrumental to access the effect of compounds on the DP inductive potential. This unprecedented approach uses the NanoLuc® Luciferase DNA system to detect bioluminescence arising from biological interactions (**Fig. 7.1**). The promoter regions of the *LEF1* and *NANOG* genes encoding transcription factors (TFs) whose expression is completely shut down upon DPCs expansion *in vitro*, were cloned upstream of the *Nluc* reporter gene. Any compound able to activate the promoter region will induce luciferase transcription and a bioluminescent reaction.

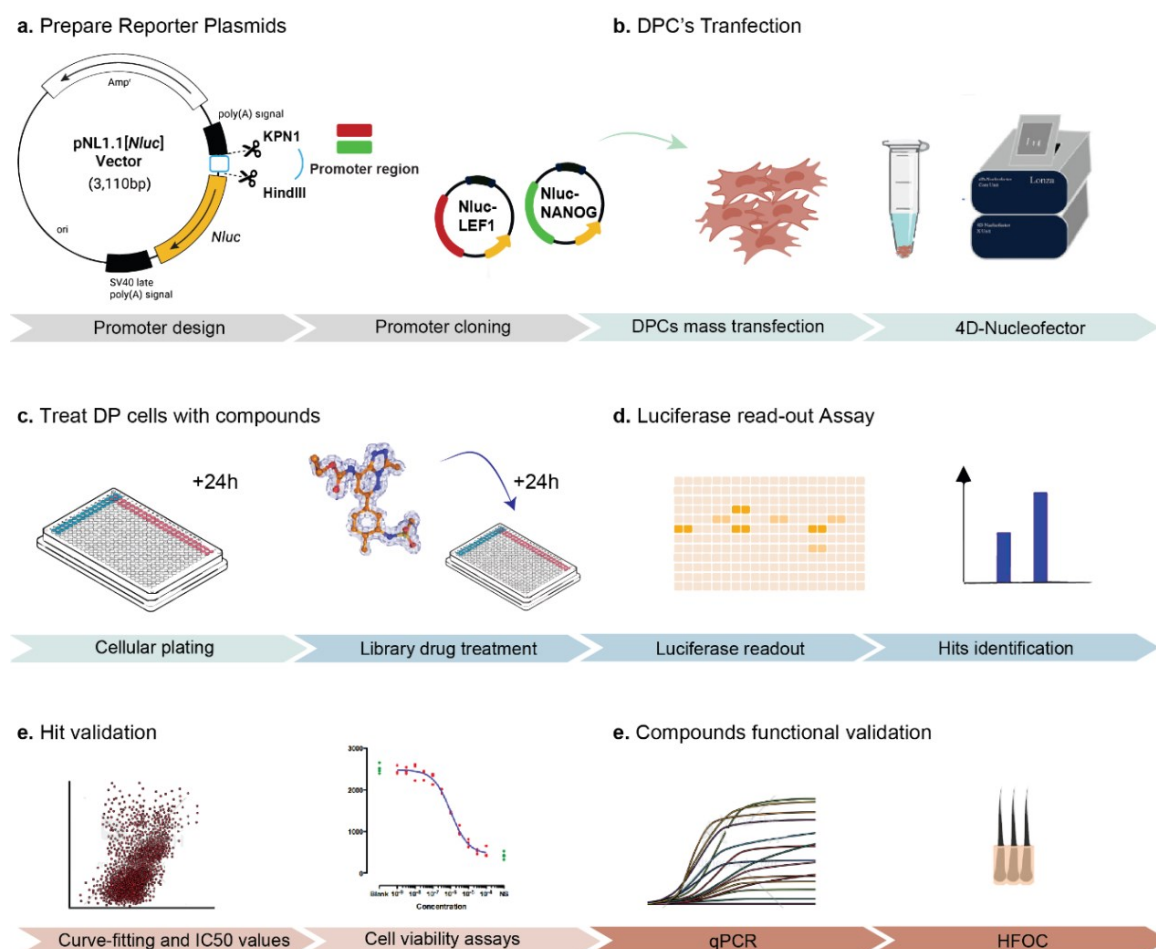


Figure 7.1. Experimental layout of the epigenetic compound library high-throughput screening.

NANOG mediates both self-renewal and pluripotency in embryonic stem cells (Mitsui et al., 2003). *NANOG* was shown to be expressed in native DP and to be repressed upon DPC's culturing (Claire A. Higgins et al., 2012). Recently, the secretome of mesenchymal stem cells genetically engineered

to overexpress *NANOG* was shown to promote HF anagenic growth (J. Park et al., 2019). *LEF1* operates downstream of the canonical WNT signaling cascade (Chen, Jarrell, Guo, Lang, & Atit, 2012). Its activity is required for HF development (Andl et al., 2002; Enshell-Seijffers et al., 2010) and critical to stimulate regeneration and differentiation (Phan et al., 2020). *LEF1* activity was also shown to be expressed in native DP and highly repressed in DPC cultures (C. A. Higgins et al., 2013). Hence, we considered these TFs ideal for the readout of drug effects in signaling cues controlling DP inductivity and HF cycle.

Bioluminescence reporter genes are robust cell-based systems to study gene regulation. To evaluate promoter activity and/or regulators of its activity, the promoter region of a gene of interest (*NANOG* and *LEF1*) is cloned upstream of the reporter luciferase gene (*Nluc*) within a plasmid, so that luciferase bioluminescence levels are measured as proxy for the promoter activity. Upon transfection of DPCs with the reporter plasmids, this system was used to screen 101 small-molecule compounds from a commercial library for their capability to induce epigenetic changes that lead to *NANOG* or *LEF1* promoter activity and *Nluc* expression. Positive hits are then validated for optimal dosage, cytotoxicity and functionality in DPC cultures and HFOCs.

Materials

Cell culture

- BINDER CO₂ tissue culture incubator (Thermo Fisher Scientific);
- Medifuge™ Small Benchtop Centrifuge (Thermo Fisher Scientific);
- Stereomicroscope (35x magnification);
- Hemocytometer
- MEM α , nucleosides, no phenol red (Gibco, cat. no. 41061037);
- Fetal Bovine Serum (Gibco, cat. no. 10270-106);
- Antibiotic Antimycotic Solution (100×) (Merck Life Science, cat. no. A5955);
- 1x TrypLE Express enzyme, phenol red (Gibco, cat. no. 12605010);
- 1x phosphate-buffered saline (PBS), pH 7.4;
- HBSS solution (Thermofisher, Gibco 14025-092)
- Gelatin from porcine skin (Merck Life Science, CAS Number: 9000-70-8);
- Collagenase D (Merck Life Science, cat. no. 11088858001);
- Stereomicroscope (EZ4W, Leica), equipped with a digital camera 5.0x mega-pixel CMOS camera;
- Sterile Dumont forceps;
- Sterile Scalpel n°11;
- Sterile glass petri dishes.

Transfection

- P3 Primary Cell 4D-Nucleofector™ X Kit;
- 4D-Nucleofector® Core Unit - Lonza Bioscience;

Screening

- Dimethyl sulfoxide (DMSO) (Merck Life Science, cat. no. D4540);
- Multidrop™ Combi Reagent Dispenser (Thermo Fisher Scientific, Inc.);
- Multidrop™ standard dispensing cassette (Thermo Fisher Scientific, Inc.);
- Multidrop™ metal tip small tube cassette (Thermo Fisher Scientific, Inc.);
- Corning® 384-well Low Flange White Flat Bottom Polystyrene TC-treated Microplates, 10 per Bag, with Lid, Sterile;
- Dual-Glo® Luciferase Assay System (Promega Corp.);
- Nano-Glo® Luciferase Assay System (Nano-Glo Luciferase Assay System (N1610, Promega Corp.);
- 384-well plate for compound preparation;
- VICTOR Nivo Multimode Microplate Reader, PerkinElmer;
- CellTiter-Glo® One Solution (CellTiter-Glo) (G7573, Promega);
- Tocriscreen Epigenetics Library (Tocris Bioscience, Cat. No. 6801);
- Virtexin-1 (CAS no. 3681-93-4, Merck);
- LiCl (CAS no. 7447-41-8, Merck).

Common equipment

- 1.5 mL snap cap low-retention microcentrifuge tubes (Thermo Scientific, cat. no. 3446)
- 15 mL conical tubes (Thermo Scientific, cat. no. 12-565-268)
- 50 mL conical tubes (Thermo Scientific, cat. no. 12-565-270)
- 5 mL serological pipettes (Fisherbrand, cat. no. 13-678-11D)
- 10 mL serological pipettes (Fisherbrand, cat. no. 13-678-11E)
- 10 µL LTS pipette tips, low retention, sterile (Neptune, cat. no. BT10.1)
- 20 µL LTS pipette tips, low retention, sterile (Neptune, cat. no. BT20.1)
- 200 µL LTS pipette tips, low retention, sterile (Neptune cat. no. BT200.1)
- 1,000 µL LTS pipette tips, low retention, sterile (Neptune cat. no. BT1000.96.1)
- T25 tissue culture flasks (Corning® cat. no. 430639)
- Corning™ Costar™ 24-well Clear TC-treated Multiple Well Plates (cat. no. CLS3526);
- T25 tissue culture flasks (Corning® cat. no. 430639).

Step-by-step protocol

1. Establishment of human DPC cultures

Human DPC cultures can be established from either balding or occipital non-balding hair grafts. Before initiating a DPC culture, training on microdissection of human DP from anagen HFs is recommended. The procedure for human DPCs isolation is described in **Chapter III (section 3.1)**. Briefly:

- 1.1) Place freshly collected occipital hair grafts in HBSS solution supplemented with 2% antibiotics-antimycotics;
- 1.2) Dissect in a sterile petri dish within 1-3h after isolation under a stereomicroscope at 35x magnification;
- 1.3) Aspirate isolated DPs from the Petri dish and incubate in pre-warmed digestion solution [0.2mg/mL collagenase D in MEM α] for 4h at 37°C with shaking;
- 1.4) Transfer 250uL of this digestion mix into a 24-well (Corning, cat. No. CLS3526) previously coated with 0.1% gelatin;
- 1.5) Add 750uL of prewarmed MEM α supplemented with 15% FBS, 2.5 mM l-glutamine and 1x antibiotic-antimycotic to each well;
- 1.6) Leave the 24-wells undisturbed for 1 week so that the DPs collapse, and DPCs start spreading out;
- 1.7) Once a reasonable density of adherent DPCs can be seen in the explant culture, replace medium every 3 days;
- 1.8) Trypsinize cells at confluency and dilute 1:4 to establish a new passage, until a maximum of 3 passages (p3) (3-4 weeks).

2. Generation of reporter plasmids

The promoter regions of the trichogenic genes *NANOG* and *LEF1* were cloned upstream of the *Nluc* reporter gene within the pNL1.1. *Cis* regulatory elements of these genes span approximately 1kb upstream of the transcription start site. **Fig. 7.2** shows the regions used for cloning. The *NANOG* promoter sequence (as reported in Xu et al., 2008) was obtained from pNANOG-Luc (Addgene plasmid #25900) by PCR amplification with the following setup: 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The *LEF1* promoter sequence was selected based on the overlap between H3K27Ac ChIP-seq and transcriptional signal in non-coding regions upstream of the *LEF1* transcription start site (**Fig. 7.2b**). The sequence was PCR amplified from purified human DPCs DNA (QIAprep Spin Miniprep Kit, Qiagen), with the following setup: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min. Primers

(Sigma-Aldrich) were designed using the Benchling online platform (www.benchling.com):
 NANOG_prom_FW: GTACGCTGGTTTCAAACCTCCTGACTTC; NANOG_prom_RV:
 AGCTTCCTGGAGTCTCTAGATT; LEF1_prom_FW:
 TAAGGTACCTCAATCACCACTCCTTCTCGC; LEF1_prom_RV:
 TATAAGCTTCAAAGTGCAGAGGAGGGTCCG.

Promoter regions were PCR amplified with a proof-reading iMax™ II DNA polymerase (25261, INtRON Biotechnology) following the manufacturer's instructions. PCR products were visualized by electrophoresis on an 1% agarose gel, and the bands excised and purified with NZYGelpure kit (MB011, NZYTech). PCR products were then cloned in the vector pNL1.1[Nluc] plasmid (N100A, Promega) digested with *KpnI* and *HindIII* (Invitrogen™ Anza™). Positive clones were confirmed by Sanger sequencing and used for plasmid purification (QIAGEN Plasmid Plus Midi Kit). Each reporter plasmid was co-transfected with the pGL4.54 [luc2/Tk] vector (Promega) in 90% confluent DPC cultures. pGL4.54 [luc2/Tk] works as internal transfection control. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

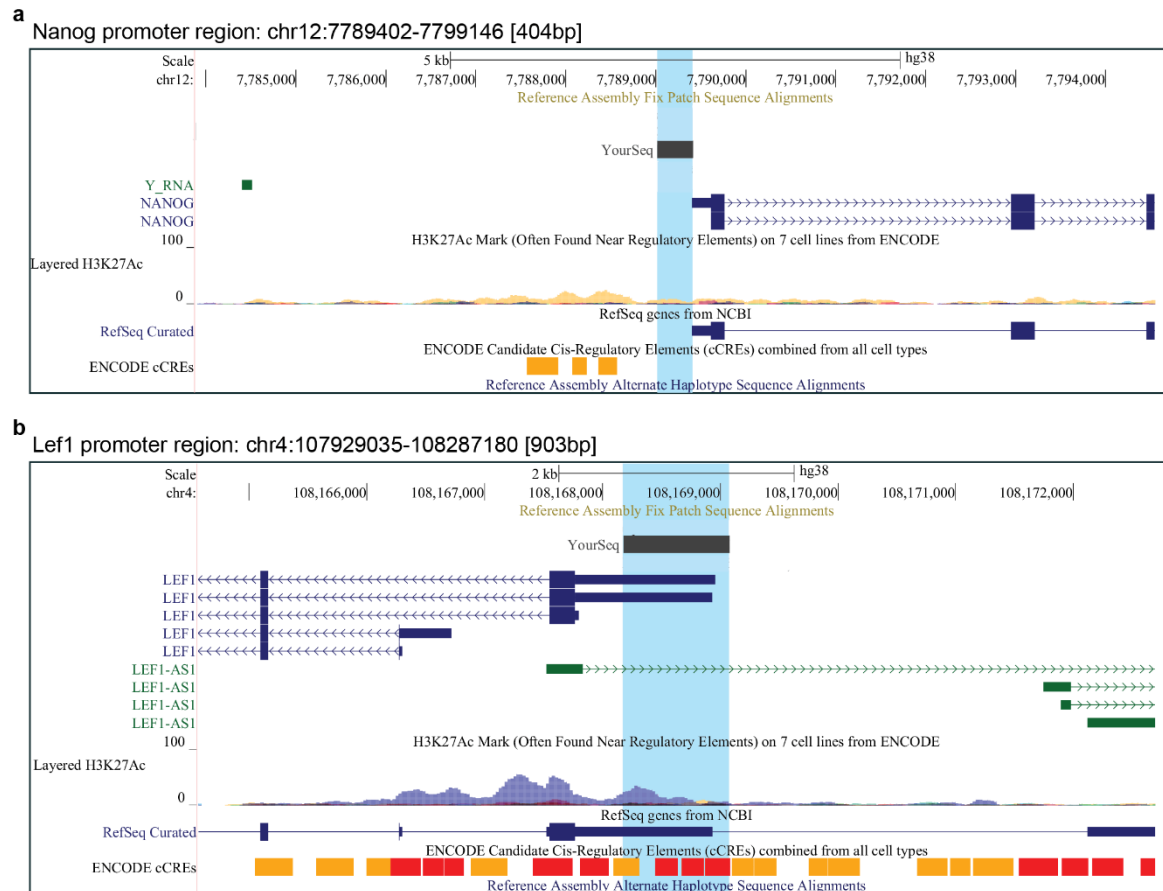


Figure 7.2. Genomic landscapes of *LEF1* and *NANOG* in the human genome according to the UCSC human genome browser (<https://genome.ucsc.edu/>). The cloned promoter regions are highlighted in blue.

3. Preparation of the epigenetic compounds' library

A library of compounds with known pharmacological targets (Tocriscreen Epigenetics Library, Tocris Bioscience) was used (**Table 7.1**). The compounds are ready-to-use from manufacturer at 10 mM in DMSO.

3.1. Prepare 384-well “source plates” to avoid freeze-thaw cycles and contamination of the library.

3.2. Considering that the half maximal effective concentration (EC₅₀) value ranges from nM to mM, prepare replica plates at 0,5 μ M and 5 μ M for the screening.

3.3. As screening positive controls, it is advisable to include compounds reported to increase the expression of the genes of interest (if such compounds are available). For this screening, we included two drugs, Virtexin-1 (Luo et al., 2018) and LiCl (Q. Wang et al., 2011), reported to stimulate the transcription of *LEF1* and *NANOG* genes, respectively.

3.4. If positive controls are not available, the screening can be performed as far as further validation assays are included to exclude false positives and false negatives. If one hit demonstrates significantly high luciferase signal early on in the screening, it may serve as positive control afterwards, including Z-factor assessment (see Hit identification and statistical analysis).

Table 7.1. Epigenetic compounds library used in this study.

Compound name	CAS number	Pharmacological target	Compound name	CAS number	Pharmacological target
Triptolide	38748-32-2	RNA Polymerase II	Hesperadin hydrochloride	-	Aurora kinase B
PJ 34 hydrochloride	344458-15-7	PARP	KU 60019	925701-46-8	ATM/ATR
IOX 2	931398-72-0	PHD2	UNC 0646	1320288-17-2	G9a/GLP
ML 228	1357171-62-0	HIF	P 22077	1247819-59-5	USP7
TC-S 7009	TC-S 7009	HIF-2 α	CP 690550 citrate	540737-29-9	JAK
GN 44028	1421448-26-1	HIF-1 α	SB 747651A dihydrochloride	1781882-72-1	MSK1
AZD 2461	1174043-16-3	PARP	UNC 1999	1431612-23-5	EZH2/EZH1
Rucaparib camsylate	1859053-21-6	PARP	UNC 2400	1433200-49-7	Negative control of UNC 1999 (Cat. No. 4904)
Resveratrol	501-36-0	SIRT1	PRT 4165	31083-55-3	E3 ubiquitin ligase
BVT 948	39674-97-0	PTP	UNC 2327	1426152-53-5	PRMT3
Sodium 4-Phenylbutyrate	1716-12-7	HDAC	UNC 0642	1481677-78-4	G9a/GLP
Valproic acid, sodium salt	1069-66-5	HDAC	AZ 20	1233339-22-4	ATR

A high-throughput screening approach towards the discovery of novel compounds that restore the dermal papilla inductive potential

CI 994	112522-64-2	HDAC1/3	Furamidine dihydrochloride	55368-40-6	PRMT1
Tranylcypromine hydrochloride	1986-47-6	LSD1	OICR 9429	1801787-56-3	WDR5
Alexidine dihydrochloride	1715-30-6	PTPMT1	MM 102	1883545-52-5	WDR5
IOX 1	5852-78-8	JMJD	WDR5 0103	890190-22-4	WDR5
GSK J1	1373422-53-7	JMJD3/UTX	EPZ 004777	1338466-77-5	DOT1L
GSK J4	1373423-53-0	JMJD3/UTX	AZD 1480	935666-88-9	JAK2
PCI 34051	950762-95-5	HDAC8	EML 425	1675821-32-5	p300/CBP
SAHA	149647-78-9	HDAC	MS 023 dihydrochloride	1992047-64-9	PRMT
GSK J2	1394854-52-4	Inactive isomer of GSK J1 (Cat. No. 4593)	GSK 591 dihydrochloride	2320953-89-5	PRMT5
LMK 235	1418033-25-6	HDAC4/5	BAY 598	1906919-67-2	SMYD2
JIB 04	199596-05-9	JMJD	TP 064	2080306-20-1	PRMT4
RN 1 dihydrochloride	1781835-13-9	LSD1	TC JL 37	1258294-34-6	TYK2
TC-E 5002	1453071-47-0	KDM2/7	JQEZ5	1913252-04-6	EZH2
GSK 2830371	1404456-53-6	Wip1 phosphatase	VH 298	2097381-85-4	E3 ubiquitin ligase VHL
GSK LSD 1 dihydrochloride	2102933-95-7	LSD1	cis VH 298	2097381-86-5	Negative control for VH 298 (Cat. No. 6156)
SirReal 2	709002-46-0	SIRT2	A 196	1982372-88-2	SUV420H1/H2
Sephin 1	1883549-35-6	PPP1R15A	PF 06726304 acetate	2080306-28-9	EZH2
S 2101	1239262-36-2	LSD2	LY 303511	154447-38-8	BET
CX08005	1256341-22-6	PTP1B	PFI 1	1403764-72-6	BET
MS 275	209783-80-2	HDAC	(+)-JQ1	1268524-70-4	BET
OG-L002	1357302-64-7	LSD1	I-BET 151 dihydrochloride	1883545-47-8	BET
Givinostat hydrochloride	199657-29-9	HDAC	Bromosporine	1619994-69-2	BRD
BRD 73954	1440209-96-0	HDAC6/8	I-CBP 112	1640282-31-0	CBP/p300 BRD
Ciclopirox	29342-05-0	HDAC	PFI 3	1819363-80-8	SMARCA2/4 and polybromo 1
ZM 447439	331771-20-1	Aurora kinase B	BAZ2-ICR	1665195-94-7	BAZ2
Ryuvidine	265312-55-8	SETD8	OF 1	919973-83-4	BRPF1B and BRPF2
Decitabine	2353-33-5	DNMT	CPI 203	1446144-04-2	BET
Temozolomide	85622-93-1	DNA	NI 57	1883548-89-7	BRPF
H 89 dihydrochloride	130964-39-5	MSK1	PFI 4	900305-37-5	BRPF1

VII

A high-throughput screening approach towards the discovery of novel compounds that restore the dermal papilla inductive potential

TCS PIM-1 1	491871-58-0	Pim kinase	BI 9564	1883429-22-8	BRD9/7
SD 1008	960201-81-4	JAK2	I-BRD9	1714146-59-4	BRD9
Mirin	1198097-97-0	ATM/ATR	(-)-JQ1	1268524-71-5	Inactive control for (+)-JQ1 (Cat no. 4499)
RG 108	48208-26-0	DNMT	NVS-CECR2-1	1992047-61-6	CECR2
BIX 01294	1392399-03-9	G9a/GLP	BAY 299	2080306-23-4	BRD1/TAF1
5-Azacytidine	320-67-2	DNMT	TP 472	-	BRD9/7
UNC 0224	320-67-2	G9a/GLP	OTX 015	202590-98-5	BET
AZ 5704	1941214-06-7	ATM	GSK 6853	202590-98-5	BRPF1
A 485	1889279-16-6	p300/CBP	L Moses dihydrochloride	-	p300/CBP- associated factor (PCAF)
EPZ 015666	1616391-65-1	PRMT5			

4. DPCs transfection with reporter plasmids

DPC cultures expanded until passage 3 (p3; see step 1. above) were used for reporter plasmid transfection. Passage 3 is recommended so that fitted dividing cells are used and sufficient cells are generated for the screening. The P3 Primary Cell 4D-Nucleofector™ X Kit and Nucleofector™ technology was used since bulk electroporation improves the transfection homogeneity. 1×10^6 cells were transfected per cuvette, according to manufacturer instructions. 7×10^6 cells are needed for the high-throughput screening (101 compounds, 2 distinct concentrations).

- 4.1. Trypsinize 90% confluent DPC cultures at p3;
 - 4.2. Count the number of live cells using a hemocytometer;
 - 4.3. Transfer the cell suspension into a 15 mL conical tube and centrifuge cells at 1200xg for 5 min;
 - 4.4. Resuspend cell pellet in amaxa's Nucleofector™ solution (amaxa, Cologne, Germany) (100uL per each 1×10^6 cells) containing the *NANOG* or *LEF1* reporter vectors;
 - 4.5. Transfer each 100uL into an electroporation cuvette (2 mm; Invitrogen, Groningen, The Netherlands);
 - 4.6. Electroporate using the CM-137 program, and according to manufacturer instructions 7. Immediately after electroporation, resuspend in culture medium (supplemented MEM α) to reach a final concentration of 7500 cells per 25uL per 384-well;
 - 4.7. Dispense 25uL per 384-well using the Multidrop™ Combi Dispense;
- Note: When a multidrop dispenser is being used, prepare about 10% of extra mixture to cover its dead volumes.*
- 4.8. Incubate cells in a standard humidified 5% CO₂ incubator at 37°C for 24h;
 - 4.9. Proceed with the compound screening as described below.

5. Library compound screening

Compounds were added to the 384-well plates containing DPC electroporated cells.

5.1. As the compound source plate is frozen, remove it from the freezer at least 1h before needed so that it stabilizes at 20°C;

5.2. Add 100nL of each compound 24h after electroporation, using the Lab Automated workstation (BioMek FX pintool), to reach a final well concentration of 5µM or 0,5 µM.

Note: Any other method of transferring compounds to a cell plate with sufficient precision may be used.

5.3. Incubate cells in a standard humidified 5%CO₂ incubator at 37°C for 24h;

5.4. Proceed with luciferase activity readout as described below.

6. Luciferase readout assay

6.1. Prepare the desired amount of a 1:50 (v/v) mix of Nano-Glo® Luciferase Assay Substrate with Nano-Glo® Luciferase Assay Buffer and vortex;

Note: Remove the reagents from the freezer and stabilize at RT at least 2h in advance

6.2. Remove the cell plates from the incubator and stabilize at RT for 15min;

6.3. Distribute 25µl of reagent mix per 384-well using the Multidrop™ Combi;

6.4. Vortex vigorously for 5 min in a microplate orbital shaker to ensure homogenization;

6.5. 3 min later, evaluate the luciferase activity using VICTOR Nivo Multimode Microplate Reader (24h timepoint after drug incubation).

7. Hit identification and statistical analysis

The low levels of *NLuc-LEF1* and *NLuc-NANOG* promoter activities under basal conditions turn this cell-based reporter assay adequate for the *in vitro* high-throughput analysis of compounds with minimal effect on promoter activity. Although some variation was found between replicates, its effect can be minimized by a higher number of technical replicates. As Vitexin and LiCl showed no effect on *LEF1* and *NANOG* promoter activities, respectively, and we dismissed them as positive controls. Therefore, to ensure the screening control quality, a Z-factor will be calculated from one positive hit identified. For hit identification, the Z-score was calculated using the following equation:

$$x = \frac{3SD_1 + 3SD_2}{\bar{x}1 - \bar{x}2}$$

where SD₁ is the standard deviation of wells treated with the hit positive compound, $\bar{x}1$ the mean value of wells treated with the positive compound, SD₂ the standard deviation of vehicle-treated control wells, and $\bar{x}2$ the mean value of control wells.

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A high-throughput screening approach towards the discovery of novel compounds that restore the dermal papilla inductive potential

In this assay, hits were primarily selected from compounds that induced an activation of promoter activity at least 3-fold higher than the SD of the control wells. For screenings with a higher number of compounds stricter selection of the hits may be needed, so that a reasonable number of compounds is further investigated for its functional activity.

Results and discussion

Using this protocol, we screened 101 epigenetic compounds and identified 27 putative hits for *NANOG* promoter activity, and 3 putative hits for *LEF1* promoter activity. Screening results can be visualized as plate heat maps (**Fig. 7.3**). The compounds were arranged as follows: compound 1 [C3 at 5 μ M, C4 at 0.5 μ M]; compound 2 [C5 at 5 μ M, C6 at 0.5 μ M], and so on. Note that the screening can be easily adjustable for big drug libraries and for testing six 384-well plates at a time. The major limitation to a scaleup is the number of early passages expanded DPCs. Still, the plate setup used in this screening, and excluding two rows and two columns on each edge of the plate to minimize edge effects, allows the screening of 720 compounds (at 2 different concentrations, without replicates) using p3 expanded DPCs from a single patient (explant cultures of 10 follicle units).

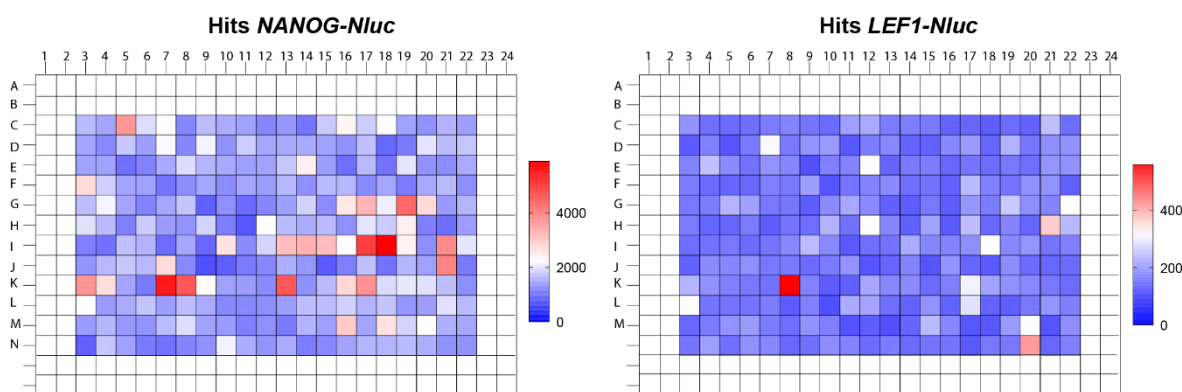


Figure 7.3. Heatmap of luciferase reporter activity upon compound screening for *NANOG* and *LEF1* promoter activators. The Heatmap was generated in GraphPad prism V9 and displays the luciferase signal intensities found in the DPCs treated with epigenetic remodeling compounds.

Several steps must be sequentially carried out to validate the hits. First, to decrease false positive rates, we confirmed by *cherry-picking* the hits by running triplicates for both 0.5 μ M and 5 μ M concentrations. EC50 values for the preliminary hits were then calculated by drug titration (2-fold dilution series) within the range of 0,0781 to 40 μ M, in triplicate. The luciferase activity was evaluated as described above. Curve-fitting and EC50 values were calculated using Prism5 (**Fig.7.4a**). Our data suggest that 6 hits proved robust in increasing *NANOG* promoter activity in DPCs. To assess cell viability in a concentration dependent manner, CellTiter-Glo 2.0 Cell Viability Assay was used, and luciferase signal normalized to vehicle-treated cells (**Fig. 7.4b**). These data

suggests that NANOG hits reach their maximal promoter response without compromising DPCS viability.

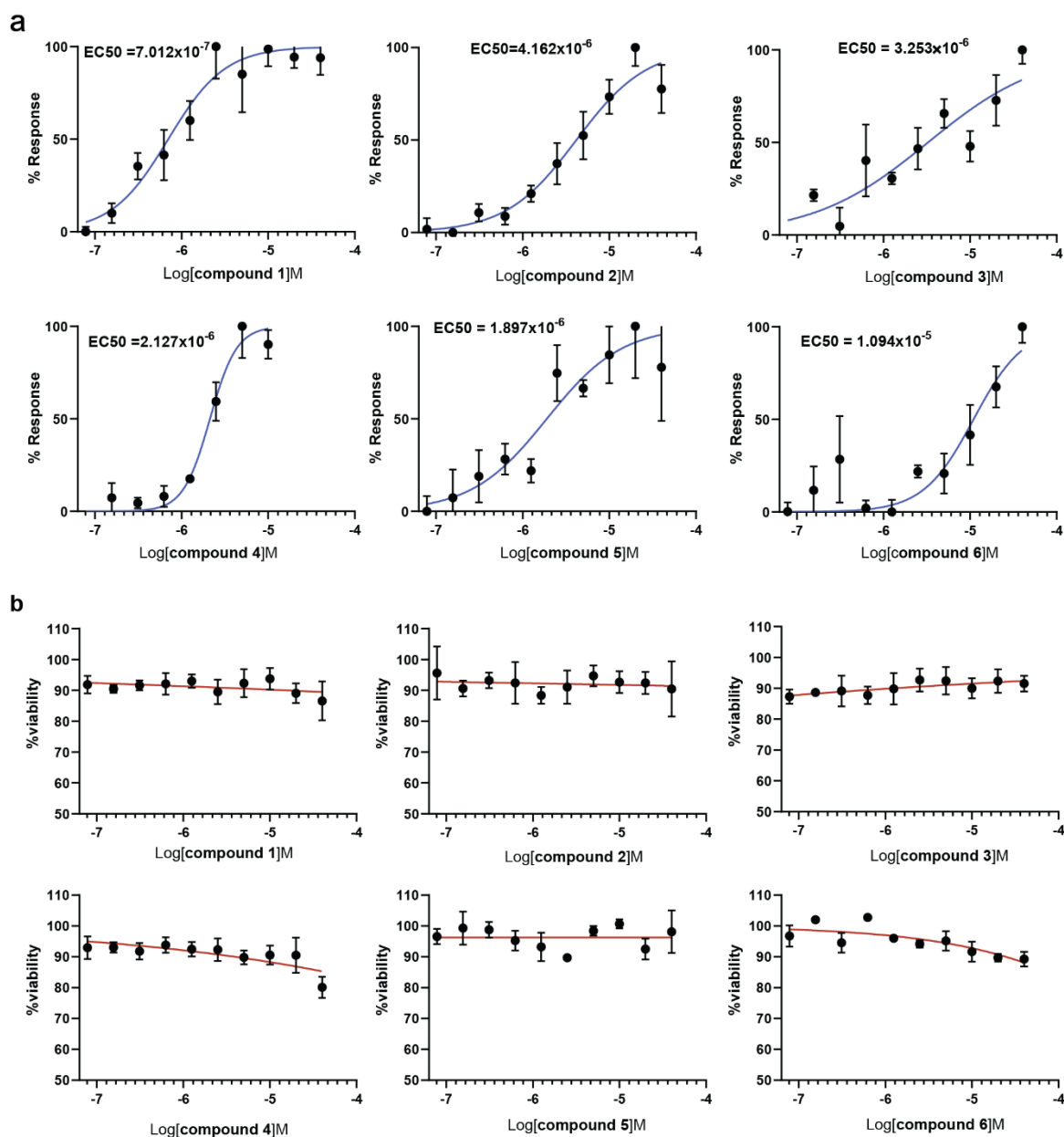


Figure 7.4. NANOG-Nluc hits validation. a) Estimated EC50 values for hits with a range of concentrations (0,0781 - 40 μ M) values in μ M $\pm$ SEM for triplicates; **b)** Drug dependent cell viability analysis of the hit compounds in DPCs; Data points showing % cell viability (mean $\pm$ SEM) from triplicates.

Although three hits were initially identified for *LEF1* promoter, it was later shown that its activity was not reproducible, and thus its study was not further pursued. New compound libraries should be later tested to find robust *LEF1* promoter activators.

From our preliminary results, we expect that increased activity of the *NANOG-Nluc* reporters should reflect upregulation of the endogenous *NANOG* gene in DPCs, and potentially, improved DP cell function and trichogenicity.

Further functional validation of the hits will be needed. First, we intend to confirm if the positive hit compounds lead to upregulation of key DP genes by performing RT-qPCR analysis (and eventually immunostaining analysis).

Next, hair follicle organ culture (HFOC) is a suitable model for studying the impact of epigenetic modulation on the delay of hair follicle growth/catagen (hair follicle regression phase). HFOC is a simple assay system to validate the impact of drugs on human hair. Hence, we expect that the hit compounds identified in this cell-based system may impact the human hair follicle cycle. More details of this *ex vivo* system are available in (Langan et al., 2015).

Ultimately, hit compounds should be discovered that are able to modulate DP hair inductive potential and therefore may play a role in the hair loss treatment. We believe that a new therapeutic approach may be developed if an efficient epigenetic reprogramming of DP cells allows them to reestablish the natural DP trichogenic signature upon *in vitro* expansion. Moreover, we envision that a compound that alone could dedifferentiate non-trichogenic fibroblast into DP hair inducing cells may be explored to trigger the anagen start and *de novo* hair growth in the balding scalp.

In summary, here we describe a rapid pipeline to identify novel transcriptional regulators of key gene function in the context of hair loss and convey important concerns that will be useful for similar screening for human cell-based assays. This protocol is easily modifiable to target other promoters of interest and may also be adapted for any cell type and 3D spheroid culture systems.

Conclusions

Potential implications for cellular reprogramming and tissue bioengineering

Retroviral induction of cells with Sox2, Oct4, and Klf4 can directly reprogram somatic cells into pluripotent stem cell state (Okita, Ichisaka, & Yamanaka, 2007). Although highly promising in a wide range of therapeutic applications, viral delivery and oncogenic potential of cellular reprogramming are suboptimal for clinical translation. One alternative is the chemical reprogramming (Guan et al., 2022). The epigenetic reprogramming could be an interesting approach for a wide range of skin-focused therapies, including hair follicle related diseases. Partial reprogramming of scalp non-hairy skin cells may restore hair inductive gene expression. Stimulating cells in their native niche (by an *in vivo* epigenetic reprogramming) would be an encouraging approach to *de novo* HF neogenesis.

Identification of such compounds would allow us to modulate gene expression without interfering with the DNA sequence and generate epigenetic-induced regeneration strategies for hair loss treatment.

Moreover, HF bioengineering strategies have so far failed due to the inability to successfully expand DP cells *in vitro*. The drug screening could accelerate the discovery of small molecules that can reset the epigenetic state of DPCs and regain key regulators of the HF induction, restoring DP identity. The success of this strategy would allow us to bypass the pluripotent state and accelerate the route to obtain the functional hair inducing cells. This strategy could potentially be used to functionally rescue the differentiated non-trichogenic DPCs into native DPCs for tissue engineering constructs. Furthermore, “epigenetic rejuvenated” DP are indeed a very promising cell source for hair loss cell replacement therapy.

Limitations

One limitation of this protocol is the use of primary cells. Stable cell lines are preferred for high-throughput screening due to their high proliferation rate and homogeneous activity. Moreover, transfection efficiency and viability are lower in primary cells (Hamm, Krott, Breibach, Blindt, & Bosserhoff, 2002), with optimization steps being required to achieve successful efficiency (40-70%). Importantly, the number of passages of primary DPCs cultures must be low to prevent *in vitro* senescence and fibrosis, but high enough to expand a sufficient number of cells. In addition, a dual reporter gene system is recommended (at least in the first optimization steps) using firefly luminescence to normalize the luciferase signal generated by the promoter activity of a gene of interest. This allows transfection efficiency and cytotoxicity to be inferred. Lastly, false hits are a limitation of the luciferase reporter system. Successful hit triage and validation are needed so that only biologically significant hits are considered (W. Xie et al., 2016).

VIII

General discussion and final remarks

Chapter 8

General discussion and final remarks

Functional cells with HF inductivity need to be multiplied in order to turn hair cloning a clinical reality. Dermal papilla has for long been the main focus of research due to its innate hair inductive capacity. However, the mechanisms behind this capacity and their loss upon *in vitro* expansion are still insufficiently understood in the human.

For years, research using mouse models has demonstrated the DP inductive potential as the prospect for hair loss cure. Mouse models have certainly contributed to the understanding of HF biology and the preclinical validation (efficacy and side effects) of hair loss treatments. The brief hair cycle experienced by the mouse HF is useful in hair research, as it enables the analysis across several regenerative loops and even during the animal's lifespan. However, the structural and functional differences between mice and human HFs have limited the robustness of mouse models to meet the human hair loss therapeutic needs. In fact, the FDA approved drugs for hair loss treatment available so far, Finasteride and Minoxidil, were identified based on their side effects on human hair growth during clinical trials, and not from studies in mice. Hence, the work presented in this thesis aims to improve the understanding of the molecular mechanics controlling human DP hair inductive capacity and explore their modulation for the development of an effective hair loss therapy. We consider that the focus of hair translational research needs to shift from animal models to humans. Therefore, in this work, human-centered research was prioritized.

Firstly, we looked into the human native DP inductive signature and its change with distinct *in vitro* conditions. Once defined the mechanisms governing the DP trichogenicity, more targeted and effective solutions can be developed for treating hair loss disorders. Although numerous studies have illustrated the transcriptional signatures of mouse and DPC cultures (Z. Deng et al., 2022; Ge et al., 2020; R. Sennett et al., 2015; Shim et al., 2022), a comprehensive transcriptional profile for the human tissue was still missing. Thus, this work developed a rigorous experimental set up to identify the human DP transcriptomic signature and the molecular mechanisms controlling the loss of DPCs regenerative potential *in vitro*. We found that DPCs lose key molecular traits associated with natural hair regeneration characterized by profound changes in *NOTCH*, *WNT*, *BMP* and *SHH* pathways (Rishikaysh et al., 2014).

Interestingly, DPC cultures overexpress the *AR* receptor and *DKK1* (a major downstream target of *AR* (Furuya et al., 2022) suggesting that *AR* signaling pathway was activated *in vitro*. Therefore, one can speculate that *in vitro* DPCs may resemble, at some extent, DHT-sensitive DPs from the

balding scalp. In fact, a comparative analysis between 2D and 3D-cultured DPCs suggested that 3D spheroids are a more representative system to comprehend the molecular mechanisms involved in androgen-induced alopecia (Y. Zhang et al., 2021). Additionally, it should be noted that the DP may consist of a heterogeneous pool of cells containing both mesenchymal progenitor and differentiated cells (Gan et al., 2022). Upon culturing, it is possible that the progenitor populations are rapidly lost or outcompeted by the differentiated cells. Additionally, anagenic DP encompasses different structures that are not present *in vitro*, namely blood vessels and a matrix structure that supports DPCs regulation and whose composition remains elusive. Therefore, full recovery of the gene expression signature of intact DP is not expected. Nevertheless, future analysis of the upstream master programs controlling DP loss of hair inductiveness will be pivotal to the discovery of new therapeutic targets.

Moreover, we uniquely compared 2D and 3D cell cultures established from DPs of the same individual. This allowed to unequivocally identify molecular changes on the DPCs fitness associated with culture systems. 2D systems are the most commonly used for *in vitro* cellular studies but they impact cells' morphology and EC environment (Kapałczyńska et al., 2018). Theoretically, 3D culture systems are able to mimic the DP structure and size, and to provide EC mechanical regulation. Several studies have suggested that 3D cultures are advantageous over 2D cultures (Abaci et al., 2018; C. A. Higgins et al., 2013; B. M. Kang et al., 2012; Lin et al., 2020). Although we observed that 3D spheroids improve the expression of DP specific markers, it still proved insufficient to restore DP trichogenic signature and modestly advantageous over 2D culture. We found that DP spheroid cultures considerably activate the expression of genes associated with culturing. These findings also highlight the relevance of identifying upstream master programs controlling DP inductiveness.

Both *in vitro* conditions abruptly disrupted the DP trichogenic gene expression, diminishing its value for hair bioengineering and/or cellular therapy for hair rejuvenation. As a matter of fact, what makes the DP so unique is its ability to signal for hair growth. For long, removal of the DP from the HF unit was shown to stop adjacent epithelial growth and HF endurance (Crounse & Stengle, 1959; Horne et al., 1986; Jahoda et al., 1996). Hence, cultured non-trichogenic DP cells are no better than other dermal cell population present in the adult skin. Importantly, advances in cellular bioprinting may accommodate superior complexity by stimulating a more physiologically apt environment (Abaci et al., 2018). Efficient methods for generating complex tissue architectures will expectedly continue to emerge. Similarly, human organoids hold great promise for the long run (Lee & Koehler, 2021). However, the multiscale production of these engineered human tissues still faces significant challenges (as described in **Chapter 1**).

The stress signaling pathways activated during *in vitro* DPC culturing are likely responsible for the repressive gene signature, perhaps by inhibiting *TCF/LEF* transcriptional activity (J. Cheng et al.,

2021). Rationally, biomechanical properties (e.g., substrate rigidity) underlying *in vitro* DP cell culture may trigger their activation into a non-regenerative phenotype and more fibrogenic lineage, in a process that almost resembles a skin fibrotic injury (Mascharak et al., 2022).

Interestingly, dermal fibroblast progenitors expressing Engrailed1 (*EN1*), Twist-related protein 2 (*TWIST2*) and platelet-derived growth factor receptor α (*PDGFR\alpha*) are suggested to differentiate into papillary and reticular fibroblasts, which are different subpopulations of fibroblasts found in human skin (Ghetti et al., 2018; Korosec et al., 2019). Intriguingly, we found *EN1*, *PDGFR\alpha* and *TWIST2* overexpressed by *in vitro* culture. So, it is possible that changes in ECM caused by *in vitro* expansion are shifting dermal fibroblast progenitors' specialization into stressed resilient fibroblasts.

Our transcriptional studies identified TGF β -Smad2 axis as a key driver of DP stress *in vitro*. Therefore, in a targeted approach, we investigated the impact of TGF β signaling inhibition as a pro-fibrotic and DP dedifferentiation blocker. Using *in vitro* cell culture drug treatments and RNA-seq analysis, we defined galunisertib as an important modulator of TGF β stress signaling and established its crosstalk to DP inductive signature. We disclosed as a potential pharmacological modulation during DP cell therapy for hair rejuvenation and in future bioengineering approaches. Transcriptome analysis identified *TGF\beta* as a downstream player of AR signaling in DP cells (Furuya et al., 2022). In AGA, AR signaling is described to activate the expression of genes such as *TGF\beta1*, *TGF\beta2* and *DKK1* in the DP, which paracrinally suppress the growth of adjacent epithelial hair matrix cells and induce catagen onset (Inui et al., 2002; Kwack et al., 2008). In a recent study, TGF β pathway inhibition coupled with Wnt/ β -catenin activation was shown to promote rat vibrissa hair growth (J. I. Kang et al., 2022).

Logically, we set out to investigate TGF β signaling inhibition in the context of human HF cycle. For that, we resorted to *ex vivo* human HFOCs and evaluated the effect of galunisertib hair shaft elongation and catagen onset. We observed that galunisertib counteracts TGF $\beta2$ activation in the DP, which translated into improved hair matrix cell proliferation required to sustain anagen (Oh et al., 2016). Importantly, these findings disclosed galunisertib as a new potential therapy against AGA, as *TGF\beta* signaling was shown to be upregulated in the DP during AGA and it contributes to the regression of surrounding blood vessels (Z. Deng et al., 2022). *AR*-mediated regression of blood vessels is a major consequence of AGA pathogenesis. Yet, further human clinical trials are needed to undoubtedly examine galunisertib therapeutic potential on the human disease context (Dinh & Wang, 2022).

Intriguingly, different studies revealed TGF β signaling pathway as a main signaling in mouse HF development (Jamora et al., 2005; Owens, Han, Li, & Wang, 2008). A very recent study suggests that SCUBE3, a non-conventional TGF β ligand, is sufficient to induce new hair growth (Y. Liu et al., 2022). Our findings, however, support that TGF β inhibition promotes epithelial matrix

proliferation. Nevertheless, this dual paradoxical effects of TGF β during HF development and in response to androgens are still a subject under study. A specific threshold switch may dictate anagen or catagen in a given background (Dinh & Wang, 2022). Therefore, we consider that the overactivation of DHT induced TGF β signaling in the balding scalp will benefit from the galunisertib treatment.

Still, the unexpected finding that galunisertib improves epithelial cells' proliferation, together with its anti-inflammatory and anti-fibrotic effects, provides therapeutic avenues to manipulate HF tissue fibrosis and scar healing. Indeed, TGF β treated DP cells may have a broad prospect in regenerative medicine (Q. Liu et al., 2022), as they constitute an adult easy access source of progenitor-like cells. A recent study reported hair shaft miniaturization during AGA and aging as a consequence of mechano-induced HFSCs depletion (Y. Xie et al., 2022). This observation raises the question whether galunisertib could also prove valuable in delaying aging hair loss.

Finally, this work introduces novel methods to accelerate hair loss drug discovery by tackling key drivers of HF regeneration. We provided this framework as a tool to uncover novel drug candidates that enable the recovery of natural DP trichogenicity. In summary, with this framework we used *NANOG* and *LEF1* as genetic probes to identify drug compounds that recover the DP transcriptional signature by modulating the epigenetic landscape.

DP progenitor-like cells accumulate epigenetic memories of their past experiences (Niall J Logan, 2021). Consequently, they can proliferate *in vitro* by temporally changing the chromatin state. Thus, we envision that non-trichogenic DP cells that reestablish their epigenetic state *in vitro* may easily recover their trichogenic functions.

Additional work is needed to functionally validate the identified hit compounds for their role in HF induction and the value of these approaches as a pre-clinical model hair loss disease.

Similar to the “partial pluripotent reprogramming” that can restore youthful gene expression (Roux et al., 2022), drug-mediated epigenetic reprogramming of DPCs can reestablish the expression of a subsets of TF that restore the hair inductive signature. Nevertheless, we do not rule out that a cocktail of epigenetic compounds may be needed to fully recover intact DP functioning.

In our future research, we will broaden the list of candidates to include compounds which target specific master regulators of hair induction.

Importantly, this work did not directly measure whether *in vitro* changes in the DPC trichogenic signature were caused by changes in their epigenetic state. Future work is thus required to determine if the epigenetic modulation of key transcriptional regulators that we observed, is sufficient to improve DPCs function or even modulate the HF cycle. Moreover, considering that DPCs migrate and incorporate into existing hair follicles when transplanted, reestablishing key DPCs' TFs may also be correlated with improved hair rejuvenation outcomes.

Future perspectives

The successful transplantation of hair inductive cells has the potential to transform regenerative medicine approaches for alopecia treatment, and open new opportunities to repair or replace damaged hair in the context of disease or aging. We speculate that trichogenic DP cells can control autocrine and paracrine factors that regulate epithelial quiescence stem cells activation, in a process akin to what happens within HF morphogenesis and HF cycling. In the near future, we anticipate that technological advancement will allow testing strategies in human cell models as the gold standard in the hair research field. Omics approaches to characterize the molecular signatures of HF cell populations, in both bald and non-bald scalp regions, should be given priority in order to accelerate bioengineering strategies. This tactic shift will help to demystify the holy grail of human HF regeneration, as well as will prevent the recurrent drawbacks in the later stages of clinical testing or the overlooking of potentially effective treatments that fail in mouse model testing.

Moreover, even though HF tissue engineering has significantly advanced in recent years, its applicability to hair regeneration is far from been clinically and aesthetically appealing. Also, although *in vivo* transplant of bioengineered HFs is able to restore HF cycling, which is critical for functional HF regeneration, the architectural and functional complexity of this dynamic mini-organ turns it considerably more difficult to reconstruct than other larger organs.

Epigenetic regulators have been investigated by its promising therapeutic potential for numerous diseases (Y. Cheng et al., 2019). Interestingly, postnatal papillary fibroblasts lose their neonatal regenerative properties by changing its epigenetic signature during development (J. Y. Kim et al., 2022). Also, aged murine hair follicle SCs (HFSCs) show a reduction in chromatin accessibility which compromised their self-renewal and differentiation capacity (Koester et al., 2021). Rationally, we may consider that epigenetic reprogramming should also be explored to recover HF regeneration in the balding scalp. Finally, anticipate that the use of these epigenetic modulator compounds may disclose exciting new opportunities for hair loss outcomes.

A business viewpoint

The limited efficacy and the adverse side effects of the mainstream treatments have fostered the investment in new products and/or treatments for AGA, translated in the observed rise in marketed products (more than \$3.5 billion a year on hair loss products, most of them ineffective) (https://www.americanhairloss.org/hair_loss_treatment/). Importantly, the recent understanding of HF biology and cycling have triggered the development of novel therapeutic and cosmetic agents aiming to reverse HF miniaturization and to promote hair growth. Recent scientific advances in tissue engineering have already made the generation of cycling HFs possible in animal models. This growing prospect for the hair loss cure and its business potential (Otberg et al., 2007), has led to a

thriving entrance of new market players. Biotech companies have sprung up with the aim of developing more efficient or definitive treatments for AGA.

Interestingly, despite the high number of emergent biotech companies fully committed to develop an efficient therapy for hair loss (**Appendix, Table 10.1**), only a few approaches are actually being explored. In general, all companies are either focused on attenuating the androgens effects on HF miniaturization or using DP (or induced DP-like cells) to rejuvenate/regenerate hair. Is this convergence towards the same biological approaches wise? For those seeking for the ultimate hair loss cure, today's interventions appear insufficient and innovative strategies to reverse the balding state are awaited.

Nowadays, the faster time-to-market for drug discovery will certainly promote pharmacological approaches, and the straightforward solution of tissue stimulation will continue to primarily fulfill the growing market needs. Autologous cell-based therapies that do not resort to genetically modified or manipulated cells will easily pursue into clinical trials as no major ethical constraints are associated. Noteworthy, the age of digitization and automation has triggered data mining and machine learning for human therapeutic purposes. Digital biotech companies can streamline genomics, metabolomics and epigenomics data to find unanticipated therapeutical targets and to prompt more efficient solutions into the market. An example is *Life Code*, an early-stage biotech company which is resorting to an artificial intelligence (AI) platform (*PetaPoly 1.0*) to explore biological pathways and uncover new therapeutic molecules.

Direct-to-consumer (DTC) medical treatment is the market trend nowadays, and hair loss clinics and treatments must take it into account. Although more complex therapeutic solutions may require a business-to-consumer (B2C) strategy, where hair loss patients visit clinical centers regularly to be monitored by a multidisciplinary team (clinicians and perhaps biomedical engineers) to successfully endure the full treatment, the DTC therapies will continue increasing to answer the self-service demand and healthcare consumerism. Alopecia patients will ever more seek for convenient and cost-effective preventative self-screen and self-treatment solutions, and this will possibly impact on the future hair loss therapies.

We envision that a promising therapeutic approach would arise from the discovery of a druggable molecular target which modulation could dedifferentiate non-trichogenic cells from the balding scalp into trichogenic entities capable of evoking native scalp trichogenic signaling, i.e., anagen and *de novo* hair growth. Considering epigenetics as the major regulator of postnatal phenotypes, epigenetic reprogramming may hold the promise for hair loss cure, and it is conceivable that alternatively to cellular reprogramming using Yamanaka transcription factors, pharmacological epigenetic remodeling therapies may arise soon.

Finally, it must be pointed out that hair follicle restoration is still a major challenge on full skin regeneration and its use for skin restoration surgery (Vig et al., 2017). Therefore, the successful hair follicle regeneration can have major outcomes which goes beyond the hair loss cure.

IX

Bibliography

Chapter 9

Bibliography

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Appendix

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Appendix

Table 10.1. Companies developing hair loss therapies (see also <https://folliclethought.com/> and <https://www.hairlosscure2020.com/>).

		Company name	Product	Developmental stage	Concept	Patent	Website
DRUG / PROTEIN THERAPY	Hair growth stimulators	Follicle Pharma	FOL100	-	Nano-emulsions containing selected pharmaceutical particles	WO2021229578A1	folliclepharma.com
		Hope Medicine	HMI-115	Phase II	Monoclonal antibody targeting the PRL receptor	-	Hopemedinc.com
		Triplehair	Therapy-07	Phase III	Combination of 3 molecules	US20160346183A1	triplehair.ca
		Follicum/ Coegin Pharma	FOL-005	Phase II A	Topical solution containing modified osteopontin	WO2015159099A1	follicum.com
		Aneira Pharma	ANR-001	Phase 1	Topical hair growth formulation	WO2021026084A1	aneirapharma.com
		TechnoDerma Medicines	TDM-105795	Phase 1	Topical new drug candidate for hair growth	WO2016023510A1	tkskin.com
		Follica	FOL-004	Phase III	Wounding device and topical drug application	WO2019232242A1	follicabio.com
		Dermaliq Therapeutics	DLQ01	Phase II A	Liquid drug targeting the prostamide E2 (PGE2) receptor	WO2021028328A1	dermalig.com
		Samumed/ Biosplice therapeutics	SM04554/D alosirvat	Clinical trials (concluded)	Topical Wnt activator solution	WO2012024404A1	biosplice.com
	Androgen blockers	Kintor Pharmaceuticals	KX-826	Phase II	Topical AR antagonists	WO2011029392A1	en.kintor.com.cn
		Kintor Pharmaceuticals	GT20029	Phase I	Gel AR protein degrader	-	en.kintor.com.cn
		Aranda Pharma	ADA-308	R&D	AR signaling small molecule inhibitor	WO2013104830A1	arandapharma.com

CELL THERAPY		Olix pharma	OLX104c	Preclinical	AR RNAi-mediated repression	WO2018155890A1 WO2018004284A1	olixpharma.com
		Cassiopea/Cosmopharma	Breezula	Phase IIa	Topical AR inhibitor	WO2016207778A	cosmopharma.com
		Moogene Medi	-	Preclinical	CRISPR/Cas9 gene editing	WO2019078611A1	moogene.com
	Cellular reprogramming	dNovo	-	Preclinical	Reprogramming skin, blood, or fat cells into HFSC	patent pending	dnovobio.com
		Stemson therapeutics	-	Preclinical	Autologous cell therapy using iPSC-derived folliculogenic cells	WO2016007522A1	stemsontx.com
	untransformed	Replicel	RCH-01	Phase III	Autologous cell therapy using dermal sheath cup cells	WO2014205142A1	replicel.com
		Hairclone	-	Preclinical	Micro-injection of autologous multiplied DP cells into the scalp miniaturized area	WO2020079441A1	hairclone.me
		EpiBiotech	EPI-001	Preclinical	Autologous dermal papilla cell therapy	WO2020079441A1	epibiotech.com
		TissUse	SHT	Preclinical	Neopapillae Smart Hair Transplant	WO2019122291A1	tissuse.com