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Optimization of the Potential of Human Adult and Embryonic Stem Cells for Skin Tissue Engineering

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ABSTRACT
Present solutions for massive skin loss are yet not capable of inducing satisfying and functional skin regeneration. Despite having the longest history of commercialization, skin analogues lack in meeting completely the demands. In fact, the long-term function of the skin equivalents could be limited by the terminal differentiation of the grafted keratinocytes. Skin Tissue Engineering (TE), together with the revolutionary use of stem cells remains as a very exciting approach to meet the challenge of large-scale skin regeneration
Human Adipose Stem Cells (hASCs), known to share the main characteristics with bone marrow mesenchymal stem cells, which can give rise to epidermal-like cells, and hESCs, recognized due their extraordinary capability of proliferation and pluripotency, are central candidates for the generation of autologous skin TE models.
As efficient approaches towards relevant skin cell lineages are still to be concisely defined, this is a major issue that has been addressed in the present PhD. Thus, different strategies, based mainly in co-culture systems with adult cells, including indirect (Conditioned media and transwells®) and direct contact approaches, have been defined to differentiate stem cells into key skin cells, keratinocytes and endothelial cells.

Additionally, Epidermal Stem Cells (EpSCs), are another natural biological alternative. However, EpSCs isolation difficulty remains, due to the lack of well-determined approaches and markers. This PhD also integrates an assemblage of strategies that have been pursued to accomplish enrichment of this multipotent fraction.
Ultimately, the fundamental goal is to establish a suitable cell niche for skin TE approaches, using the defined cells. *Gellan gum*, a thermoresponsive hydrogel is herein proposed to mimic dermis mechanical features.

INTRODUCTION

Despite having the longest history of commercialization, skin analogues lack in completely meeting the regeneration demands (Priya *et al.*, 2008). In fact, the long-term function of the skin equivalents could be limited, among other factors, by the terminal differentiation of the grafted keratinocytes. The main ambition of this work-plan is to develop novel skin Tissue-engineered constructs that stimulate regeneration rather than dressing of full-thickness skin wounds.
Stem Cells have emerged as a powerful tool for treat a wide range of diseases, namely for Skin TE, providing an exclusive unlimited source of biological material. The potential of hMSCs and hESCs for skin regeneration has been studied, aiming at guarantying an active source of biological material, crucial for full-thickness skin defects and aiming at clinical application.
Adult MSCs integrate several mammalian tissues and although their primary function is homeostasis maintenance, they also seem to still express pluripotency markers, and appear to participate in the regeneration of other tissues than mesenchyme (Riekstina *et al.*, 2009; Trottier *et al.*, 2008). hASCs in particular, are

highly attractive due to their abundance and readily accessibility to subcutaneous adipose tissue and to the features shared with other MSCs. Besides that, hASCs can be used in autologous approaches, are easy to expand *in vitro* and present a great differentiation potential, including towards the endothelial and neuronal lineages, generated from the same germ layer as epidermis (Anghileri *et al.*, 2008).

hESCs, derived from the inner cell mass of pre-implantation embryos at blastocyst stage, are natural favourite candidates for obtaining the main skin cell types. The most obvious factors rely on their immortality capacity, self-renewal *ad* infinitum and differentiation into all components of the embryonic germ layers and subsequently all cell types that comprise human tissues (Stojkovic *et al.*, 2004). Thus, hESCs can potentially provide an extraordinary source of cells for TE together with a great insight into early embryonic development.

Skin itself is a large reservoir of stem cells, namely Epidermal Stem Cells (EpSCs), which are a naturally privileged sub-population in the skin tissue. Strategies to promote enrichment of this fraction are being currently undertaken (Watt, 1998), however there is still a need of efficient strategies to obtain this epidermal fraction. Specific markers of EpSCs are yet a controversial issue, however, an effort has been performed towards the characterization of these cells and to reunite techniques that would allow a better population purification (Jones and Watt, 1993; Kaur and Li, 2000; Li *et al.*, 1998; Watt, 1998). A class of candidate EpSCs markers includes β1-integrin (Watt, 1998) and the hemidesmossomal α6-integrin (Kaur and Li, 2000), as well as early keratin markers, such as K19, K14 and K15 (Lane *et al.*, 1991).

Gellan gum is an FDA-approved natural linear anionic extracellular polysaccharide secreted by *Sphingomonas paucimobilis*. It is characterized for its unique gelling behavior that involves temperature-dependent hydrogen bonding and cation-induced electrical incorporation. Studies in simulated physiological environment have proven the long-term fate of the gellan-based constructs to be compatible with the human body, which underlines its potential in TE approaches(Oliveira *et al.*, 2009).

MATERIALS AND METHODS

Establishment of hESCs Culture

SHEF3 hESC line was obtained from University of Sheffield, through UK Stem Cell Bank, and cultured in Mouse Embryonic Fibroblasts (MEFs), together with a rich culture medium that includes knockout serum replacement and basic fibroblast growth factor-2. Cells in every 3 passages were characterized by immunocytochemistry for pluripotency markers, namely Sox2, Oct3/4, Nanog, SSEA-4 and TRA-160.

Differentiation into keratinocytes

This particular study exploited three methodologies, based on both indirect and direct co-cultures of primary keratinocytes and hASCs or with hESCs, aiming at inducing commitment towards keratinocyte lineage precursors. In the indirect contact experiments, hASCs/hESCs were both exposed to conditioned media (CM) resulting from keratinocytes metabolic activity and cultured with primary keratinocytes using Transwell® Inserts. Cell differentiation was followed at days 7, 14 and 21 by immunocytochemistry using specific epidermal cell markers such as p63 and CK14. Additional gene expression analysis by real-time RT-PCR was performed for these main epidermal cell markers.

Boost and Characterization of Epidermal Stem Cells Fraction

Human primary keratinocytes (hKC) were isolated from human adult skin, after informed

patient consent, and following a protocol previously optimized under the scope of this PhD project. Different methods of culture/purification were applied, aiming at EpSCs fraction enrichment.
Rho-Associated Protein Kinase (Rock) Inhibitor Y- 27632 was firstly administered to freshly hKC cultures to increase EpSC number (Terunuma *et al.*). Consecutive selective methods such as rapid adherence to β1-integrin ligand in collagen type IV (Watt, 1998) and immunomagnetic separation methods were then performed to establish populations based in the α6/CD71 expression. In order to study the outcome of the proposed strategies, their clonogenic capacity, growth rate and long-term proliferation was compared to non-EpSCs enriched populations. CFUs assay, flow cytometry analysis and immunocytochemistry were then performed, focusing on the effect of the treatments, over expression rate of early epidermal markers keratins 19/5/14 and correlated with α6/CD71 sub-populations. Methodologies were refined based on the analysis of a significant number of skin samples.

Gellan Gum – hydrogels from dermal regeneration

Gellan gum powder was mixed with distilled water under constant stirring at room temperature to obtain a final concentration of 1% (w/v). The solution was progressively heated to 90ºC, under which complete and homogeneous dispersion of the material was obtained. The solution was kept at this temperature during 20-30 minutes, and after letting the temperature decrease until 42ºC, hASCs were ressuspended in medium and added in the gellan gum solution. The solution was casted into cylindrical moulds and allowed it to rest at room temperature for 2-5 minutes to form a solid gel.

RESULTS AND CONCLUSIONS

hASCs and hESCs cultured in epidermal differentiating conditions have shown a dependence with the tested culture conditions. Depending on the strategy chosen to induce differentiation, both hASCs and hESCs progressively assumed phenotypic characteristics that seem to indicate alteration of cell behaviour and differentiation.
Furthermore, as skin stem cells namely Epidermal Stem Cells (EpSCs) are a naturally privileged sub-population in the skin tissue, strategies to promote enrichment of this fraction are being currently undertaken (Watt, 1998). This comprehends extensive characterisation and employment of substrata/chemical stimuli, along with selective methods. These approaches allow the identification of the most promising source and methodology to obtain stem cells sub-populations to be further combined with polymeric scaffolds in novel Skin TE constructs.
Ultimately, the central goal is to establish the most appropriate cell niche for skin TE approaches, using the exploited cell sources. *Gellan gum*, a thermoresponsive hydrogel (Fig 1), is herein proposed to mimic dermis mechanical features.

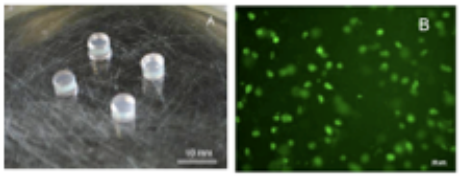

Fig. 1 (A) Thermoresponsive hydrogel, Gellan Gum. (B) Human Dermal Fibroblasts encapsulated in gellan gum membranes, after 3 days of culture, labelled with Calcein-AM, that stains live cells.

In addition to the obtained stem-derived skin cells, EpSCs and adult cells from human skin will be assembled with gellan-gum to form a skin tissue engineered construct (Fig.4B) aiming at treating full-thickness wounds.
The suitability of the proposed strategies will be assessed *in vitro* and validated *in vivo* by evaluating the regeneration efficiency in a mouse

full-thickness model. Ultimately, it will be possible to compare the potential of committed skin cells, stem-derived cells and pre-committed cells, the EpSCs.

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Mariana Cerqueira was born in 1983 in Porto, Portugal, where in 2006 concluded her BSc in Biology, in the Faculty of Sciences of the University of Porto. She is currently in her third year of PhD in *Tissue Engineering, Regenerative Medicine and Stem Cells* under supervision of Prof. Rui Reis, at 3B's Research Group. Part of her PhD project was carried out between June-09 and February-10, at *Stem Cell Laboratory*, King's College London, UK under co-supervision of Dr. StephenMinger.