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Phenotypical characterization of 6–21-week gestational age human dermis and epidermal cell isolation methods for *in vitro* studies on epidermal progenitors

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ABSTRACT

Cell banked epidermal skin progenitor cells have the potential to provide an "off-the-freezer" product. Such cells may provide a skin donor area-independent cell-spray grafting therapy for the treatment of burns. We first characterized fetal skin samples of gestational ages ranging from 6 to 21 weeks. As the results suggest that the phenotypic differentiation occurs after 10 weeks, which may complicate follow-up *in vitro* studies, we developed and compared different cell isolation techniques for human fetal skin-derived epithelial cells from tissue ages 6 to 9 weeks. We initially screened seven methods of characterization, concluding that two methods warranted further investigation: incubating the epidermal tissue in Petri-dishes with culture medium for spontaneous cell outgrowth, and wiping the epidermal tissue onto a dry Petri-dish culture surface followed by adding culture medium. Non-controllable culture contamination with dermal cells was the reason for excluding the other five methods. The results suggest that epidermal cells can be isolated from tissue exhibiting a single homogeneous layer of $CK15^{+}$ basal keratinocytes up to week 9. At later gestational ages, the ongoing skin differentiation results in a multi-layer basal structure and progenitors associated with the hair bulb would have to be considered. Spraying the resulting cells with a clinical spray device was successfully demonstrated in an *in vitro* model.

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Abbreviations: BSA, bovine serum albumin; CK, cytokeratin; EGF, epidermal growth factor; DAPI, 4[1],6-diamidino-2-phenylindole; FCS, fetal calf serum; FACS, fluorescence activated cell scan, flow cytometry; HEK, human epidermal keratinocytes; IF, immunofluorescence; IHC, immunohistochemistry; KGF, keratinocyte growth factor; PBS, phosphate buffered saline; rhIGF, recombinant human insulin-like growth factor; rhEGF, recombinant human epidermal growth factor; SD, standard deviation.

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Conclusion: Gestational age 6–9 weeks epidermal human fetal skin cells from the basal layer can be reproducibly isolated and transferred into culture for studies on the development of skin cell transplantation therapies.

1. Introduction

Therapies are needed to substitute current mesh-skin grafting for deep partial-thickness burns. [1,2]. Various approaches for autologous skin cell grafting have been described previously, though controversially discussed [3–9].

Single cell [10] spray-transplantation techniques currently in development are typically performed with cultured adult autologous epidermal cells [11] or freshly isolated adult epidermal cells, prepared on-site in the operating room [12,13] immediately before grafting. Given the controversy in the literature on the suitability of differentiated keratinocytes, research was initiated on providing autologous skin progenitors [14,15]. However, since the potency of epidermal basal keratinocytes has been demonstrated through the ability to regenerate the entire epidermis *in vivo* and *in vitro* [16], the fetal counterpart in the basal structure consisting only of a one-cell layer should be considered for therapy development. While scar-free skin wound healing by fetal cells is described in the early *in vivo* gestational ages, these cells were less intensely studied *in vitro* and we found no literature describing isolation methods for *in vitro* studies for fetal keratinocytes derived before the 12th gestational week [17].

Our project hypothesis is that for experimental studies on advancing skin cell grafting, human fetal epidermal skin cells can be reproducibly isolated and transferred into culture. Here, the age of the donor tissue should play an important role. We first performed histology and immunofluorescence (IF) microscopy studies on human fetal skin tissue of gestational ages 6–21 weeks, in comparison with adult skin, in order to identify suitable donor tissue ages associated with only a single layer of epithelial cells. As the results suggest that the phenotypic differentiation occurs after 10 weeks, which may complicate follow-up *in vitro* studies, we then developed and compared different cell isolation techniques for human fetal skin-derived epithelial cells from tissue ages 6 to 9 weeks. A development and comparison of seven isolation techniques for human fetal skin derived epithelial cells was performed. We report our isolation method development and discuss the pros and cons of these methods. A demonstration of cell spray feasibility using a clinical skin cell sprayer was performed in order to identify the capability of the resulting cells for further therapy development.

2. Materials and methods

2.1. Skin tissue

The tissue donations were obtained from patients of the Allegheney Health Reproductive Center, Pittsburgh, PA, USA. Samples from medically indicated abortions were considered. The work was approved by the Institutional Review Board of the University of Pittsburgh. Tissue procurement was initiated only after obtaining written informed consent from each donor. Tissue gender and donor characteristics were not provided. Specimens were not obtained in cases where genetic skin diseases or infectious diseases (including HIV and hepatitis A–C) were diagnosed. The age of the donated human fetal skin tissue specimens ranged from 6 to 21 weeks of gestation. Two age groups were investigated, ranging from 6 to 9 weeks for fetal cell isolation and tissue characterization, as well as 10–21 weeks for tissue characterization. Adult skin from cosmetic surgery tissue donations was used as a control for tissue histology ($n = 6$). Prior to cell isolation, the specimens were exposed to storage times of 3–5 h at 4 °C in PBS (Invitrogen, Carlsbad, CA, USA) solution containing 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 2.50 μg/ml Amphotericin B (Invitrogen).

2.2. H&E histology on tissue samples

Adult or fetal tissue was fixed in 4% formaldehyde and embedded in paraffin. After deparaffination and dehydration in gradual series of ethanol, 3 μm sections were stained with hematoxylin and eosin (BioOptica, Milan, Italy). Cell microscopy was performed using a Nikon Eclipse 50i microscope with a Nikon DS Fi1 camera and software for image acquisition (Nikon, Tokyo, Japan).

2.3. Immunohistochemistry on tissue samples

The 3 μm sections were rinsed with PBS and an antigen retrieval heat-induced step with citrate buffer pH 6.0 was performed. After washing in PBS, the sections were incubated in methanol with addition of 3% H_2O_2 to deactivate the endogenous peroxidases and blocked with PBS 0.5% Tween 20 (PBS-T, SIGMA, St. Louis, MO, USA), containing 3% bovine serum albumin (BSA) (SIGMA, St. Louis, MO, USA) for 1 h at room temperature. After overnight incubation with the primary antibody at 4 °C in a humid chamber, staining with the secondary antibody was conducted by using the Vectastain Elite ABC Kit and the DAB Substrate Kit for peroxidase (Vector Laboratories, Burlingame, CA, USA). Cells were counterstained with Harris hematoxylin (BioOptica, Milan, Italy). Primary antibodies used were CK15 (clone EPR1614Y, Abcam, Cambridge, MA, USA) as marker of basal epidermal cells and CK5 (Abcam, Cambridge, MA, USA) as marker for differentiated keratinocytes. Vimentin (clone V9, Millipore, Billerica, MA, USA) was used as marker for dermal cells and non-keratinocyte contaminations in culture. Cell observation was performed using a Nikon Eclipse 50i microscope with a Nikon DS Fi1 camera and software for image acquisition (Nikon, Tokyo, Japan).