THE ISOLATION OF AMNION STEM CELLS AND CHARACTERIZATION OF ITS POTENTIAL IN SKIN REGENERATION

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INTRODUCTION

Amnion derived stem cells has been recognised as an alternative source of stem cells for cell-based therapy and regenerative medicine. Amnion from the term placenta is easily isolated without much ethical issue since placenta is usually discarded after delivery. Since amnion derived cells are of fetal origin, it is immunoprivilege that has the potential for skin wounds repair such as in burn treatments (Maral *et al.,* 1999). So far, the potential of using amnion tissue in skin regeneration has not been fully exploited. This is due to the lack of fundamental knowledge in amnion stem cells differentiation pathway towards skin tissue.

OBJECTIVES

In this project, we characterised the stem cell properties at the cellular and molecular levels. The knowledge acquired then enabled us to use these stem cells for skin differentiation. The ultimate goal in this project is to use the newly acquired knowledge to construct a clinically reliable skin substitute for the treatment of skin wounds such as burn injuries and diabetic ulcers.

METHODOLOGY

Human amnion epithelial cells characterisation

Human amnion was obtained from term placenta with informed consent from the healthy mothers. The human amnion epithelial cells (HAECs) were isolated using a differential trypsin-EDTA digestion method with constant agitation at 37ºC. Effects of basal medium, PBS and normal saline as transport buffer were evaluated. The cell viability was checked using tryphan blue exclusion dye and haemocytometer. The freshly isolated HAECs were cultured in F12:DMEM (1:1) supplemented with 10% fetal bovine serum (FBS) and 10ng/ml epidermal growth factors (EGF). Upon confluence, HAECs were sub-cultured to passage 4.

The characteristics of HAECs in serial passage were determined using quantitative PCR (epithelial and stemness gene expressions), cell surface antigen analysis, immunocytochemistry, growth kinetics and morphological features. The stemness genes of interest included Oct-4, Sox-2, FGF-4, Rex-1, Nanog3, Nestin, FZD-9, ABCG-2, BST-1 and vimentin; whereas the epithelial genes group consisted of CK3, CK1, CK16, CK18, CK19, CK14, CK13, P63 and Involucrin. Furthermore, HAECs cultured at P2 were analysed for its *in vitro* differentiation to adipocytes, osteocytes and neuron.

To evaluate the effects of EGF and keratinocytes growth factors (KGF), HAECs at passage 1 (P1) were cultured in reduced serum (2% serum) with various concentrations of either EGF or KGF (0, 5, 10, 20, 30 and 50ng/ml). HAECs were cultured for 7 days. Morphology and growth kinetics were assessed and determined. Quantitative gene expression was then determined.

Human amnion mesenchymal cells characterisation

Denuded amnion after HAECs isolation was digested with 0.3% collagenase for 3-4 hours at 37ºC. The freshly isolated human amnion mesenchymal cells (HAMs) were cultured in FD+10% FBS. They were culture-expanded (1:4) until passage 5. The serial passage HAMs (at passages 0, 1, 3, and 5) were analyzed for its stemness (Oct-4, Sox-2, FGF-4, Rex-1, Nanog3, Nestin, FZD-9, ABCG-2 and BST-1) and angiogenic (PECAM-1, eNOS, vWF, VEGF receptor 2, PGF, HGF and angiopoeitin-1) gene expression, CFU-F assay, growth kinetics and morphological features. CD markers analysis; adipogenic, osteogenic and neuron differentiation of HAMs at P5 were also determined.

Development of skin substitutes from human amnion derived cells *in vitro*

HAECs at P1-P3 and HAMs at P5 were used for skin substitute development. Human plasma was collected, sterile-filtered and stored at -20ºC until used. Dermis-like substitutes were formed by mixing HAMs with 1ml of human plasma and $CaCl_2$ to initiate the fibrin polymerization. HAECs were then seeded on top of the fibrin containing HAMs. These skin substitutes were either remained in the submerged culture or cultured at the air-liquid interface for a period of 1, 2, 3 weeks before they were fixed in 10% formalin for hematoxylin staining and immunohistochemistry analysis or in 3% glutaraldehyde for scanning and transmission electron microscopic analysis. Human keratinocytes and dermal fibroblasts were cultured concurrently and act as a positive control.

Engraftment of skin-like substitutes into nude mice

The skin substitutes formed from human amnion derived cells were transplanted into nude mice after 3-5 days in submerged culture. After 1, 2 and 3 weeks of transplantation, the skin substitutes were harvested and fixed in either 10% formalin for hematoxylin staining and immunohistochemistry analysis or in 3% glutaraldehyde for scanning and transmission electron microscopic analysis. Skin substitutes were used as control.

RESULTS

Amnion yielded more than 5.09×10^7 HAECs with F12:DMEM being the best transport buffer. HAECs had a polygonal shape at P0 and became larger and flattened epithelial features at P4. They have a declined growth rate after serial passage. HAECs expressed CD9, CD44, CD73, CD 90 and HLA-A, B, C but not CD31, CD34, CD45, CD 117 and HLA-DR, DP, DQ after serial passage. CD44, CD90 and HLA-A, B, C significantly increased while CD9 significantly decreased after serial passage. Also, cultured HAECs expressed the stemness and epithelial genes. After serial passage, CK3, CK19 and involucrin gene expressions were upregulated, but p63, CK1 and CK14 gene expressions were downregulated. Sustained gene expressions for integrin β1 and CK18 were observed. On the other hand, Oct-3/4, Sox-2, Nanog3, Rex-1, FGF-4, and FZD-9 decreased after serial passage; BST-1 and ABCG-2 increased from P0 to P1 and then decreased from P1-P4; but nestin and vimentin increased after serial passage. For the *in vitro*

differentiation, it showed positive staining with Oil Red O staining and Alizarin Red staining for the adipogenic and osteogenic differentiation respectively. Whereas for the neural differentiation, it showed strong positive staining against anti-NSE and also demonstrated significant increased in the gene expression for NF-M, β-III Tubulin and MAP-2. It has CFE of 5.7% at P2 with colony forming frequency of 1 colony per 23 HAECs plated.

With the addition of EGF, HAECs displayed polygonal shape and they became flattened and more dispersed after day 4 of culture. The dose-response profile of EGF on the proliferation of cultured HAECs showed that 10ng/ml EGF was the optimal concentration for the proliferation of HAECs. The epithelial genes expression: CK1, CK14, CK18 and involucrin, were downregulated with increasing EGF concentration. Similarly, the proliferation marker genes expression: p21, p53 and pRb, were also downregulated.

Dose-response profiles of KGF on cultured HAECs showed the highest proliferation at 10ng/ml KGF. Quantitative RT-PCR results showed that HAECs cultured in KGF medium differentially expressed the epithelial genes (CK1, CK3, CK13, CK14, CK16, CK18, CK19, Integrin β1, Involucrin and P63) compared to control.

Cultured HAMCs displayed an intermediate epitheloid-fibroblastoid morphology at an initial culture and the fibroblastoid features became more pronounced in later passages. They showed high clonogenic activity and faster proliferation at later passages. Quantitative RT-PCR showed the cultured HAMCs expressed the stemness genes; Oct-4, Sox-2, FGF-4, Rex-1, Nanog3, Nestin, FZD-9, ABCG-2 and BST-1. Their expression reduced following passages. Similarly, the angiogenic genes; PECAM-1, eNOS, vWF, VEGF receptor 2, PGF, HGF and angiopoeitin-1, were expressed until passage 5 and were downregulated in serial passages. For adipogenic and osteogenic differentiation, HAMs showed positive staining with Oil Red O staining and with Alizarin Red staining which showed calcium deposition. HAMs showed significant increased in neural specific β-tubulin III and upregulated BDNF, NF-M and MAP-2 via quantitative RT-PCR.

The development of skin substitutes *in vitro* and *in vivo* showed the amnion stem cell has high potential for skin wounds treatment.