HAIR FOLLICLE BULB DEVELOPED AS 3D SCAFFOLD FREE MICROTISSUE: **3D HF-MTS**

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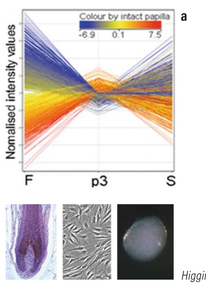
INTRODUCTION AND AIM OF THE STUDY

The hair follicle, a skin appendage, is a self-renewing "mini-organ" presenting several biological functions, such as skin repairing during re-epithelisation, dispersion of sweat and sweat gland products (e.g. pheromones and sebum). Moreover, it has a psychological and social importance in our society, so the study of its physiology and the development of hair-care products shows an increasing interest.
Hair follicle undergoes to a continuous cycles of growth (anagen phase) and regression (catagen phase) which imply deep morphological and physiological modifications. The renewing process starts in the bulb from dermal papilla (DP), a spherical core of mesenchymal cells, and subsequently involves the surrounding epithelial cells of the follicle. The molecular mechanisms underlying the HF cycle are complex and not fully understood but it is clear that the epithelial–mesenchymal interactions, and in particular the cross-talk between dermal papilla (DP) fibroblast and the keratinocytes of the outer root sheath (ORSK) play a pivotal role.

A DPF-ORSK co-culture model allows to investigate the mechanisms that regulate the HF cycle and to study the effects of active compounds in pre-clinical research. As reported in the study of Higgins et al. (2013, PNAS), dermal papilla cells deeply modify their gene expression profile when cultured as monolayer, but the transcriptional pattern can be partially restored when they are cultured as 3D spheroids. This draws the attention to the fact that the spatial distribution of the cells is fundamental in order to produce a verisimilar model and the use of spheroidal co-culture of DPF and ORSK is the optimal method, because it doesn't include scaffold, to create a "proto-follicle".
The aim of the present study was to create a spheroidal 3D model of hair follicle bulb (**3D HF-MTS**) based on the "hanging drop" method and to investigate its relevance and reliability for research studies and applications in cosmetics and dermatology. A multi-parametric approach was adopted in order to characterise the model at morphological and molecular level and to have a comprehensive view of its evolution during culture time.

The culture of derma papilla fibroblast in 3D spheroids partially restores the transcriptional profile of intact dermo-papilla (22% of totally differentially regulated transcripts) after passages in monolayer.

Fig.1



Higgins et al., 2013 — *Ohyama et al. 2012*

Fig.1a. Courtesy of Higgins et al. 2013. Transcriptional profile in ex vivo human hair follicle (F), dermal papilla cells in monolayer at passage (p3) and dermal papilla 3D spheroids (S).

Fig.1b. Courtesy of Ohyama et al. 2012. Schematic illustration of the outcome of gene ontology analysis of DP signature genes, enriched for those with binding functions, including signalling molecules, enzyme activity regulators and transcription factors. Previously unreported genes encoding membrane and extracellular matrix molecules were also detected

	PROS	CONS
Animal model	• Well established model for research study (possibility of knock out, gene silencing…)	• Ethical concern on animal experimentation • Low predictive power
***Ex vivo* Human Hair Follicle**	• Good predictive power • Possibility to analyse hair growth	• Low availability • Low through-put analysis • High inter - individual variability
***In vitro* mono or co-culture 2D Human Models**	• High through-put analysis • Low variability • Easy to be standardized	• Intrinsic limitation for primary cells due to limited number of passage in culture • Simplified structure • No 3D interactions • Poor predictive power
***In vitro* 3D scaffold free Human Models**	• Complex spatial structure recapitulating in vivo features • 3D cell interactions and endogenous extra cellular matrix (ECM) production • Low variability • Good predictive power	• Intrinsic limitation for primary cells due to limited number of passage in culture • Extremely well trained scientist

METHODS AND EXPERIMENTAL DESIGN

Human Hair Follicle Dermal Papilla Cells (HDPC) and Human Hair Follicle Keratinocytes (HHFK) (supplier: Innoprot-Spain) have been used to reconstruct proto-hair microtissue using the hanging drop method into GRAVITY^{PLUS} plates from InSphero (Fig.2a). The **3D HF-MTS** have been further transferred in GRAVITY^{TRAP} for long term cultivation in a 7-days time course experiment.
According to a dynamic and multi-parametric approach the **3D HF-MTS** have been harvested at different time points (Fig.2b) and characterized on the basis of the following parameters:

- **Viability by ATP measure** (Cell titer Glow, Promega)
- **Histo-morphological analysis**: performed by Hematoxilin-Eosin (Histoline) staining of 7µm formalin fixed paraffin embedded (FFPE) sections
- **Immunostaining**: performed on FFPE sections using anti-human CK6 (Sigma) primary antibody and DAB chromogenic detection method (Life-technologies) or anti-human COLIV (Santa Cruz) and fluorescent secondary antibody to monitor the presence and the maintenance of type-specific features. All the images have been captured using Leica Instruments (DM2500 microscope or SP2 confocal microscope) and LASX software.
- **Gene expression**: total RNA has been extracted from 8 3D HF-MTs per sample (RNAqueous kit, Life-technologies) and retro transcribed (RNAqueous kit, Life-technologies). The produced cDNA has been used for relative RTqPCR analysis by ΔΔCt methods using BMP2, FGF7, LAMC3 and GAPDH (as reference gene) TaqMan Assay and TaqMan Master mix in ABI7500 system Life-technologies). RTqPCT allowed to verify the **restoration of transcriptional profile** of specific genes, in comparison with 2D culture.

PCR MARKERS			
DERMAL PAPILLA	FGF7	Fibroblast Growth Factor 7	Higgins et al. 2013
	BMP2	Bone Morphogenetic Factor 2	Higgins et al. 2013
	LAMC3	Laminin Gamma 3	Higgins et al. 2013
IHC MARKERS			
HF KERATINOCYTES	CK6 (Cytokeratin 6)	Hair Follicle Cytokeratin (IRS6)	Havlickova et al. 2009
ADHESION MARKER	Collagen IV	Basal lamina collagen	Jahoda et al. 2012

Fig.2a

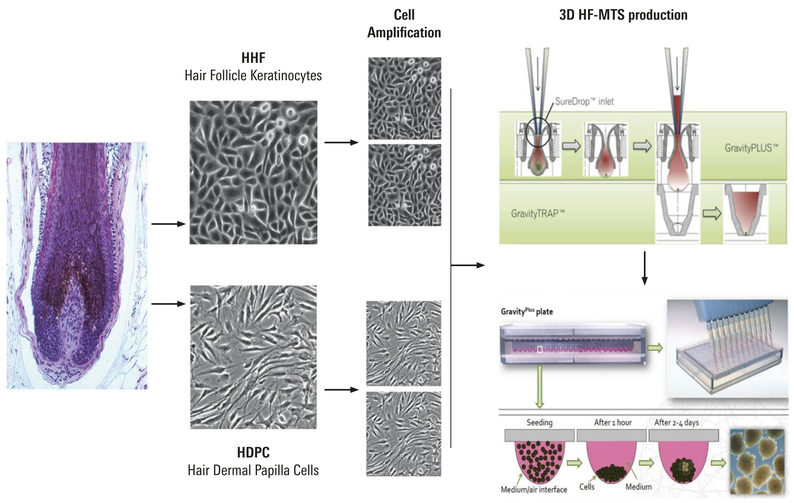


Fig.2 a. Production of Proto-hair Microtissues (3D HF-MTS) using cells from human hair follicle. After cultivation of cells in monolayer, Mts have been developped by co-culturing HHFK and HDPC following an optimized procedure based on hanging drop method and using InSphero plates.

Fig.2b

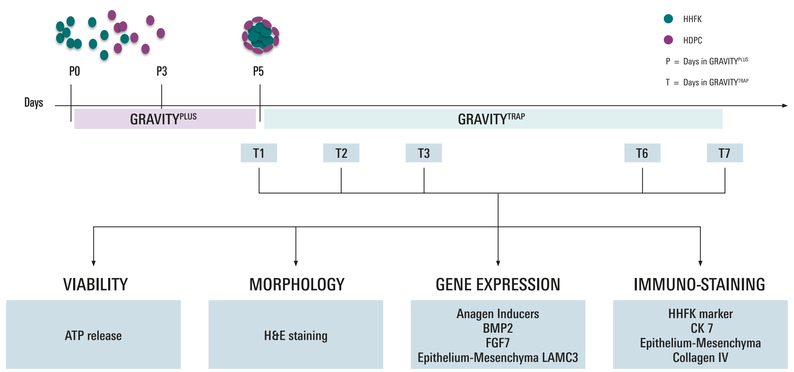


Fig.2 b. Experimental Design of time course experiment for 3D HF-MTS

RESULTS

Fig.3

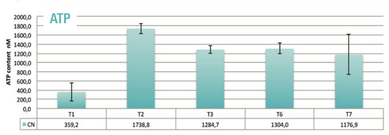


Fig.3 ATP (nM) content in 3D HF-MTS during 7 days of culture. Data are expressed as Mean and Standard Deviation (n=5 biological replicates)

Fig.4

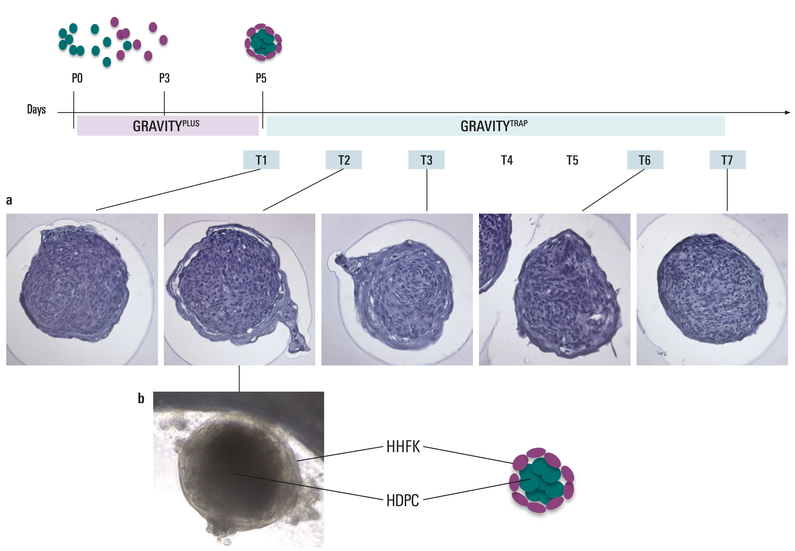


Fig.4 a. Hematoxilin-Eosin staining of 3D HF-MTS proto-hair at different days of culture. The spatially distincted and complex structure of the **3D HF-MTS** is evident. Magnification 20X

Fig.4 b. Bright Field Image of **3D HF-MTS** at T2 of co-culture, 4X Magnification. HHFK are disposed in a multi-layered translucent stratum surrounding the central dense HDPC core

Fig.5

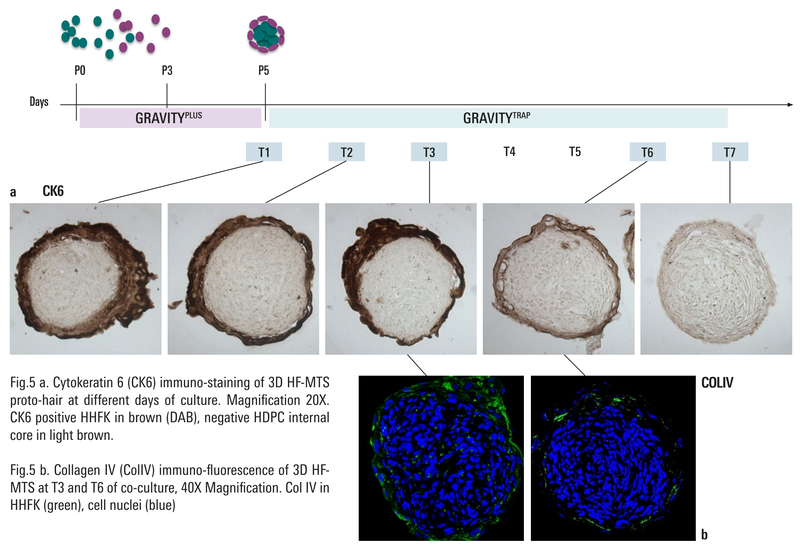


Fig.5 a. Cytokeratin 6 (CK6) immuno-staining of 3D HF-MTS proto-hair at different days of culture. Magnification 20X. CK6 positive HHFK in brown (DAB), negative HDPC internal core in light brown.

Fig.5 b. Collagen IV (ColIV) immuno-fluorescence of 3D HF-MTS at T3 and T6 of co-culture, 40X Magnification. Col IV in HHFK (green), cell nuclei (blue)

Fig.6a. Expression levels of Anagen Inducers genes

The anagen promoters BMP2 and FGF7 are strongly over-expressed in 3D HF-MTS in comparison with monolayer HDPC indicating a genetic profile of 3D models closer to in vivo situation. Their expression shows a time dependent evolution during the time course, collocating the developed model in a anagen like-phase with a possible regression starting at day 7.

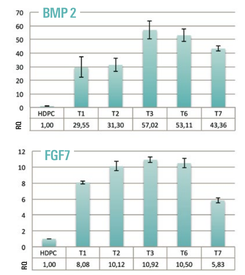


Fig. 6 Analysis of relative expression by q-RT PCR of anagen inducers (a) and marker of epithelium-mesenchyma (b) genes in 3D proto-hair during the time course experiment in comparison with Dermal Papilla fibroblast cells cultured in 2D. Data are expressed as relative quantification (RQ) mean and standard deviation (n=3)

Fig 6b. Expression level of Epithelium-Mesenchyma Interaction Marker

Also the epithelium-mesenchyma interaction, mediated by Laminin C (LAMC3) is restored in 3Ds spheroid in comparison with 2D culture and presents an evolution during the period with an increase up to day 6 and a subsequent decrease at day

CONCLUSIONS 1: VIABILITY AND MTS MORPHOLOGY

Viability of microtissue increases after at least 24h in gravity TRAP (T2) and stabilize until T6 (120H) indicating a good maintenance of metabolic functions.
At day 7 the viability values are less reproducible and with higher variability indicating a dynamic behaviour of the proto-hair and a possible starting of a regressive phase.

The morphological analysis has revealed, as expected, a pluri-stratified structure of 3D HF-MTS in which a core of dermal papilla fibroblast is surrounded by a multi-layered stratum of follicular keratinocytes. The thickness of the epithelial stratum increases until day 3 and then stabilizes until day 6. At day 7 a pluristratification is slightly visible and a decrease in thickness seems to occur.
As expected, the expression of CK6 is strong and specific in the epithelial layers composed by follicular keratnocytes (Havlickova 2009). The intensity of the staining and the thickness of the epithelial layers are marked and stable until day 3 then decrease until day 6. At day 7 no epithelial layer are stained.
Collagen IV is the major component of basal which anchor epithelial strata on connective tissue and is present in hair follicle in the basement membrane zone between derma papilla and follicular keratinocyte (Jahoda 2012). In proto-hair it is localized in follicular keratinocytes and its expression decrease from day 3 to day 6.

CONCLUSION 2: 3D SPHEROID AS A MODEL OF A CYCLING HAIR BULB

In conclusion, we have developed a proto-hair model -3D HF-MTS that presents a well-defined tridimensional structure with a precise spatial distribution of the 2 cell types and a functional epithelium-mesenchyma interactions. It can be cultured up to 7 days, mimicking the hair bulb and presenting morphological features similar to an active (anagen-like) dermal papilla and a potential regression starting from day 7.
In particular gene expression results have shown a time dependent development of the fully developed hair bulb model that better mimic intact dermal papilla in comparison with traditional 2D culture and the temporal evolution of our in vitro model compared to in vivo hair follicle cycle.
This project is currently under development in order to confirm these preliminary results, to better characterize the model for its dynamic evolution during an extended culture time and finally to investigate the rising of the regressive phase (catagen-like).
The biological relevance of established 3D HF-MTS model will be validated by testing reference molecules.

ACKNOWLEDGMENTS: the authors are grateful to Unifarco SpA for authorizing the use of Col IV localization IF images on control 3D HF-MTS

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