ABSTRACT N. 654

Dermopapilla: a self-renewing mini-organ reproduced in 3D scaffold free spheroids

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INTRODUCTION

The hair follicle (HF) is a self-renewing "mini-organ" which undergoes to continuous cycles of growth and regression, following a precise scheme in which a complex and fine-tuned interaction of signals induces deep metabolic and morphologic changes driving a growth phase (anagen), a regression (catagen) and a final quiescence (telogen). A 3D scaffold-free dermopapilla spheroid system has been developed and thanks to its high responsiveness to external stimuli it has been optimized to recapitulate 2 different metabolic contexts: physiological with an active growth phase (anagen) and regressive mirroring a forced involution model (catagen-telogen phase). An advanced model with higher morphological and functional similarity to *in vivo* was developed as vascularized system by co-culturing with microvascular endothelial cells (HMVEC) and extending to new potential applications:

- To screen the activity of compounds on different phases of HF cycle with an unique biological relevance
- To investigate drug metabolism and side effects on HF and microvasculature detoxifying activity
- Mechanistic studies mimicking HF cell cycle and diseases modelling

MATERIALS AND METHODS

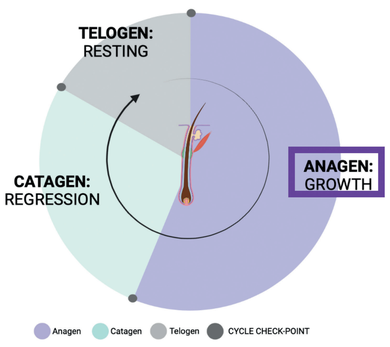
Spheroids were produced (Patent PO 5838 IT) with 5000-10.000 fibroblast (HDPF) alone or in co-culture with HMVEC (ratio 3:1) in Cnt-PR-F medium or in mix with EGM-2MV Medium (1:3 ratio) or with Human Hair Follicle Keratinocites (Ratio 5:1) in CnT-3D medium. Spheroids were transferred in culture plates and cultivated up to 14 days. For the forced involution model, TGFb1 10 ng/ml was applied to dermopapilla spheroids for 72H to force the anagen-catagen transition, then FGF18 10-100 ng/ml was applied for 24H mimicking a quiescent state. Vascularized dermopapilla was characterized with transcriptomic analysis of WNT pathway by nCounting NanoString© and at morphological level (on FFPE section Immunostaining: CK6 for epithelial compartment, Fibronectin, Versican and Collagen IV for dermopapilla and CD31 for endothelium). The vascularized system was exposed to Minoxidil (10µM), as reference of hair growth inducer.

RESULTS

DERMOPAPILLA IN PHYSIOLOGICAL HOMEOSTASIS (ANAGEN)

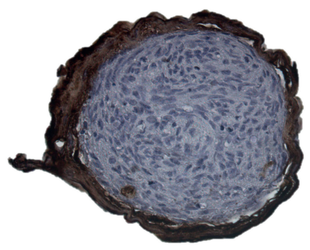
The ORA© dermopapilla model alone or with keratinocytes (Fig.1B) can be maintained in culture for up to 14 days in homeostatic conditions mimicking an anagen phase (Fig.1A).
Compared to a bidimensional model the dermopapilla system has expressed biomarkers related to the follicle growth phase such as BMP2 and FGF7 and in the presence of keratinocytes, a stable epithelial-mesenchyme cross talk is established as suggested by LAMC3 expression.

1A



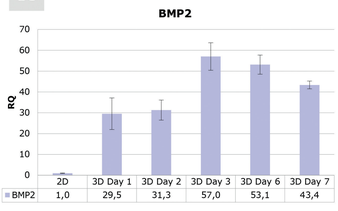
1B Spatial distribution of hair follicle keratinocytes and dermopapilla fibroblasts

(IHC ON FFPE SECTION)



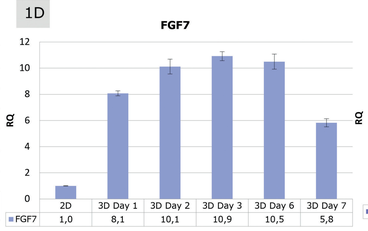
CK6 (HF KERATINOCYTES) HEMATOXYLIN (HF DERMAL PAPILLA)

1C



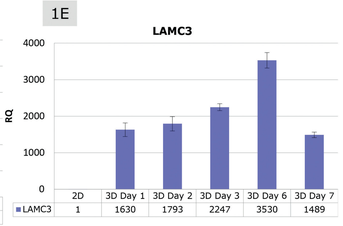
	2D	3D Day 1	3D Day 2	3D Day 3	3D Day 6	3D Day 7
BMP2	1,0	29,5	31,3	57,0	53,1	43,4

1D



	2D	3D Day 1	3D Day 2	3D Day 3	3D Day 6	3D Day 7
FGF7	1,0	8,1	10,1	10,9	10,5	5,8

1E



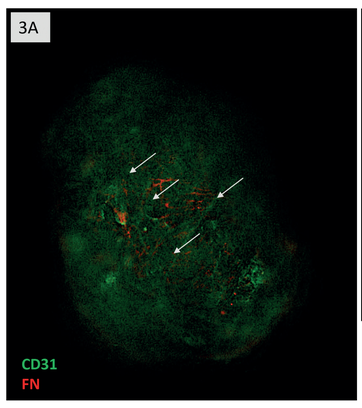
	2D	3D Day 1	3D Day 2	3D Day 3	3D Day 6	3D Day 7
LAMC3	1	1630	1793	2247	3530	1489

Fig. 1. A. Dermopapilla with keratinocytes maintained in homeostatic conditions mimicking anagen phase. **B.** Immunostaining of dermopapilla core (nuclei, blue hematoxilin) surrounded by keratinocytes stained by Keratin 6, CK6, (brown). **C-E** Gene expression analysis by RTqPCR in 7 days spheroid culture of anagen markers FGF7 and BMP2 and LAMC3 protein of basal lamina of hair matrix.

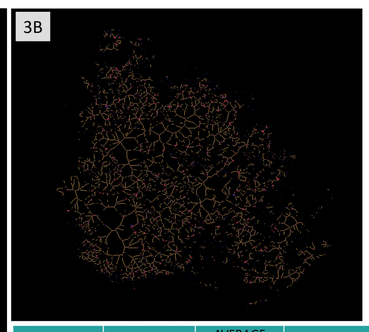
VASCULARIZED DERMOPAPILLA

The morphological characterization of vascularized dermopapilla was performed at day 5 and day 10 of culture to assess the establishment of the co-culture, the morphological organization of endothelial cells in the dermopapilla core (Fig. 3A-B) and to determine difference in transcriptional profile of the vascularized model compared to the dermopapilla (Fig. 3C).

3A



3B



N° BRANCHES (AVEARGE)	N° JUNCTIONS (AVERAGE)	AVERAGE BRANCH LENGHT	N° TRIPLE POINTS (AVERAGE)
3744	1591	1483.6	1153

3C

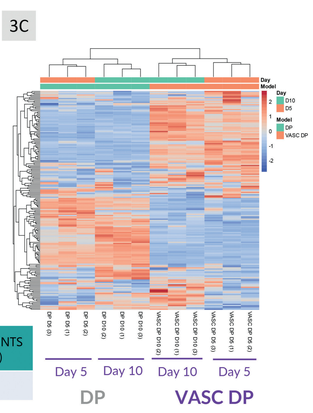


Fig.3 Morphology of vascularized dermopapilla spheroid (whole mount).

A. Immunostaining of endothelial cells with CD31 (green) and dermopapilla core with fibronectin (FN, red), a high-molecular weight glycoprotein of the dermal extracellular matrix, primitive vessel formation (white arrow).

B. 3D rendering and element counting of vascular branching. Skeletonize 3D Fiji plug-in on CD31 on endothelial cells has revealed: branched pattern with clear zones of branch continuity and segments interconnection, and more peripheral small segments. The presence of triple points indicate a physiological vessel-like formation.

C. Nanostring results on WNT codeset for standard dermopapilla (DP) and vascularized models at day 5 and day 10 of culture. Gene Set Analysis (Direct GSA) showed that the pathways connected to cell cycle and specific genes related to HF phases are predominant in standard dermopapilla, as a constitutive process in the core that generates signals for proliferation, while those related to adhesion and migration are more expressed in vascularized model as well as the WNT-regulators related to angiogenesis / vasculogenesis.

FORCED INVOLUTION MODEL

To investigate the progression of hair follicle cycle and, in particular, the involution related to anagen-catagen transition, the dermopapilla model was pushed in a catagen-like state by exposure during 72H to TGFβ1 followed by FGF18 to induce quiescence (catagen-telogen), reducing the expression on anagen related biomarkers (Fig. 2).

Marker	Function	Effect of TGFβ1 versus NC	Effect of FGF18 versus TGFβ1
FGF7	Fibroblast Growth Factor 7: highly expressed during anagen phases, driving hair elongation. It's expression decrease in regressive catagen phase	Downregulation (catagen-like)	Downregulation (telogen-like)
CCND1	Cyclin 1: proliferation gene expressed during anagen growth	Downregulation (catagen-like)	Downregulation (telogen-like)
WNT5B	WNT/beta catenin pathway is strictly related to anagen rising. Its expression is increased during telogen to anagen transition	N.A.	Downregulation (telogen-like)

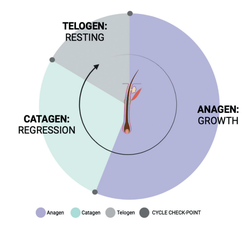


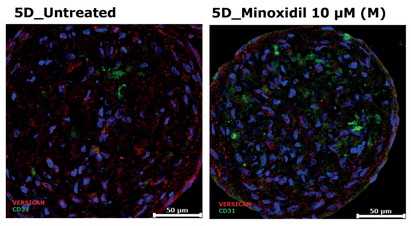
Fig. 2. Forced involution in dermopapilla model by sequential application of TGFβ1 to induce anagen-catagen transition and FGF18 for quiescente state.

The results after exposure to TGFβ1 highlight the transition from anagen-like phase to catagen and FGF18 furtherly decreases FGF7 inducing a quiescent-like state characterized by the downregulation of WNT5B, a key regulator of WNT/beta catenin pathway essential to hair follicle growth, and the downregulation of the proliferation marker CCND1.

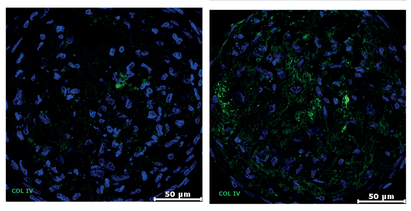
MODEL VALIDATION BY HAIR GROWTH INDUCER

Minoxidil[1] is a drug widely used to counteract hair loss due to its action on vascular compartment, the involvement in DHT conversion in androgenic alopecia and its efficacy in stimulating dermopapilla matrix proteins such as Versican, collagens.
To validate the responsiveness of the vascularized model the spheroids were treated with Minoxidil 10 µM during 5 days (Fig.4).
[1]Bejaoui, M. et al. 3D Spheroid Human Dermal Papilla Cell as an Effective Model for the Screening of Hair Growth Promoting Compounds: Examples of Minoxidil and 3,4,5-Tri-O-caffeoylquinic acid (TCQA). Cells 2022, 11, 2093

4A



4B



4C

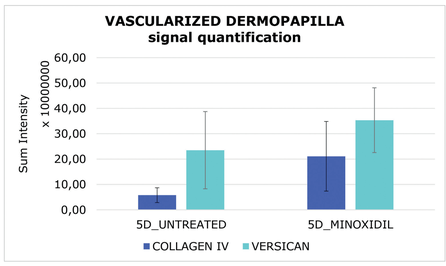
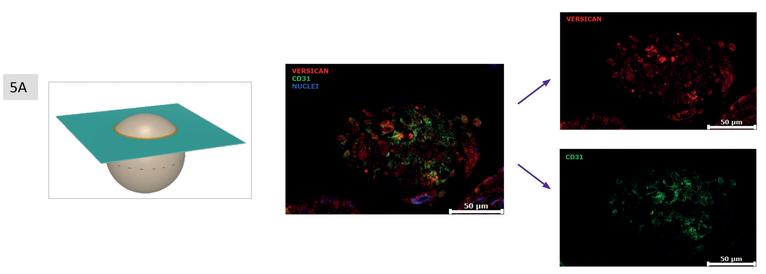


Fig. 4 Histological analyses on FFPE sections of vascularized dermopapilla treated with Minoxidil 10 µM for 5 days.
A IHC/IF of CD31 (green) and Versican (red), nuclei DAPI (blue)
B IHC/IF of Collagen IV (green) nuclei DAPI (blue)
C Signal quantification

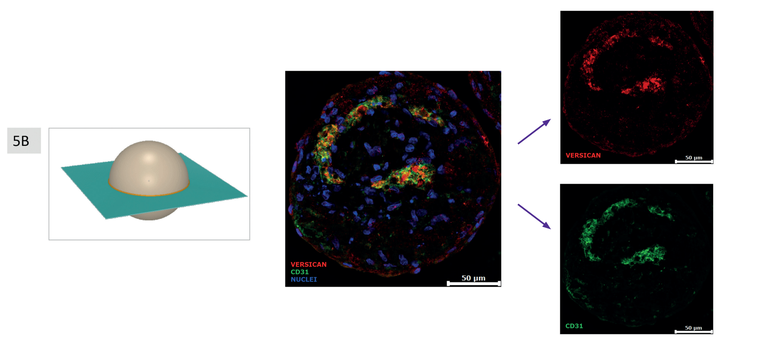
Versican is highly expressed during anagen and is part of the extracellular matrix proteins that bind and stabilize the hair follicle dermopapilla by connection of basal lamina of keratinocytes of outer root sheath. Minoxidil has significantly increased Versican as well as Collagen IV expression confirming its well know efficacy in boosting the extracellular matrix deposition, typical of HF active growth phase.

3D SPATIAL DISTRIBUTION, ORGAN-LIKE FUNCTIONALITY AND MORPHOLOGICAL PRECISION

5A



5B



VERSICAN: a key adhesion molecule involved in ECM interaction and fibroblast differentiation; it is highly expressed during the anagen phase.

CD31: expressed on endothelial cells, its overexpression suggests the formation and development of new blood vessels (angiogenesis) through cell-cell adhesion.

Fig. 5 Planes section of dermopapillary spheroids.
A) Secant not passing through the center of the spheroids
Paraffin-embedded dermopapillary spheroids sectioned in the apical part where the endothelial cells (CD31 signal) are more concentrated. Nuclei are rare in this spheroids area while Versican expression appears high.

B) Secant passing through the center of the spheroids
Centrally sectioned paraffin-embedded dermopapilla spheroids; endothelial cells have penetrated into the microtissue forming new vessels, as shown by the CD31 signal. Versican signal appears concentrated around the newly formed vessel.

CONCLUSIONS

The physiological and morphological complexity of the hair follicle and hair cycle as a mini-organ has been described with different applications:

- Dermopapilla and Hair Follicle: the first mimics the basal core of hair follicle in growing phase, expressing the key marker of anagen and maintaining homeostasis during 1 week. The second is a co-culture with keratinocytes that has shown an interesting interaction between epithelium and mesenchyme compartments. These models represent the simplest systems to investigate active compounds able to promote anagen phase and the cross-talk with the keratinocytes, increase hair growth as well as identify a specific drug side effect on hair growth.
- Regressive Dermopapilla: we succeed in mirroring the physiological signals that push the progression of hair follicle toward catagen and then telogen transforming the model in a regressive and quiescent dermopapilla in which anagen markers are progressively repressed. This system is unique to investigate biological properties of compounds indented to reduce or delay hair follicle growth or promote hair follicle loss.
- Vascularized Dermopapilla: an efficient endothelial network around the dermopapilla core has created an advanced tool for the investigation and screening of actives with the aim to ameliorate the transport of nutrients and the elimination of catabolites which are fundamental for healthy hair development.

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