EFFECTIVE USE OF MESENCHYMAL STEM CELLS IN HUMAN SKIN SUBSTITUTES GENERATED BY TISSUE ENGINEERING

M.A. Martin-Piedra[1,2], C.A. Alfonso-Rodriguez[1], A. Zapater[1], D. Durand-Herrera[1,2], J. Chato-Astrain[1], F. Campos[1,2], M.C. Sanchez-Quevedo[1,2], M. Alaminos[1,2,*] and I. Garzon[1,2]

[1] Tissue Engineering Group, Department of Histology, Faculty of Medicine, University of Granada
[2] ibs.GRANADA, Biohealth Institute, Granada, Spain

Abstract

Mesenchymal stem cells (MSCs) can differentiate toward epithelial cells and may be used as an alternative source for generation of heterotypical artificial human skin substitutes, thus, enhancing their development and translation potential to the clinic. The present study aimed at comparing four types of heterotypical human bioengineered skin generated using MSCs as an alternative epithelial cell source. Adipose-tissue-derived stem cells (ADSCs), dental pulp stem cells (DPSCs), Wharton's jelly stem cells (WJSCs) and bone marrow stem cells (BMSCs) were used for epidermal regeneration on top of dermal skin substitutes. Heterotypic human skin substitutes were evaluated before and after implantation in immune-deficient athymic mice for 30 d. Histological and genetic studies were performed to evaluate extracellular matrix synthesis, epidermal differentiation and human leukocyte antigen (HLA) molecule expression. The four cell types differentiated into keratinocytes, as shown by the expression of cytokeratin 10 and filaggrin 30 d post-grafting; also, they induced dermal fibroblasts responsible for the synthesis of extracellular fibrillar and non-fibrillar components, in a similar way among each other. WJSCs and BMSCs showed higher expression of cytokeratin 10 and filaggrin, suggesting these cells were more prone to epidermal regeneration. The absence of HLA molecules, even when the epithelial layer was differentiated, supports the future clinical use of these substitutes – especially ADSCs, DPSCs and WJSCs – with low rejection risk. MSCs allowed the generation of bioengineered human skin substitutes with potential clinical usefulness. According to their epidermal differentiation potential and lack of HLA antigens, WJSCs should preferentially be used.

Introduction

In recent years, tissue engineering has developed as an alternative regenerative medicine approach for the clinical treatment of severe skin diseases, as it allows the efficient treatment of large and deep skin conditions (Garzon *et al.*, 2015; Kober *et al.*, 2016). Numerous models of human skin, generated by tissue engineering, have been developed to date (Beaudoin Cloutier *et al.*, 2017; Bhowmick *et al.*, 2017; Forouzandeh *et al.*, 2010; Hartmann-Fritsch *et al.*, 2016; Klar *et al.*, 2018; Larouche *et al.*, 2016; Lloyd *et al.*, 2015; Pensalfini *et al.*, 2017; Reuter *et al.*, 2017; Strong *et al.*, 2017; Vig *et al.*, 2017). However, few clinical trials have been conducted to test the biological security (phase I and II assays) of human skin models (Boa *et al.*, 2013; Boyce *et al.*, 2006; Boyce *et al.*, 1999; Boyce *et al.*, 2017; Germain *et al.*, 2018; Moravvej *et al.*, 2016; Yamada *et al.*, 2012). Most of the clinically efficient skin models are organotypic structures, consisting of a dermal substitute of dermal fibroblasts immersed in biocompatible scaffolds and an epidermal layer of keratinocytes seeded on top of the stroma. These orthotypic models show high feasibility for epidermal differentiation and maturation because differentiation of epidermal cells strongly depends on epithelial-mesenchymal interactions (Smola *et al.*, 1993).

Development of organotypic human skin substitutes is strongly dependent on the isolation and

culture of autologous cells, isolated and expanded in culture from healthy donor skin. However, healthy skin biopsies are sometimes difficult to obtain, especially when the patient is in a critical condition due to large-scale burns and concomitant infections. In addition, a major limitation of this technique is the time required for the isolated cells to generate a sufficient number of cells for tissue engineering, especially in the case of keratinocytes. On the other hand, epidermal keratinocytes are terminally differentiated cells that are difficult to handle in culture in the absence of feeder layers, proliferate slowly and tend to dedifferentiate in culture (Garzon *et al.*, 2015; Kraft *et al.*, 2012). For all these reasons, alternative cell sources allowing for the generation of human skin substitutes, without the drawbacks associated with skin biopsies, are needed for an optimal clinical translation (Bhardwaj *et al.*, 2017).

Mesenchymal stem cells (MSCs) are adult cells with proliferation and differentiation capabilities, showing adherence to plastic, expression of specific surface markers and multipotent differentiation potential into adipocyte, chondrocyte and osteoblast lineages (Dominici *et al.*, 2006; Kim and Park, 2017; Pittenger *et al.*, 1999). MSCs are excellent candidates for tissue engineering applications due to their unique properties. MSCs have been described in a wide variety of human adult tissues (Gimble and Guilak, 2003). However, the four main sources of MSCs in humans are adipose-tissue-derived stem cells (ADSCs) (Zuk *et al.*, 2002), dental pulp stem cells (DPSCs) (Gronthos *et al.*, 2000), umbilical cord Wharton's jelly stem cells (WJSCs) (Wang *et al.*, 2004) and bone marrow stem cells (BMSCs) (Friedenstein *et al.*, 1976; Pittenger *et al.*, 1999). In addition to adipocyte, chondrocyte and osteoblast differentiation potential, MSCs have epithelial differentiation capability and heterotypical models of artificial tissues could be generated using MSCs as alternative cell sources (Garzon *et al.*, 2013). In this regard, preliminary reports support the epithelial differentiation capability of ADSCs (Baer *et al.*, 2013) and human tooth-derived stem cells (Dogan *et al.*, 2015). In turn, WJSCs are efficiently differentiated into cornea epithelial cells (Garzon *et al.*, 2014) and human skin as well as oral mucosa keratinocytes (Garzon *et al.*, 2013). BMSCs, the most characterised MSC source, can differentiate into several types of epithelial cells, including skin keratinocytes (Kokubun *et al.*, 2016; Mahdavishahri *et al.*, 2012; Mendez *et al.*, 2014; Wang *et al.*, 2014; Zhang *et al.*, 2012). Although these previous reports support the epithelial potential of MSCs, there are no studies comparing the keratinocytic differentiation capability of the four main MSC sources for skin tissue engineering.

When compared with skin keratinocyte cultures, MSCs have important advantages, including accessibility, high proliferation rate and easy culturing, as well as very low risk of malignant transformation after allogeneic transplantation as compared to embryonic stem cells (ESCs) or induced pluripotent stem cells (iPS) (Kim and Park, 2017). In addition, one of the striking properties of MSCs, from a translational point of view, is that these cells are immunoprivileged due to the low expression of human leukocyte antigen (HLA) complex molecules and may have immunomodulatory properties (Castro-Manrreza and Montesinos, 2015; Losk *et al.*, 2016; Shi *et al.*, 2011). Several studies have demonstrated that MSCs can be grafted into a host patient with a low rejection risk (Casiraghi *et al.*, 2008; Ma *et al.*, 2017; Reinders *et al.*, 2013). In this sense, development of human skin models using allogeneic MSCs differentiated into epidermal keratinocytes could prevent the above-described shortcomings derived from generation of autologous skin models. Thus, easier and faster generation of heterotypical models could be an advantageous approach for the translation of these advanced-therapy products as a therapeutical choice.

The aim of the present study was to compare four different models of heterotypical human bioengineered skin generated by using ADSCs, DPSCs, WJSCs and BMSCs as alternative epithelial cell sources. These models were evaluated *in vitro* or *in vivo* to determine the potential clinical usefulness of heterotypical human skin substitutes.

Materials and Methods

Generation of MSC primary cell cultures
To generate primary cell cultures of human dermis fibroblasts, skin biopsies were obtained from healthy donors. To generate primary cultures of ADSCs, WJSCs and BMSCs, small biopsies were obtained from adipose tissue, umbilical cord and bone marrow, as previously described (Alfonso-Rodriguez *et al.*, 2015; Cejka *et al.*, 2016; Dori *et al.*, 2017). To generate DPSC cultures, extracted sound third molars were obtained (Garzon *et al.*, 2017). In all cases, tissues were obtained from healthy donors subjected to surgical procedures not related to the present study. All patients gave informed consent and the study was approved by the Research and Ethics Committee of the Health System of Granada Hospitals, Spain.

Upon arrival at the laboratory, tissues were washed in phosphate-buffered saline (PBS), dissected to isolate tissues rich in MSCs and enzymatically digested for 6 h at 37 °C under slight agitation with a 2 mg/mL solution of *Clostridium histolyticum* type I collagenase (Gibco). Isolated human dermal fibroblasts and ADSCs, DPSCs and BMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10 % foetal bovine serum (Sigma-Aldrich) and 1 % antibiotic and antimycotics (100 U/mL penicillin G, 100 mg/mL streptomycin and 0.25 mg/mL anphoterycin B; Sigma-Aldrich) under standard culture conditions. WJSCs were cultured in AmnioMAX™ medium (Gibco), as previously reported (Alfonso-Rodriguez *et al.*, 2015). Culture medium was changed three

times a week and cells were dissociated with 0.25 % trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) before reaching confluence.

Generation of human heterotypic skin (HHS)
HHS was developed using 24 mm Transwell® porous inserts with polycarbonate membranes with a pore size of 0.4 µm (Costar, Corning Inc.). Briefly, a stromal substitute was generated using a mixture of human fibrin obtained from frozen plasma (Human Tissue Bank of Granada, Spain) and 0.1 % type VII agarose in PBS. To produce a 5 mL dermal substitute, 3.8 mL of human plasma were added to 375 µL of DMEM with 500,000 cultured skin fibroblasts. To prevent gel fibrinolysis, the mixture was supplemented with 75 µL of tranexamic acid (Amchafibrin, Fides Ecopharma, Barcelona, Spain). Then, 500 µL of 1 % $CaCl_2$ were added to induce fibrin polymerisation, followed by immediate addition of 250 µL of melted concentrated agarose (2 % in PBS) at 40 °C. The final concentration of agarose in the stromal substitute was 0.1 %. Finally, the mixture was rapidly aliquoted on porous inserts and allowed to solidify at 37 °C for 2 h. 24 h after the stroma had solidified, 250,000 MSCs were seeded on top of the stromal layer to generate an epithelial-like layer consisting of each MSC type in order to generate ADSC-HHS, DPSC-HHS, WJSC-HHS and BMSC-HHS substitutes. All HHS were cultured for 7 d submerged in culture medium and cultured for 21 d following an air-liquid culture technique (Garzon *et al.*, 2014) to induce epithelial differentiation (4 weeks in total). Heterotypic artificial skin substitutes were cultured in keratinocyte inductive medium consisting of a 3 : 1 mixture of DMEM and HAM's F12 culture medium supplemented with 10 % foetal bovine serum (FBS), 1 % antibiotic/antimycotic solution, 24 µg/mL adenine, 0.4 µg/mL hydrocortisone, 5 µg/mL insulin, 10 ng/µL epidermal growth factor and 1.3 ng/mL triiodothyronine (all reagents were purchased from Sigma-Aldrich).

In vivo evaluation
To evaluate them *in vivo*, HHS were grafted in 6-week-old immune-deficient athymic nude *Foxn1nu* mice (Envigo, Huntingdon, UK) (Fig. 1). Briefly, mice were anesthetised using a mixture of 0.001 mg/g of body-weight of acepromazine (Calmo-Neosan, Boehringer Ingelheim, Ingelheim am Rhein, Germany) and 0.15 mg/g of body-weight of ketamine (Imalgene 1000, Merial labs, Barcelona, Spain). Then, a skin

area of 20 mm in diameter was surgically removed from the dorsal area of each animal and the HHS was trimmed to the same diameter. HHS samples were grafted at the injury site using absorbable suture material. A plastic ring was implanted at the injury borders and sutured using the same suture material to prevent the host skin from shrinking; the plastic ring was carefully removed after 10 d post-grafting. Only one graft HHS was implanted per mouse. Mice were euthanised 15 or 30 d after implantation of the bioengineered tissues, for histological analysis of grafted HHS and surrounding native skin. 4 mice per MSC group (ADSCs, DPSCs, WJSCs and BMSCs) were used.

Histological analyses
Control native human skin (NHS) and HHS kept *in vitro* (for 1, 2 and 4 weeks) and grafted *in vivo* (for 15 and 30 d) were fixed in 4 % formaldehyde for 24 h at 4 °C and embedded in paraffin. 5 µm-thick tissue sections were stained with haematoxylin-eosin for histological analysis. 5 independent sample replicates were analysed per group.

To evaluate the synthesis of extracellular matrix (ECM) components in the bioengineered dermis, the presence of collagen fibres, glycosaminoglycans (GAGs) and proteoglycans was assessed by histochemistry. First, the presence of collagen fibres was evaluated by picrosirius red histochemistry using Sirius red F3B (Sigma-Aldrich) for 30 min, as previously described (Alfonso-Rodriguez *et al.*, 2014). Fibre organisation was assessed by polarised light microscopy. To identify the presence of glycoproteins in the HHS, the Schiff periodic acid histochemical method (PAS), with Harris's haematoxylin counterstaining for 1 min, was used. Finally, samples were incubated in alcian blue histochemical solution for 30 min and counterstained with nuclear fast red solution for the detection of extracellular proteoglycans (Alfonso-Rodriguez *et al.*, 2014).

Epithelial layer analysis was performed by immunofluorescence for the epidermal markers cytokeratin 5 (CK5), cytokeratin 10 (CK10) and filaggrin (FGN). Briefly, tissue sections were deparaffinised and rehydrated before antigen retrieval with citrate buffer for 20 min at 95 °C. Then, samples were prehybridised with normal horse serum (Vector Laboratories) for 1 h and incubated with prediluted anti-CK5 and anti-CK10 (Master Diagnostica, Granada, Spain) or 1 : 50 anti-FGN (Abcam) primary
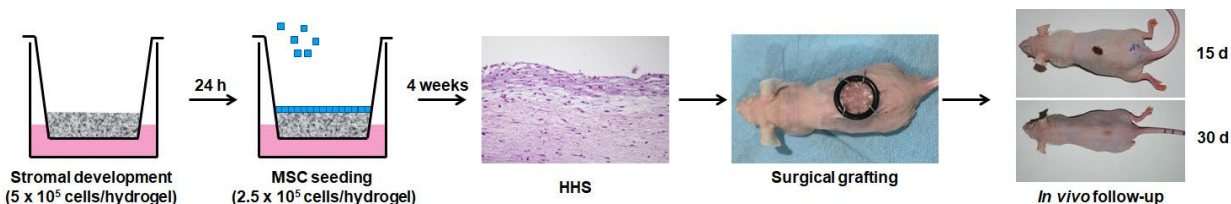

Fig. 1. Generation of heterotypical bioartificial human skin substitutes by tissue engineering and surgical grafting in animal models for *in vivo* evaluation.

antibodies overnight at 4 °C. Anti-mouse secondary antibodies conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich) were used at a 1 : 500 dilution. Sections were counterstained with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

To identify the presence of major histocompatibility complex molecules, routine immunohistochemical methods were used. First, samples were dewaxed and rehydrated and endogenous peroxidase was quenched with H_2O_2. Then, tissues were prehybridised and incubated with primary antibodies against type I HLA (HLA I) and type II HLA (HLA II) diluted at 1 : 200 (Santa Cruz Biotech). Positive signal was visualised using 3, 3-diaminobenzidine (DAB; Vector Laboratories) and tissue sections were counterstained for 15 s with Harris's haematoxylin.

In all cases, light microscopy images were obtained using an Eclipse 90i microscope (Nikon). Results of the histochemical and immunohistochemical analyses were semi-quantitatively analysed as previously described (Garzon *et al.*, 2014; Garzon *et al.*, 2009b). Staining was categorised as strong (+++), mild (++), slight (+) or negative (−). The expression was also stated following the topograpycal location of the signal as basal layer ([a]), spinosum layer ([b]), granulosum layer ([c]), cornified layer ([d]) or all layers ([e]). In all cases, average results corresponding to the analysis of 5 replicate samples were obtained.

Preliminary gene expression analysis

To determine the putative differentiation potential of each MSC type, ADSCs, DPSCs, WJSCs and BMSCs were kept in culture in basal medium (DMEM supplemented with 10 % foetal calf serum and antibiotics-antimycotics) or epithelial induction medium (QC) for 28 d. Then, total RNA was isolated from each cell culture using RNeasy Mini Kit (Qiagen) and retrotranscribed to cDNA using a reverse transcriptase (Superscript II, Life Technologies) and a T7-oligo(dT) primer. Biotinylated cRNA was generated using a T7 RNA polymerase and biotin-11-uridine-5'-triphosphate (Enzo Diagnostics, Farmingdale, NY, USA), chemically fragmented and hybridised to Affymetrix Human Genome U133 plus 2.0 oligonucleotide arrays (Affymetrix Inc., Santa Clara, CA, USA) for 6 h at 45 °C. 4 samples were analysed per group ($n = 4$). Expression levels found for each probe-set in the array were classified as present (P), marginal (M) or absent (A) for each sample by using the Affymetrix Suite software (Affymetrix Inc.). Affymetrix NetAffx Analysis Center (Affymetrix Inc.) was used to identify all genes in the array with a putative epithelial-cell-related function. All probe-sets showing an expression increase after QC induction (for example, an absent gene in cells cultured with basal medium that became present upon QC culturing) were selected for each MSC type. To determine the gene functions associated with
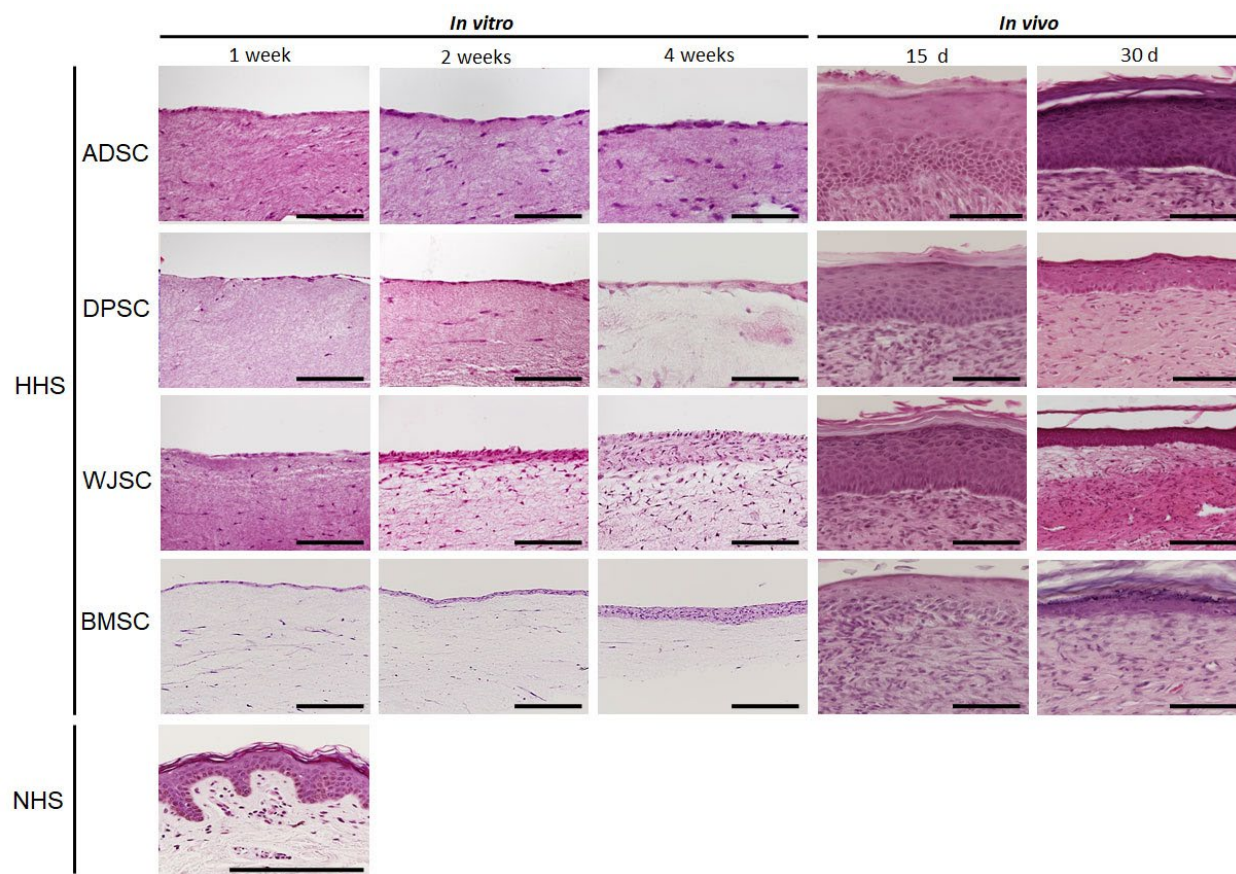

Fig. 2. Haematoxylin and eosin histological analysis of NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.

selected probe-sets, BiNGO gene ontology analysis (Ghent University, Belgium) was used as previously described (Maere *et al.*, 2005).

Results

Gene expression analysis

Preliminary gene expression analysis suggested that QC differentiation medium induced the expression of several genes in the 4 MSC types evaluated (Raw Data file 1). Out of the 54,613 array probe-sets, 4,351 (7.97 % of all probe-sets) became upregulated upon induction in ADSCs, with 172 being identified as epithelial genes. For WJSCs, 3,670 probe-sets (6.72 %) were upregulated, with 184 corresponding to epithelial genes. When BMSCs were analysed, 2,913 probe-sets (5.33 %) were upregulated, with 103 being identified as epithelial genes. Finally, 2,545 probe-sets (4.66 %) were upregulated in DPSCs, with 106 corresponding to epithelial genes. BiNGO gene ontology analysis identified 98 gene functions associated with all probe-sets activated in ADSCs, 76 in WJSCs and 23 in BMSCs and DPSCs (Raw Data 2 file). Although

most 'GO' gene functions corresponded to unspecific cell components and cell physiology, some of these functions were associated with cell adhesion and cell-cell junctions.

In vitro and *in vivo* histological analysis

In vitro generation of HHS substitutes using ADSCs, DPSCs, WJSCs and BMSCs allowed for the development of an epithelial-like layer from the first week of culture in all study groups (Fig. 2). HHS generated using transdifferentiated ADSCs and DPSCs were able to form an epithelium-like structure composed of a single cell layer along the 4 weeks of *in vitro* culture without any sign of terminal differentiation.

Grafting of HHS for the treatment of skin wounds did not generate any signs of inflammation, necrosis or other harmful effects. After 15 d post-grafting, wound diameter showed an average contraction of 45 % as compared to the original injury for all experimental groups and 61.7 % after 30 d (Fig. 1). However, after *in vivo* grafting, both cell populations induced the generation of up to 10 epithelial-like layers after 15 and 30 d. Haematoxylin-eosin histological
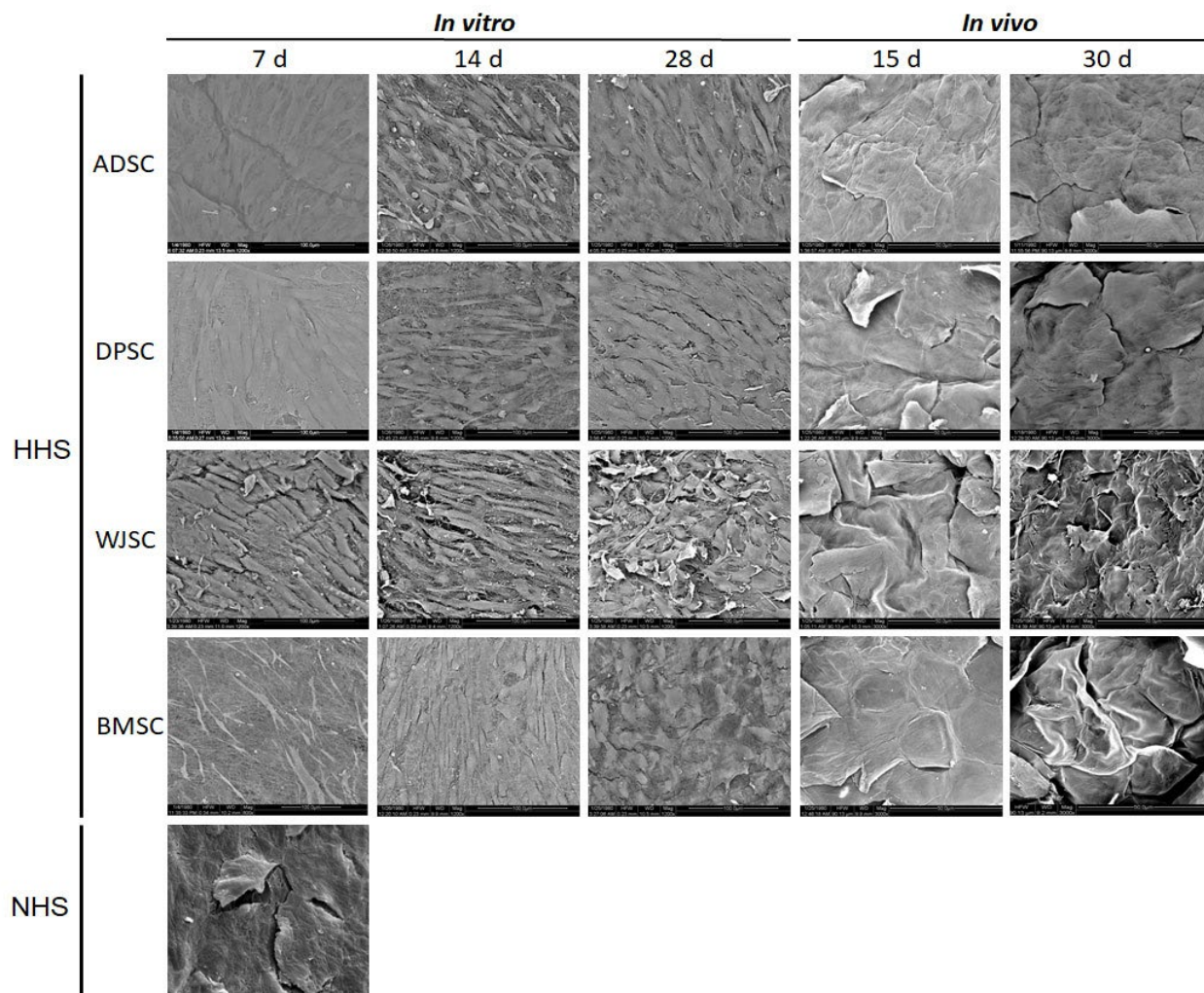

Fig. 3. Analysis of the epithelial surface of NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d.

staining revealed the presence of basal, spinosum, granulosum and cornified strata both in ADSCs and DPSCs once grafted *in vivo*, being similar to NHS. In addition, non-dermal papillae and epithelial rete ridges were identified *in vitro* or *in vivo*. When WJSCs and BMSCs were used as epithelial substitutes, a single layer was observed after 1 week *in vitro*. In contrast, after 14 d, the epithelial-like tissue formed more than 2 layers. The number of formed layers was time-dependent, with the identification of more than 6 layers after 28 d in both WJSCs and BMSCs, more being formed when WJSCs were used. Strikingly, all cell layers were morphologically similar and no signs of stratum differentiation were found *in vitro*. After *in vivo* implantation, epithelial cells showed a high degree of organisation and differentiation in basal, spinosum, granulosum and cornified strata, as in the case of NHS, although rete ridges and dermal papillae were not identified. The analysis of the epithelial surface of the different HHS, as determined by scanning electron microscopy (SEM), demonstrated that the MSCs used as epithelial-like substitute *in vitro* had the typical spindle-shape, elongated morphology of native MSCs (Fig. 3). However, cells tended to partially change their morphology and acquire a more polygonal shape with time in culture, especially in the case of WJSCs and BMSCs at day 28. Once grafted *in vivo*, the four MSC types showed a clear morphological change and cells became clearly flat and polygonal, showing keratinisation and desquamation signs, especially after 30 d of *in vivo* development.

In vitro and *in vivo* evaluation of the dermal substitute

The dermal substitute was mainly composed of cellular and extracellular components. A spindle-shape cell population was found in all *in vitro* HHS substitutes and the cell number tended to increase once grafted *in vivo* (Fig. 2).

The ECM is mainly composed of fibrillar and non-fibrillar molecules. In terms of fibrillar components, collagen fibres were identified by picrosirius red staining (Fig. 4, Table 1). *In vitro* development did not show any sign of mature collagen synthesis in ADSC, DPSC, WJSC or BMSC HHS samples. However, *in vivo* grafting resulted in the expression of collagen fibres, mainly in WJSCs after 15 d and in DPSCs, WJSCs and BMSCs after 30 d, with similar amount and fibre organisation as compared to NHS. However, ADSCs showed mild expression of collagen from day 15.

Regarding the non-fibrillar components of ECM, GAGs staining by PAS was negative in all *in vitro* samples. Furthermore, *in vivo* implantation of HHS using MSCs resulted in mildly positive expression of GAGs at 30 d in DPSC, WJSC and BMSC HHS and positive signal in ADSC HHS at the basement membrane. Interestingly, the staining signal found in ADSCs corresponding to day 30 was comparable to that of NHS (Fig. 5, Table 1).

Alcian blue staining detected proteoglycans as other important non-fibrillar components of the ECM (Fig. 6, Table 1). In this sense, *in vitro* development of HHS resulted in a lack of expression of these molecules. Once HHS samples were implanted in an animal model, proteoglycans synthesis reached intermediate levels of expression in ADSCs, DPSCs and WJSCs after 15 d. BMSCs revealed stronger synthesis with similar levels to NHS after 15 d. Moreover, at 30 d post-implantation, all MSC groups showed high expression of proteoglycans with amounts similar to NHS.
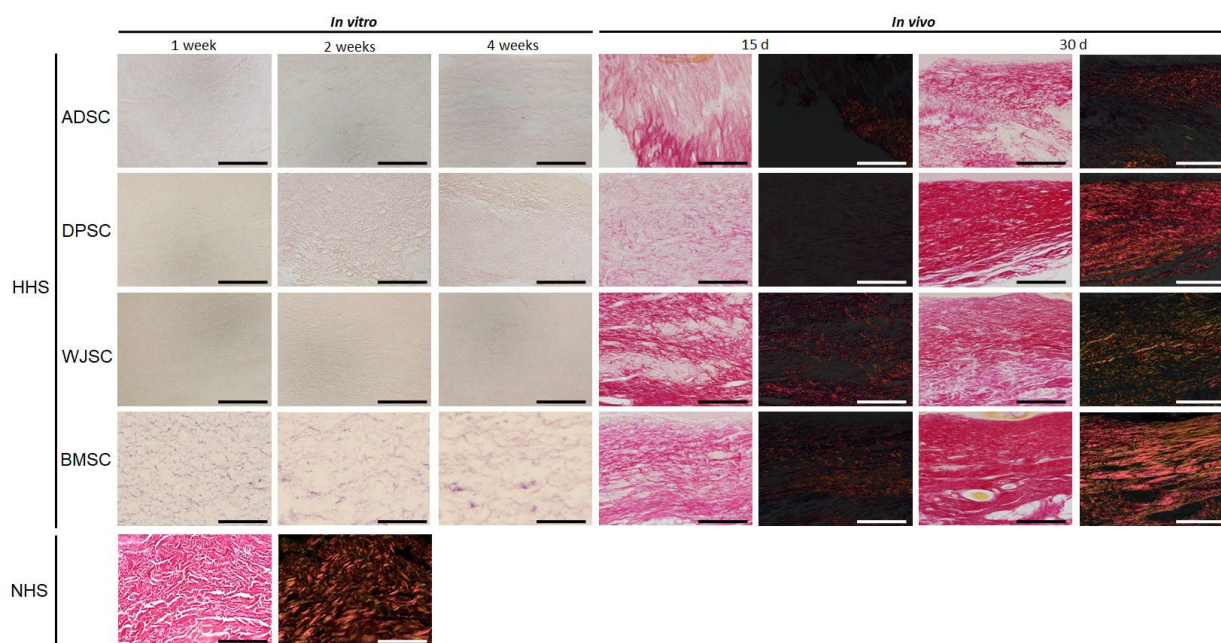

Fig. 4. Picrosirius red histochemical analysis of NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.

Table 1. Semiquantitative analysis of ECM components, epithelial markers and HLA antigens in native and heterotypical bioengineered human skin. The well-organised collagen fibres of the ECM components are indicated by an asterisk. The staining reaction was categorised in each sample as strong (+++), mild (++), slight (+) or negative (−). For epithelial markers (CK5, CK10 and FGN), the positive expression was stated as basal layer ([a]), spinosum layer ([b]), granulosum layer ([c]), cornified layer ([d]) or all layers ([e]). [§] indicates that the expression was mainly detected at basal stratum, but it could be present with lower intensity in other strata.

			NHS	HHS	*In vitro*			*In vivo*	
					1 week	2 weeks	4 weeks	15 d	30 d
Extracellular matrix	Collagen fibres	ADSCs	+++ (*)		−	−	−	++	++
		DPSCs			−	−	−	+	+++(*)
		WJSCs			−	−	−	+++(*)	+++(*)
		BMSCs			−	−	−	++(*)	+++(*)
	GAG	ADSCs	++		−	−	−	−	++
		DPSCs			−	−	−	−	+
		WJSCs			−	−	−	−	+
		BMSCs			−	−	−	−	+
	Proteoglycans	ADSCs	+++		−	−	−	++	+++
		DPSCs			−	−	−	++	+++
		WJSCs			−	−	−	++	+++
		BMSCs			−	−	−	+++	+++
Epithelial markers	CK5	ADSCs	+++[a,§]		−	−	−	++[e]	++[e]
		DPSCs			−	−	−	++[e]	++[e]
		WJSCs			−	−	+[e]	++[a§]	++[a§]
		BMSCs			+[e]	+[e]	+[e]	++[a§]	++[a§]
	CK10	ADSCs	+++[c,d]		−	−	−	−	+[c]
		DPSCs			−	−	−	−	+[c]
		WJSCs			−	−	+[e]	++[c,d]	++[c,d]
		BMSCs			−	+[e]	+[e]	−	++[c,d]
	Filaggrin	ADSCs	+++[d]		−	−	−	+[c,d]	++[c,d]
		DPSCs			−	−	−	+[c,d]	++[c,d]
		WJSCs			−	−	−	+[c,d]	+++[d]
		BMSCs			−	−	−	−	++[c,d]
Immunogenic potential	HLA I	ADSCs	+++		−	−	−	−	−
		DPSCs			−	−	−	−	−
		WJSCs			−	−	−	−	−
		BMSCs			−	+	+	−	−
	HLA II	ADSCs	++		−	−	−	−	−
		DPSCs			−	−	−	−	−
		WJSCs			−	−	−	−	−
		BMSCs			−	−	−	+	+

***In vitro* and *in vivo* expression of skin epithelial markers**

CK5 expression was negative *in vitro* in ADSCs and DPSCs and positive in all layers after *in vivo* implantation. Moreover, WJSCs were slightly positive for CK5 expression after 4 weeks of *in vitro* development and signal notably increased after 15 and 30 d of implantation, resembling the expression pattern of native human skin – with positive expression mainly in the basal layer (Fig. 7, Table 1). Finally, BMSCs showed a slight expression of CK5 at 1, 2 and 4 weeks in all layers *in vitro* and mimicked the expression pattern of native human skin *in vivo*. The expression of CK10 in HHS samples was negative for ADSCs and DPSCs after 4 weeks of *in vitro* culture. Interestingly, a slight expression of CK10 was detected in all layers of BMSC samples after 2 weeks of *in vitro* development and after 4 weeks *in vivo*. Similar expression levels were achieved in WJSCs after 4 weeks (Fig. 8, Table 1). Once grafted *in vivo*, WJSCs were the first MSC population able to generate epithelium-like cells expressing mild levels of CK10 in the cornified and granulosum layers 15 d post-implantation, which was maintained up to 30 d. At the end of the study, BMSCs and DPSCs showed a similar mild expression pattern of CK10, whereas ADSCs revealed a lower CK10 signal, confined to the granulosum layer.

According to FGN expression, *in vitro* analysis did not show any sign of epithelial maturation (Fig.
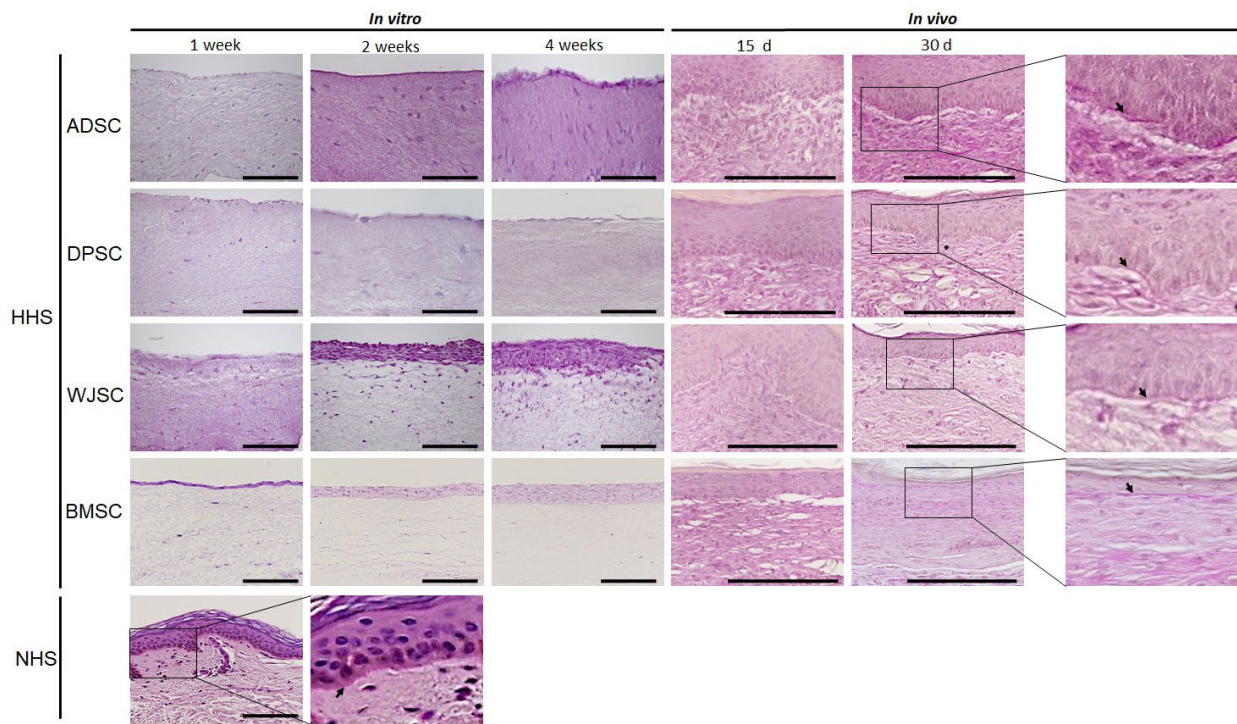
Fig. 5. PAS histochemical analysis of NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm. Black arrows point to GAGs in the basement membrane.
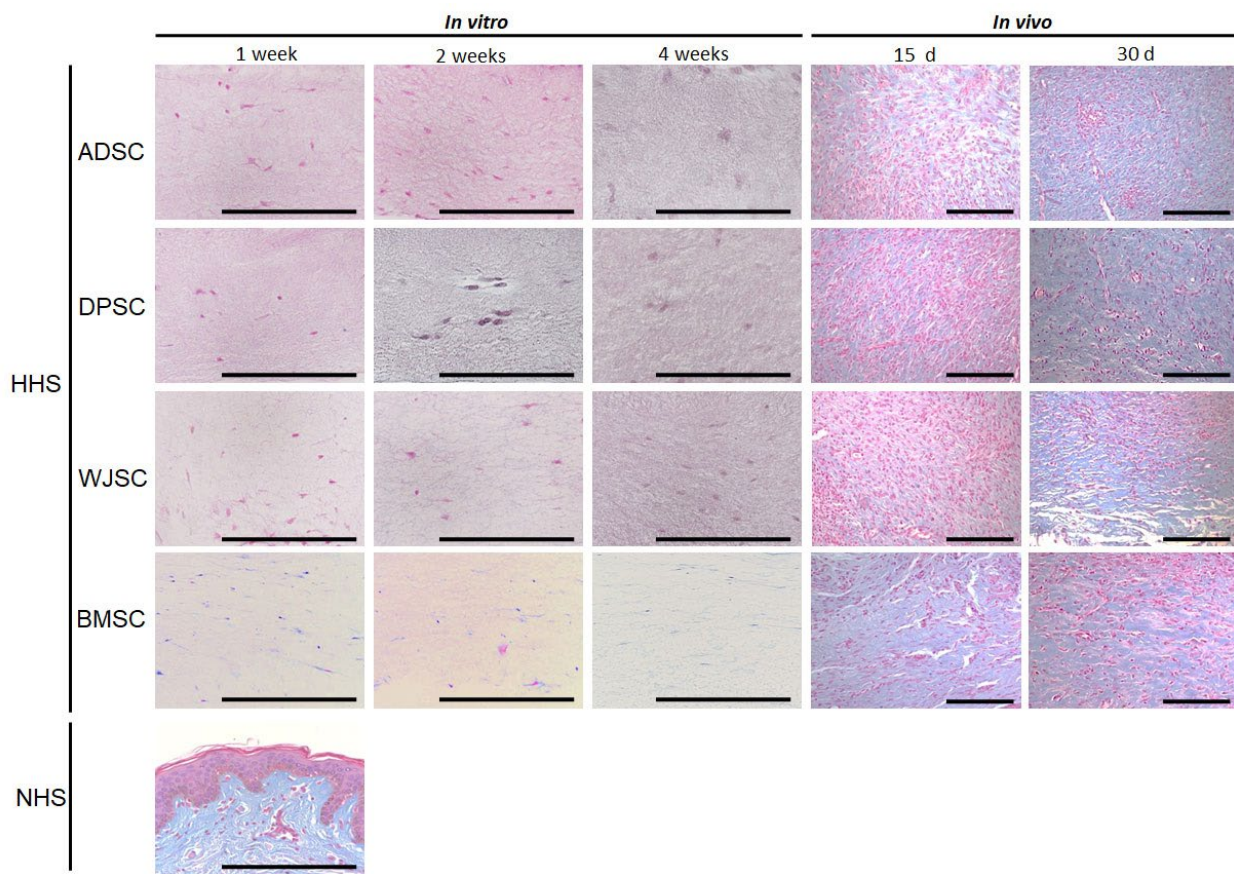

Fig. 6. Alcian blue histochemical analysis of NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.
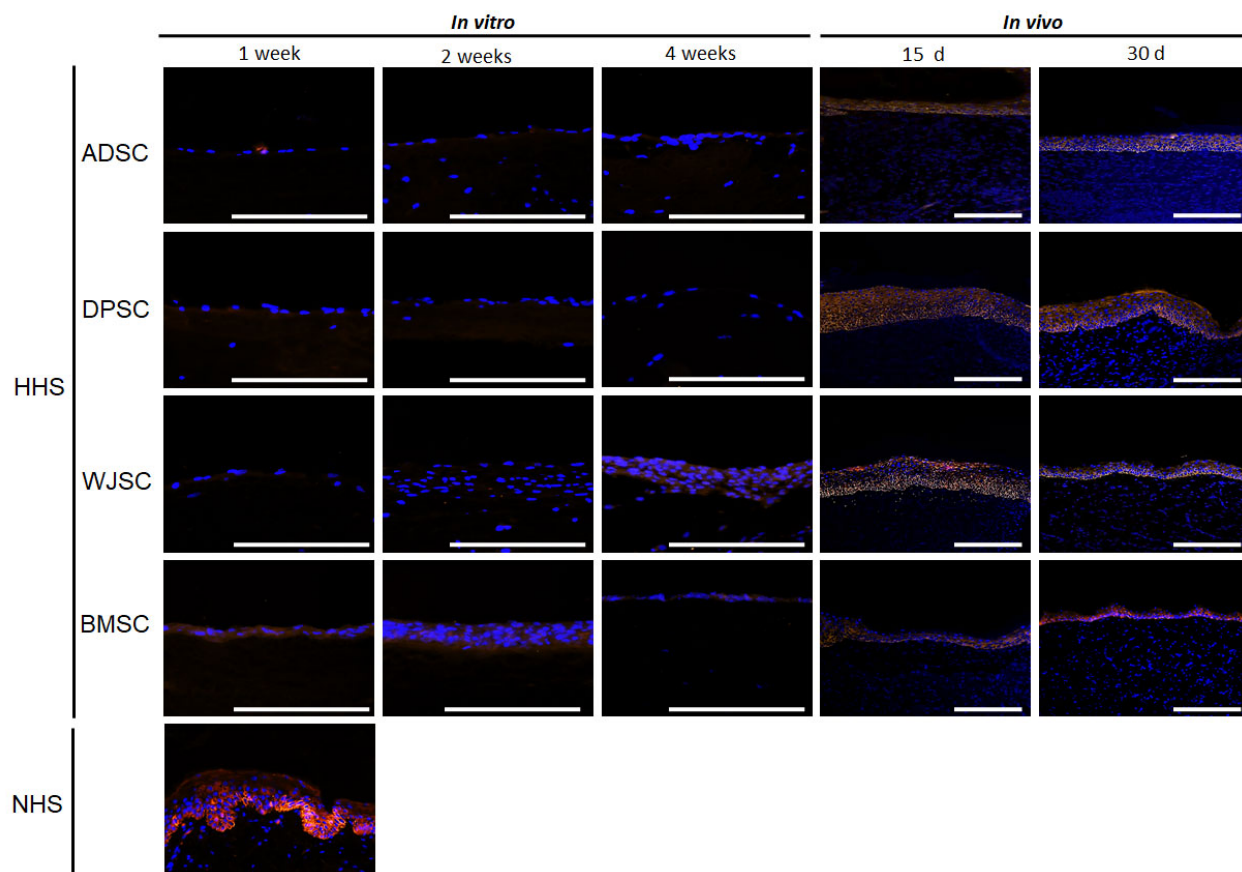
Fig. 7. Immunofluorescent analysis of CK5 in NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.

9, Table 1). Nonetheless, samples corresponding to ADSCs, DPSCs and WJSCs showed a slight FGN expression in cornified and granulosum layers after 15 d of *in vivo* implantation and all samples had a mild expression after 30 d of *in vivo* grafting, except for WJSCs, which reached similar expression at the cornified layer mimicking NHS.

In vitro and *in vivo* immunogenical evaluation of HHS samples

The immunogenical potential of transdifferentiated MSCs was evaluated by the detection of HLA complex molecules. MSCs used to generate HHS models revealed low expression of HLA I molecules evaluated by immunohistochemistry in BMSCs and a lack of HLA II in all MSC groups *in vitro*. Once grafted, transdifferentiated MSCs were negative in all groups both at 15 and 30 d for HLA I. BMSCs showed a mild expression of HLA II after 15 and 30 d post-implantation (Fig. 10 and 11, Table 1).

Discussion

The skin is the largest organ of the human body. It serves as an effective barrier between the organism and the environment and is involved in the regulation of body temperature and fluid loss (Proksch *et al.*,

2008). Among the main conditions severely affecting the human skin, burns are serious therapeutical situations for which a fully valid management approach is not clinically available. In fact, treatment of deep burn-wounds is based on the use of autografts, which are not available in cases where large areas of skin are affected. Recently, tissue engineering has emerged as a therapeutic alternative, allowing the generation of autologous full-thickness skin substitutes for the treatment of deep burn-wounds and showing promising results (Llames *et al.*, 2004). Development of heterotypical artificial skin models would contribute to overcome the drawbacks associated with the generation of bioengineered skin.

The use of MSCs as alternative cell sources offers several potential advantages such as availability, proliferation and differentiation capability with low immunogenic potential (Koppula *et al.*, 2009; Weiss *et al.*, 2008). The lack of immunological reaction makes these cells excellent candidates for cellular therapy in an allogeneic setting (Le Blanc and Ringden, 2005). However, a comparative study focussed on the differentiation potential of the four main sources of MSCs into skin epithelium has not been carried out to date. The present study approach was to determine the capability of each of the four MSC types to be induced using a specific epithelial conditioning medium in two-dimensional (2D) culture systems.
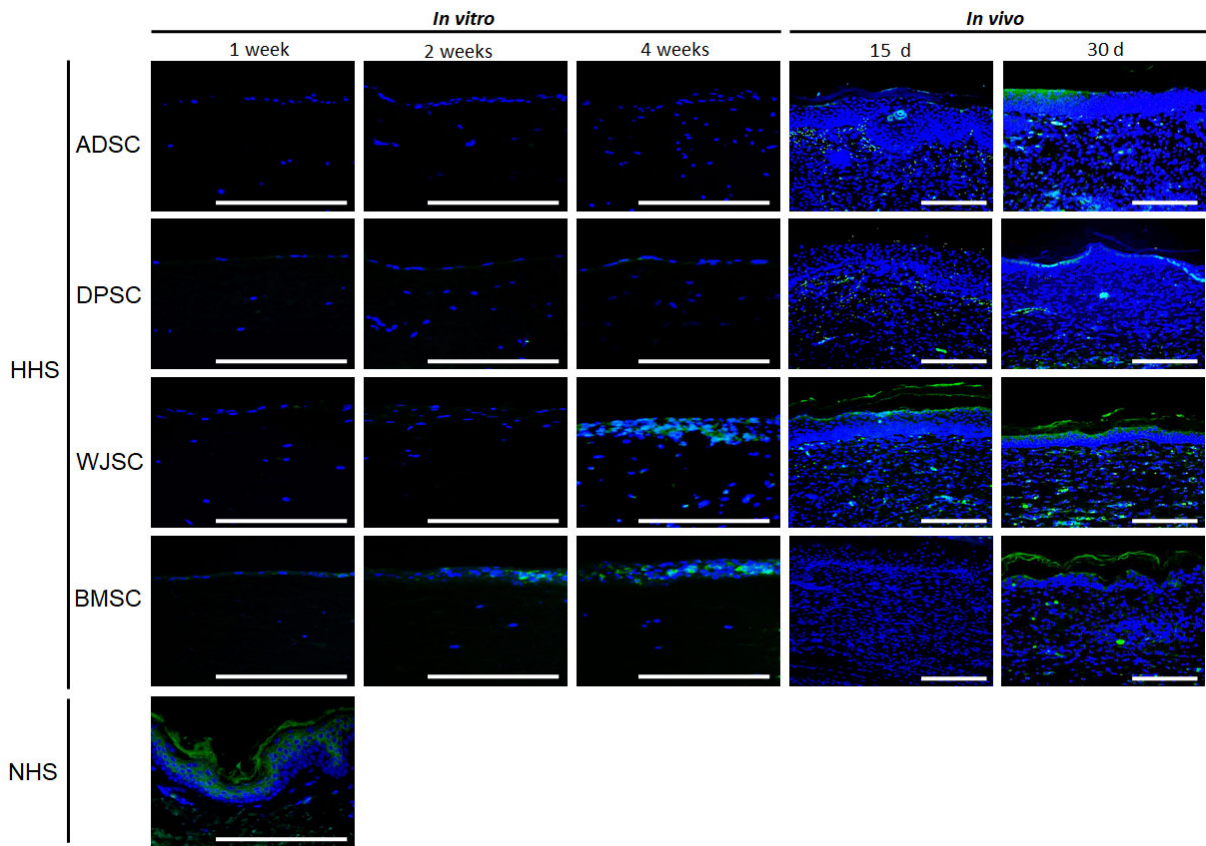
Fig. 8. Immunofluorescent analysis of CK10 in NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.
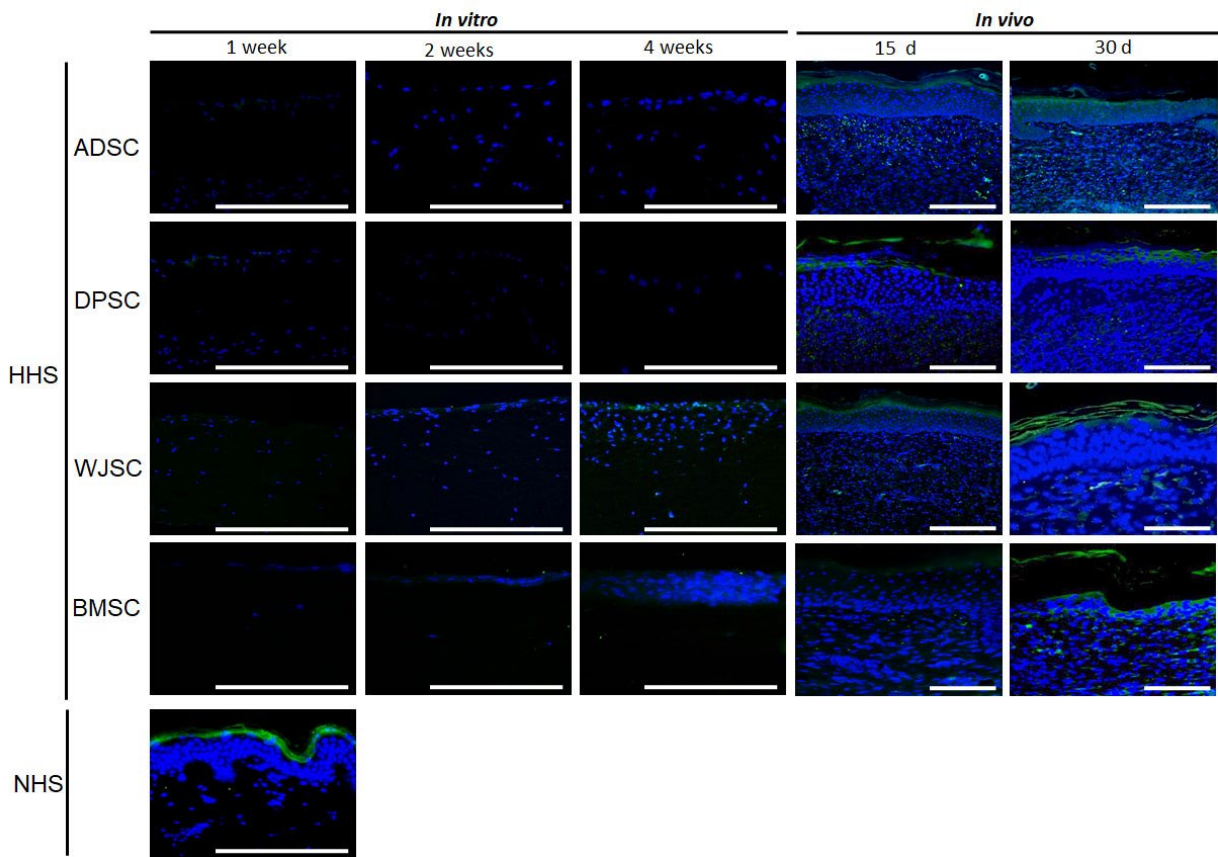

Fig. 9. Immunofluorescent analysis of FGN in NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.

Results showed a small number of induced genes. ADSCs and WJSCs showed larger percentages of induced genes. However, BMSCs and DPSCs were able to induce a series of genes with a putative role in epithelial cell function, such as cell adhesion. These results supported the use of the four MSC types in tissue engineering, given their epithelial differentiation capability, as previously demonstrated (Zhang *et al.*, 2012). In the present work, novel models of bioengineered skin generated by using four MSC types were described. In agreement with previous reports, suggesting that heterotypical bioengineered tissues have low levels of differentiation while kept *in vitro* (Garzon *et al.*, 2013; Garzon *et al.*, 2009a), *in vitro* tissues had very few cell layers on top of the dermis substitute – although the number of cell layers tended to increase with time – and a complete absence of differentiation markers, such as rete ridges and chorial papillae or keratinisation.

Expression of CK is one of the main features of human epidermal cells. For this reason, the first CK analysed was CK5, which is paired with CK14 and is a marker of basal proliferative keratinocytes that act as epithelial stem cells (Kim *et al.*, 2002). Results showed that WJSCs and BMSCs were able to express small amounts of this marker under *in vitro* conditions, suggesting that these cell types could respond to epithelial inductive medium more efficiently than ADSCs and DPSCs. However, the *in vivo* setting was able to induce the expression of

CK5 in all samples, but the expression pattern tended to resemble the preferentially basal expression of native skin only in the two cell types that showed the highest differentiation potential *in vitro*, WJSCs and BMSCs. On the other hand, HHS generated using WJSCs and BMSCs showed mild expression of CK10 in most epithelial-like cells. The slight expression of CK10 under *in vitro* conditions could be linked to an early epithelial differentiation stage. However, when tissue samples were implanted *in vivo*, differentiation was enhanced and stratification organisation was more evident. The proper set-up of basal cells may enhance proliferation and cell differentiation into epithelial-like tissue. An interesting phenomenon was that BMSCs showed slight CK10 expression *in vitro*, but this expression was initially negative *in vivo* and became positive at 30 d. This finding may be explained as an adaptive process of the cells, which could partially lose their original orientation *in vivo* and, thus, minimise their expression profile until the epithelium became fully differentiated. These results, related to the *in vitro* differentiation potential of WJSCs and BMSCs, were in agreement with previous work demonstrating their keratinocytic differentiation capability (Garzon *et al.*, 2013) and with the fact that ADSCs and DPSCs have not yet been differentiated into skin keratinocytes – although reports have suggested that these cell types may have epithelial differentiation capability in the presence of inductive medium (Baer *et al.*, 2013; Dogan *et al.*,
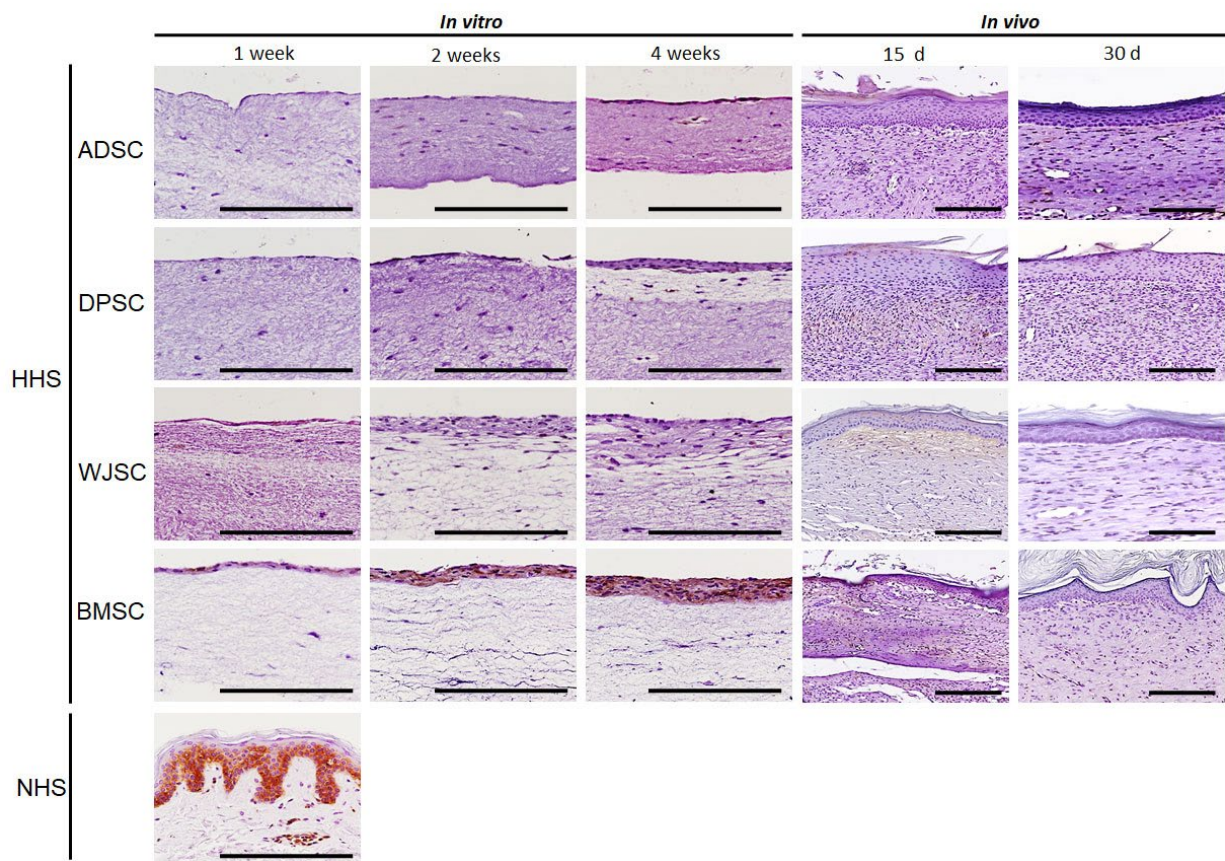

Fig. 10. Immunohistochemistry analysis of HLA I in NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.

2015). In the present study, both WJSCs and BMSCs were able to develop a larger number of cell layers at the *in vitro* stage, which coincided with the early cytokeratin expression. Therefore, the use of a combined trans-differentiation protocol based on epithelial conditioning medium, 3D systems with dermal cells supporting epithelial-mesenchymal interaction and air-liquid culture technique could partially induce *in vitro* epidermal differentiation of some of the studied MSC types but failed to achieve terminal differentiation.

In vivo grafting was performed by using a plastic ring standardised model (Fang and Mustoe, 2008; Galiano *et al.*, 2004) to minimise and control wound contraction. Previous studies have described contraction in mice as a common physiological phenomenon after skin grafting. Park *et al.* (2014) compared the use of splinted *versus* non-splinted models and demonstrated that the wound generated in the skin of the animal tends to fully shrink and contract after 11 d in non-splinted models, whereas splinted models reduce the contraction rate to near 60 % after 11 d (Gebremeskel *et al.*, 2018; Park *et al.*, 2014). In the present study, the wound contraction percentage reached 61.7 % after 30 d, suggesting a reduction of the wound contraction that may improve re-epithelisation with the grafted tissue. These results are in accordance with a study reporting that use of silicone splints around dorsal wounds may attenuate

contraction but that the final graft size is typically around 40-50 % (Galiano *et al.*, 2004).

Once grafted *in vivo*, MSCs were subjected to additional differentiation signals depending on the local and systemic inductive factors generated by the host. This resulted in higher differentiation in all cases, with the formation of terminally differentiated epithelial cell layers and other differentiation markers. Previous reports have demonstrated that the *in vivo* environment is necessary for the cells to fully develop and differentiate, which does not occur *in vitro* (Garzon *et al.*, 2009a; Garzon *et al.*, 2013). One of the challenges of bioartificial tissues grafted in immunodeficient animals is demonstrating the presence of the human cells in the graft and determining the fate of the implanted cells. In the present work, human-specific antibodies were used to demonstrate that cells in the regenerated skin area were of human nature. Future studies should analyse cell migration and stability *in vivo* by using high-definition cell-tracking techniques (Bulte and Daldrup-Link, 2018).

The *in vitro* analysis of the dermal substitute showed a complete absence of the fibrillar and non-fibrillar ECM components analysed, which tended to appear *in vivo*. In general, the artificial dermis grafted *in vivo* was able to synthesise and generate abundant amounts of collagen fibres and reached the levels of the control except for ADSCs, suggesting that the type
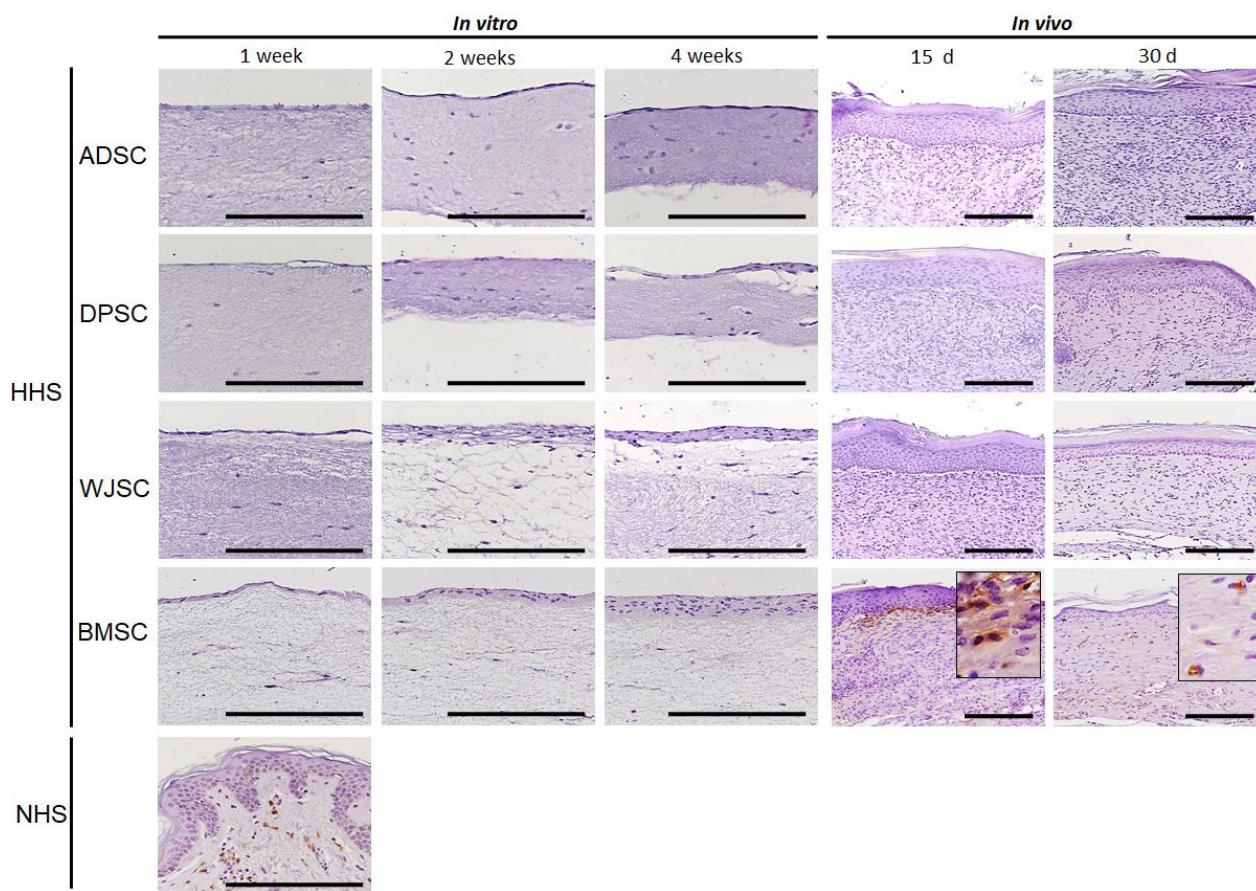

Fig. 11. Immunohistochemistry analysis of HLA II in NHS and HHS generated using ADSCs, DPSCs, WJSCs and BMSCs kept *in vitro* for 1, 2 and 4 weeks and grafted *in vivo* for 15 and 30 d. Scale bar: 200 μm.

of MSCs used as epithelial substitute may influence the biosynthetic activity of dermal fibroblasts. The presence of collagen fibres is very important to keep the biomechanical properties of the human skin (Aziz *et al.*, 2016) and its presence in the dermal substitute could favour the adequate physiology and mechanical behaviour of the artificial skin. In this regard, the fibrin-agarose bioartificial human tissue substitutes share some biomechanical similarities [G′ modulus ranging between 30 and 70 Pa and G′′ between 40 and 50 Pa (Rodriguez *et al.*, 2012)] with native human tissues, although their biomechanical properties are not completely biomimetic to control tissues.

Regarding the non-fibrillar ECM components of the dermal substitute, results showed that the four types of HHC were able to synthesise GAGs, although only ADSCs were able to reach the expression levels found in the control tissues. However, proteoglycans were very abundant in the four MSC types after 30 d of development, suggesting that this important dermal component was easily generated by the dermal fibroblasts independently of the type of epithelial substitute found on top. Previous work showed that GAGs and proteoglycans are important components of the skin ECM that may facilitate wound healing, cell adhesion, migration, proliferation, metabolism, differentiation, survival and growth factor activity (Ghatak *et al.*, 2015). Therefore, their presence in the bioengineered skin should be a requisite for these tissues to be clinically useful and previous fibrin-agarose human bioartificial skin models display high expression of these components *in vivo* (Martin-Piedra *et al.*, 2017).

Results of the epithelial substitute showed that the highest epithelial differentiation potential corresponded to WJSCs and BMSCs according to CK10 expression and WJSCs according to FGN. Along with the larger number of cell layers in WJSC-based HHS, these findings would confirm previous results showing the skin-epithelium differentiation potential of WJSCs (Garzon *et al.*, 2013). In contrast with the other three MSC types studied, WJSCs were obtained from new-born umbilical cords. This perinatal cell source could retain some of the proliferation and differentiation capabilities of embryonic cells, which could explain their potential in tissue regeneration. WJSCs are easily accessible and have been extensively used in tissue engineering and regenerative medicine without the risk of tumorigenesis (Joerger-Messerli *et al.*, 2016). However, the other three MSC types also displayed differentiation potential as epithelial markers, as determined by CK10 and FGN expression analysis, and showed high expression of proteoglycans and collagen (especially DPSCs and BMSCs). Future works should determine if these four MSC types may have the potential to fully differentiate into skin epidermis by using other inductive factors or clues, other *in vivo* models or longer follow-up periods.

One possible issue related to the use of MSCs differentiated towards human epithelial keratinocytes is the possibility that immunopriviliged undifferentiated MSCs (Weiss *et al.*, 2008) might acquire immunogenicity under certain conditions, including epidermal differentiation. In fact, upregulation of major histocompatibility complex (MHC)-II antigens is detected after gamma-interferon induction in MSC cultures, which could point towards this possibility (Le Blanc and Ringden, 2005). In the present work, the four analysed MSC types were initially negative for several HLA I and HLA II markers in the HHS models. However, preliminary epithelial differentiation found in BMSC-HHS kept *in vitro* for 2 and 4 weeks (as determined by CK10 immunostaining) coincided with a slight expression of HLA I antigens. Nevertheless, *in vivo* grafting resulted in a complete absence of HLA I signal in all HHS types, although HLA II became slightly positive in BMSC-HHS after 15 and 30 d of *in vivo* development. The transient expression of HLA I in BMSCs kept *in vitro* remains intriguing and future studies should be performed to elucidate this issue. One important concern is the possibility that HHS cells could express HLA markers after full differentiation, as previously suggested for bone and cartilage tissue substitutes (Lee *et al.*, 2012; Lohan *et al.*, 2014; Lohan *et al.*, 2017). The lack of information related to HLA expression on MSCs differentiated towards epithelial cell lineages makes necessary to perform future studies using longer follow-up times.

Altogether, these results confirm the absence of immunogenic HLA markers in bioengineered human substitutes generated using ADSCs, DPSCs and WJSCs. Although the epithelial layer was differentiated and would support the future clinical use of these substitutes, BMSC-based HHS could become immunogenic for HLA II upon induction. For this reason, the preferential use of HHS based on ADSCs, DPSCs and WJSCs instead of BMSCs would be recommendable. Therefore, these results would support the use of these types of MSCs for the generation of heterotypical models of human skin, but also other bioengineered tissues or organs such as human cornea and oral mucosa.

For the clinical transference of these bioengineered skin substitutes, several steps should be achieved, including exhaustive preclinical quality control analyses fulfilling the requirements of the European Medicines Agency.

Conclusions

Results demonstrated the partial epithelial differentiation capability of ADSCs, DPSCs, WJSCs and BMSCs, which could be used for the generation of bioengineered human skin substitutes with potential clinical usefulness. However, WJSCs demonstrated the highest differentiation capability and lack of HLA

I and II expression upon induction. Therefore, WJSCs could be used for the generation of human skin substitutes. However, future studies should assess the functional properties of the skin regenerated by these substitutes and its long-term differentiation potential.

References

Alfonso-Rodriguez CA, Garzon I, Garrido-Gomez J, Oliveira AC, Martin-Piedra MA, Scionti G, Carriel V, Hernandez-Cortes P, Campos A, Alaminos M (2014) Identification of histological patterns in clinically affected and unaffected palm regions in dupuytren's disease. PLoS One **9**: e112457. DOI: 10.1371/journal.pone.0112457.

Alfonso-Rodriguez CA, Gonzalez-Andrades E, Jaimes-Parra BD, Fernandez-Valades R, Campos A, Sanchez-Quevedo MC, Alaminos M, Garzon I (2015) *Ex vivo* and *in vivo* modulatory effects of umbilical cord Wharton's jelly stem cells on human oral mucosa stroma substitutes. Histol Histopathol **30**: 1321-1332.

Aziz J, Shezali H, Radzi Z, Yahya NA, Abu Kassim NH, Czernuszka J, Rahman MT (2016) Molecular mechanisms of stress-responsive changes in collagen and elastin networks in skin. Skin Pharmacol Physiol **29**: 190-203.

Baer PC, Doring C, Hansmann ML, Schubert R, Geiger H (2013) New insights into epithelial differentiation of human adipose-derived stem cells. J Tissue Eng Regen Med **7**: 271-278.

Beaudoin Cloutier C, Goyer B, Perron C, Guignard R, Larouche D, Moulin VJ, Germain L, Gauvin R, Auger FA (2017) *In vivo* evaluation and imaging of a bilayered self-assembled skin substitute using a decellularized dermal matrix grafted on mice. Tissue Eng Part A **23**: 313-322.

Bhardwaj N, Chouhan D, Mandal BB (2017) Tissue engineered skin and wound healing: current

strategies and future directions. Curr Pharm Des **23**: 3455-3482.

Bhowmick S, Rother S, Zimmermann H, Lee PS, Moeller S, Schnabelrauch M, Koul V, Jordan R, Hintze V, Scharnweber D (2017) Biomimetic electrospun scaffolds from main extracellular matrix components for skin tissue engineering application - the role of chondroitin sulfate and sulfated hyaluronan. Mater Sci Eng C Mater Biol Appl **79**: 15-22.

Boa O, Cloutier CB, Genest H, Labbe R, Rodrigue B, Soucy J, Roy M, Arsenault F, Ospina CE, Dube N, Rochon MH, Larouche D, Moulin VJ, Germain L, Auger FA (2013) Prospective study on the treatment of lower-extremity chronic venous and mixed ulcers using tissue-engineered skin substitute made by the self-assembly approach. Adv Skin Wound Care **26**: 400-409.

Boyce ST, Kagan RJ, Greenhalgh DG, Warner P, Yakuboff KP, Palmieri T, Warden GD (2006) Cultured skin substitutes reduce requirements for harvesting of skin autograft for closure of excised, full-thickness burns. J Trauma **60**: 821-829.

Boyce ST, Kagan RJ, Meyer NA, Yakuboff KP, Warden GD (1999) The 1999 clinical research award. Cultured skin substitutes combined with integra artificial skin to replace native skin autograft and allograft for the closure of excised full-thickness burns. J Burn Care Rehabil **20**: 453-461.

Boyce ST, Simpson PS, Rieman MT, Warner PM, Yakuboff KP, Bailey JK, Nelson JK, Fowler LA, Kagan RJ (2017) Randomized, paired-site comparison of autologous engineered skin substitutes and split-thickness skin graft for closure of extensive, full-thickness burns. J Burn Care Res **38**: 61-70.

Bulte JWM, Daldrup-Link HE (2018) Clinical tracking of cell transfer and cell transplantation: trials and tribulations. Radiology **289**: 604-615.

Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, Cavinato RA, Todeschini M, Solini S, Sonzogni A, Perico N, Remuzzi G, Noris M (2008) Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory t cells. J Immunol **181**: 3933-3946.

Castro-Manrreza ME, Montesinos JJ (2015) Immunoregulation by mesenchymal stem cells: biological aspects and clinical applications. J Immunol Res **2015**: 394917. DOI: 10.1155/2015/394917.

Cejka C, Cejkova J, Trosan P, Zajicova A, Sykova E, Holan V (2016) Transfer of mesenchymal stem cells and cyclosporine a on alkali-injured rabbit cornea using nanofiber scaffolds strongly reduces corneal neovascularization and scar formation. Histol Histopathol **31**: 969-980.

Dogan A, Demirci S, Sahin F (2015) *In vitro* differentiation of human tooth germ stem cells into endothelial- and epithelial-like cells. Cell Biol Int **39**: 94-103.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating

A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy **8**: 315-317.

Dori I, Petrakis S, Giannakopoulou A, Bekiari C, Grivas I, Siska EK, Koliakos G, Papadopoulos GC (2017) Seven days post-injury fate and effects of genetically labelled adipose-derived mesenchymal cells on a rat traumatic brain injury experimental model. Histol Histopathol **32**: 1041-1055.

Fang RC, Mustoe TA (2008) Animal models of wound healing: utility in transgenic mice. J Biomater Sci Polym Ed **19**: 989-1005.

Forouzandeh F, Jalili RB, Hartwell RV, Allan SE, Boyce S, Supp D, Ghahary A (2010) Local expression of indoleamine 2,3-dioxygenase suppresses T-cell-mediated rejection of an engineered bilayer skin substitute. Wound Repair Regen **18**: 614-623.

Friedenstein AJ, Gorskaja JF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol **4**: 267-274.

Galiano RD, Michaels Jt, Dobryansky M, Levine JP, Gurtner GC (2004) Quantitative and reproducible murine model of excisional wound healing. Wound Repair Regen **12**: 485-492.

Garzon I, Martin-Piedra MA, Alaminos M (2015) Human dental pulp stem cells. A promising epithelial-like cell source. Medical hypotheses **84**: 516-517.

Garzon I, Martin-Piedra MA, Alfonso-Rodriguez C, Gonzalez-Andrades M, Carriel V, Martinez-Gomez C, Campos A, Alaminos M (2014) Generation of a biomimetic human artificial cornea model using Wharton's jelly mesenchymal stem cells. Invest Ophthalmol Vis Sci **55**: 4073-4083.

Garzon I, Martin-Piedra MA, Carriel V, Alaminos M, Liu X, D'Souza RN (2017) Bioactive injectable aggregates with nanofibrous microspheres and human dental pulp stem cells: a translational strategy in dental endodontics. J Tissue Eng Regen Med **12**: 204-216.

Garzon I, Miyake J, Gonzalez-Andrades M, Carmona R, Carda C, Sanchez-Quevedo Mdel C, Campos A, Alaminos M (2013) Wharton's jelly stem cells: a novel cell source for oral mucosa and skin epithelia regeneration. Stem Cells Transl Med **2**: 625-632.

Garzon I, Sanchez-Quevedo MC, Moreu G, Gonzalez-Jaranay M, Gonzalez-Andrades M, Montalvo A, Campos A, Alaminos M (2009a) *In vitro* and *in vivo* cytokeratin patterns of expression in bioengineered human periodontal mucosa. J Periodontal Res **44**: 588-597.

Garzon I, Serrato D, Roda O, Del Carmen Sanchez-Quevedo M, Gonzales-Jaranay M, Moreu G, Nieto-Aguilar R, Alaminos M, Campos A (2009b) *In vitro* cytokeratin expression profiling of human oral mucosa substitutes developed by tissue engineering. Int J Artif Organs **32**: 711-719.

Gebremeskel L, Bhoumik D, Sibhat GG, Tuem KB (2018) *In vivo* wound healing and anti-inflammatory

activities of leaf latex of aloe megalacantha baker (xanthorrhoeaceae). Evid Based Complement Alternat Med **2018**: 5037912. DOI: 10.1155/2018/5037912.

Germain L, Larouche D, Nedelec B, Perreault I, Duranceau L, Bortoluzzi P, Beaudoin Cloutier C, Genest H, Caouette-Laberge L, Dumas A, Bussiere A, Boghossian E, Kanevsky J, Leclerc Y, Lee J, Nguyen MT, Bernier V, Knoppers BM, Moulin VJ, Auger FA (2018) Autologous bilayered self-assembled skin substitutes (SASSs) as permanent grafts: a case series of 14 severely burned patients indicating clinical effectiveness. Eur Cell Mater **36**: 128-141.

Ghatak S, Maytin EV, Mack JA, Hascall VC, Atanelishvili I, Moreno Rodriguez R, Markwald RR, Misra S (2015) Roles of proteoglycans and glycosaminoglycans in wound healing and fibrosis. Int J Cell Biol **2015**: 834893. DOI: 10.1155/2015/834893.

Gimble J, Guilak F (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy **5**: 362-369.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. Proc Natl Acad Sci U S A **97**: 13625-13630.

Hartmann-Fritsch F, Biedermann T, Braziulis E, Luginbuhl J, Pontiggia L, Bottcher-Haberzeth S, van Kuppevelt TH, Faraj KA, Schiestl C, Meuli M, Reichmann E (2016) Collagen hydrogels strengthened by biodegradable meshes are a basis for dermo-epidermal skin grafts intended to reconstitute human skin in a one-step surgical intervention. J Tissue Eng Regen Med **10**: 81-91.

Joerger-Messerli MS, Marx C, Oppliger B, Mueller M, Surbek DV, Schoeberlein A (2016) Mesenchymal stem cells from Wharton's jelly and amniotic fluid. Best Pract Res Clin Obstet Gynaecol **31**: 30-44.

Kim HJ, Park JS (2017) Usage of human mesenchymal stem cells in cell-based therapy: advantages and disadvantages. Dev Reprod **21**: 1-10.

Kim HJ, Tinling S, Chole R (2002) Expression patterns of cytokertins in cholesteatomas: evidence of increased migration and proliferation. J Korean Med Sci **17**: 381-388

Klar AS, Michalak K, Bottcher-Haberzeth S, Reichmann E, Meuli M, Biedermann T (2018) The expression pattern of keratin 24 in tissue-engineered dermo-epidermal human skin substitutes in an *in vivo* model. Pediatr Surg Int **34**: 237-244.

Kober J, Gugerell A, Schmid M, Zeyda M, Buchberger E, Nickl S, Hacker S, Ankersmit HJ, Keck M (2016) Wound healing effect of conditioned media obtained from adipose tissue on human skin cells: a comparative *in vitro* study. Ann Plast Surg **77**: 156-163.

Kokubun K, Pankajakshan D, Kim MJ, Agrawal DK (2016) Differentiation of porcine mesenchymal stem cells into epithelial cells as a potential therapeutic application to facilitate epithelial regeneration. J Tissue Eng Regen Med **10**: E73-83.

Koppula PR, Chelluri LK, Polisetti N, Vemuganti GK (2009) Histocompatibility testing of cultivated human bone marrow stromal cells - a promising step

towards pre-clinical screening for allogeneic stem cell therapy. Cell Immunol **259**: 61-65.

Kraft R, Herndon DN, Al-Mousawi AM, Williams FN, Finnerty CC, Jeschke MG (2012) Burn size and survival probability in paediatric patients in modern burn care: a prospective observational cohort study. Lancet **379**: 1013-1021.

Larouche D, Cantin-Warren L, Desgagne M, Guignard R, Martel I, Ayoub A, Lavoie A, Gauvin R, Auger FA, Moulin VJ, Germain L (2016) Improved methods to produce tissue-engineered skin substitutes suitable for the permanent closure of full-thickness skin injuries. Biores Open Access **5**: 320-329.

Le Blanc K, Ringden O (2005) Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant **11**: 321-334.

Lee JH, Park HJ, Kim YA, Lee DH, Noh JK, Kwon CH, Jung SM, Lee SK (2012) Differentiation and major histocompatibility complex antigen expression in human liver-derived stem cells. Transplant Proc **44**: 1113-1115.

Llames SG, Del Rio M, Larcher F, Garcia E, Garcia M, Escamez MJ, Jorcano JL, Holguin P, Meana A (2004) Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin. Transplantation **77**: 350-355.

Lloyd C, Besse J, Boyce S (2015) Controlled-rate freezing to regulate the structure of collagen-glycosaminoglycan scaffolds in engineered skin substitutes. J Biomed Mater Res B Appl Biomater **103**: 832-840.

Lohan P, Coleman CM, Murphy JM, Griffin MD, Ritter T, Ryan AE (2014) Changes in immunological profile of allogeneic mesenchymal stem cells after differentiation: should we be concerned? Stem Cell Res Ther **5**: 99. DOI: 10.1186/scrt488.

Lohan P, Treacy O, Griffin MD, Ritter T, Ryan AE (2017) Anti-donor immune responses elicited by allogeneic mesenchymal stem cells and their extracellular vesicles: are we still learning? Front Immunol **8**: 1626. DOI: 10.3389/fimmu.2017.01626.

Losk K, Vaz-Luis I, Camuso K, Batista R, Lloyd M, Tukenmez M, Golshan M, Lin NU, Bunnell CA (2016) Factors associated with delays in chemotherapy initiation among patients with breast cancer at a comprehensive cancer center. J Natl Compr Canc Netw **14**: 1519-1526.

Ma T, Wang X, Jiang D (2017) Immune tolerance of mesenchymal stem cells and induction of skin allograft tolerance. Curr Stem Cell Res Ther **12**: 409-415.

Maere S, Heymans K, Kuiper M (2005) Bingo: A cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics **21**: 3448-3449.

Mahdavishahri N, Moghatam Matin M, Fereidoni M, Yarjanli Z, Banihashem Rad SA, Khajeh Ahmadi S (2012) *In vitro* assay of human gingival scaffold in differentiation of rat's bone marrow mesenchymal

stem cells to keratinocystes. Iran J Basic Med Sci **15**: 1185-1190.

Martin-Piedra MA, Garzón I, Gómez-Sotelo A, Garcia-Abril E, Jaimes-Parra BD, López-Cantarero M, Alaminos M, Campos A (2017) Generation and evaluation of novel stromal cell-containing tissue engineered artificial stromas for the surgical repair of abdominal defects. Biotechnol J **12**. DOI: 10.1002/biot.201700078,

Mendez JJ, Ghaedi M, Steinbacher D, Niklason LE (2014) Epithelial cell differentiation of human mesenchymal stromal cells in decellularized lung scaffolds. Tissue Eng Part A **20**: 1735-1746.

Moravvej H, Hormozi AK, Hosseini SN, Sorouri R, Mozafari N, Ghazisaidi MR, Rad MM, Moghimi MH, Sadeghi SM, Mirzadeh H (2016) Comparison of the application of allogeneic fibroblast and autologous mesh grafting with the conventional method in the treatment of third-degree burns. J Burn Care Res **37**: e90-95.

Park SA, Teixeira LB, Raghunathan VK, Covert J, Dubielzig RR, Isseroff RR, Schurr M, Abbott NL, McAnulty J, Murphy CJ (2014) Full-thickness splinted skin wound healing models in db/db and heterozygous mice: implications for wound healing impairment. Wound Repair Regen **22**: 368-380.

Pensalfini M, Ehret AE, Studeli S, Marino D, Kaech A, Reichmann E, Mazza E (2017) Factors affecting the mechanical behavior of collagen hydrogels for skin tissue engineering. J Mech Behav Biomed Mater **69**: 85-97.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science **284**: 143-147.

Proksch E, Brandner JM, Jensen JM (2008) The skin: an indispensable barrier. Exp Dermatol **17**: 1063-1072.

Reinders ME, de Fijter JW, Roelofs H, Bajema IM, de Vries DK, Schaapherder AF, Claas FH, van Miert PP, Roelen DL, van Kooten C, Fibbe WE, Rabelink TJ (2013) Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. Stem Cells Transl Med **2**: 107-111.

Reuter C, Walles H, Groeber F (2017) Preparation of a three-dimensional full thickness skin equivalent. Methods Mol Biol **1612**: 191-198.

Rodriguez IA, Lopez-Lopez MT, Oliveira AC, Sanchez-Quevedo MC, Campos A, Alaminos M, Duran JD (2012) Rheological characterization of human fibrin and fibrin-agarose oral mucosa substitutes generated by tissue engineering. J Tissue Eng Regen Med **6**: 636-644.

Shi M, Liu ZW, Wang FS (2011) Immunomodulatory properties and therapeutic application of mesenchymal stem cells. Clin Exp Immunol **164**: 1-8.

Smola H, Thiekotter G, Fusenig NE (1993) Mutual induction of growth factor gene expression by

epidermal-dermal cell interaction. J Cell Biol **122**: 417-429.

Strong AL, Neumeister MW, Levi B (2017) Stem cells and tissue engineering: regeneration of the skin and its contents. Clin Plast Surg **44**: 635-650.

Vig K, Chaudhari A, Tripathi S, Dixit S, Sahu R, Pillai S, Dennis VA, Singh SR (2017) Advances in skin regeneration using tissue engineering. Int J Mol Sci **18**: pii: E789. DOI: 10.3390/ijms18040789.

Wang C, Zhu H, Sun Z, Xiang Z, Ge Y, Ni C, Luo Z, Qian W, Han X (2014) Inhibition of wnt/beta-catenin signaling promotes epithelial differentiation of mesenchymal stem cells and repairs bleomycin-induced lung injury. Am J Physiol Cell Physiol **307**: C234-244.

Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, Fu YS, Lai MC, Chen CC (2004) Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells **22**: 1330-1337.

Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, Troyer D, McIntosh KR (2008) Immune properties of human umbilical cord Wharton's jelly-derived cells. Stem cells (Dayton, Ohio) **26**: 2865-2874.

Yamada N, Uchinuma E, Kuroyanagi Y (2012) Clinical trial of allogeneic cultured dermal substitutes for intractable skin ulcers. J Artif Organs **15**: 193-199.

Zhang WB, Cheng MJ, Huang YT, Jiang W, Cong Q, Zheng YF, Xu CJ (2012) A study *in vitro* on differentiation of bone marrow mesenchymal stem cells into endometrial epithelial cells in mice. Eur J Obstet Gynecol Reprod Biol **160**: 185-190.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell **13**: 4279-4295.

Discussion with Reviewer

Reviewer: What are next steps for the development of this bioengineered skin substitute to obtain an epidermis similar to human skin with terminally differentiated epithelial cell layers on the entire surface?

Authors: The promising results of the study lead to the hypothesis that the HHS generated could have future clinical translation potential. Therefore, next steps should include fulfilling the requirements of the European Medicines Agency for Advanced Therapies, including the preclinical characterisation at the biomechanical, physiological and optical (UV absorbance, speckle pattern) levels. In addition, improving the current HHS model by incorporating vascular progenitors in order to achieve vascularised models is in progress.

Reviewer: Several studies have shown the re-expression of HLA upon differentiation of stem cells (Lee *et al.*, 2012; Lohan *et al.*, 2014). Could the authors comment on the possibility that HLA could be re-expressed when the epidermis will be fully differentiated?

Authors: This is an important issue as HHS cells could express HLA markers after full differentiation, as described for bone and cartilage tissue substitutes (Lee *et al.*, 2012; Lohan *et al.*, 2014; Lohan *et al.*, 2017). However, the lack of information related to HLA expression on epithelial differentiation of MSCs point out the importance to perform further research in this field.

Editor's note: The Scientific Editor responsible for this paper was Mauro Alini.