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Enrichment in c-Kit improved differentiation potential of amniotic membrane progenitor/stem cells

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ABSTRACT

Introduction: Human term placenta has attracted increasing attention as an alternative source of stem cells for regenerative medicine since it is accessible without ethical objections. The amniotic membrane (AM) contains at least two stem cell types from different embryological origins: ectodermal amniotic epithelial stem cells, and mesodermal mesenchymal stromal cells. Among the second group we studied the characteristics of amniotic mesenchymal cells (AMC) versus the ones enriched for the commonly used surface marker c-Kit (amniotic progenitor/stem cells-ASC), a stem cell factor receptor with crucial functions in a variety of biological systems and presents in early progenitors of different origin, as been already demonstrated in the enriched chorionic stem cells.
Methods: After isolation, cells from the amniotic membranes (amniotic cells-AC) were selected for c-Kit (ASC) and compared these cells with c-Kit unselected (AMC), evaluating the expression of other stem cell markers (Oct-4, Tra-1-81, SSEA-4), CD271 and Slug.
Results: Immunofluorescence analysis showed that ASC cells exhibited greater stem cell marker expression and included more CD271 and Slug positive cells. This was consistent with the interpretation that c-Kit enriched AC show greater stemness capacity compared to c-Kit unselected AMC.
Discussion: AMC and ASC can both differentiate into various cell types including adipogenic, osteogenic, chondrogenic, neurogenic and hepatic lineages, but the enrichment in c-Kit improved stemness and differentiation potential of ASC.

1. Introduction

Stem cells are defined by their unique capacity to differentiate into multiple cell lineages and tissue types. Besides their established potency hierarchy they also show a developmental hierarchy based on the specific stages of ontogenesis: embryonic, fetal and adult. Fetal stem cells can derive either from the fetus or extra-embryonic structures of fetal origin which are usually discarded following birth [1], such as umbilical cord blood, amniotic fluid, Wharton's jelly and placenta, containing the amniotic membrane, chorion and decidua [2]. These tissues are a rich source of broadly multipotent stem cells with immunosuppressive properties, that make them an exciting new approach for treatment of disease [3–5] and an accessible resource for regenerative medicine [6].

Cells derived from human placenta and fetal membranes exhibit several features of early embryonic stem cells (ESC) regarding plasticity [7] and expression of stem cell markers [8]. Their

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differentiation potential, either *in vitro* or *in vivo*, recapitulates features of plasticity residing between pluripotent and multipotent stem cells [2]. This stem cell source has considerable advantages over other sources of stem cells, such as adult or bone marrow-derived, since these do not require patients' biopsies associated with their collection and use, they have low immunogenic profile and lack tumorigenicity [4,9,10].

The amniotic membrane (AM) is the innermost layer of the gestation sac and consists of a thin epithelial layer, a thick basement membrane and an avascular stroma [11]. It arises prior the gastrulation from embryonic epiblast cells that are also destined to develop into the three primary fetal germ layers: the endoderm, the mesoderm, and the ectoderm [9]. In pregnancy, AM cells are separated from the embryo or fetus by the amniotic fluid. It is possible that signaling which normally regulate cell differentiation in the embryo are not present in the extra-embryonic tissue during development and therefore some cells may retain their stem or stem cell-like capabilities until term [13]. In addition, the phenotype of stem cells originating from this tissue source is affected by the development stage at which they are isolated [9,14].

Several protocols have been established for AM cell isolation, primarily based on mechanical separation of the amnion from the chorionic membrane and subsequent enzymatic digestion [15–18]. Two cell types of different embryological origin are present in AM: amniotic epithelial cells, derived from embryonic ectoderm, and amniotic mesenchymal cells of mesodermal origin [2]. AM mesenchymal stem cells share similar phenotypic characteristics to those derived from adult sources [20] but in common with stem cells derived from the amniotic fluid they have a high proliferation rate and multilineage differentiation potential [21,22].

The presence of mesenchymal cell markers (CD73, CD105, CD90) [2,21,23], pluripotency markers, including Oct-4, SOX2 and Nanog [9,24–26] and embryonic stem cell markers, such as c-Kit (CD117), SSEA-4, TRA-1-60 and TRA-1-81 [27] also established the potential of these AM derived cells. Nevertheless, it remains unclear whether the human AM harbors true pluripotent stem cells, or a mixed population of multipotent progenitor cells.

The expression/activation of c-Kit in different cells lines is associated with the expression of a specific member of the Snail Family, Slug, a zinc-finger transcriptional factor during epithelial to mesenchymal transition (EMT) [28]. EMT plays crucial roles in the formation of the body plan and in the differentiation of multiple tissues and organs; it contributes to tissue repair, but it can adversely cause organ fibrosis and promotes carcinoma progression through a variety of mechanisms [29]. In cells with migratory and invasive properties, EMT induces stem cell characteristics, prevents apoptosis and senescence, and contributes to immunosuppression. Thus, the mesenchymal state is associated with the capacity of cells to migrate to distant organs and to maintain stemness [30].

Recent evidence suggests that cells that undergo EMT acquire stem cell-like properties [31,32] and retain the expression of several transcription factors, including Oct-4 and Nanog whilst showing a mesenchymal phenotype [30].

In a previous study [33], we described that a mesenchymal population of chorionic cells enriched for c-Kit (CSC) better maintained some of the characteristics of the primitive embryonic layers from which they originate. The differentiation capability of these cells was improved by c-Kit enrichment, showing an intermediate potential between pluripotent ESCs and multipotent adult stem cells. We hypothesized that enrichment for c-Kit would have a similar effect in AM derived progenitor/stem cells. In this study we investigated the characteristics of c-Kit enriched ASCs, by assessing their immunophenotypical profile and differentiation potential and examined a possible correlation between c-Kit positive cells and Slug during passages.

2. Materials and methods

2.1. Human term placental collection

Fresh human term placentae ($n = 6$ from XY fetus, gestational age 38 ± 1 weeks of pregnancy, mean ± SD) were obtained from healthy pregnant women at the time of elective Caesarian section delivery. Informed written consent was obtained from all patients (Table 1). The study had ethical approval (NRES Committee London – Bentham. Study title: Amniotic fluid and placental stem cells. REC reference: 08/H0714/87). After delivery, the umbilical cord was trimmed and an X-shaped incision was cut into the placental tissue. The amniotic membrane (AM) was separated from the chorion by gentle peeling, cut into small pieces (3 cm^2) and rinsed with cold phosphate buffer saline (PBS) to cleanse of blood contamination [34].

2.2. FISH analysis to confirm fetal origin of AM

One sample of each freshly isolated AM was fixed in 4% paraformaldehyde in PBS, processed, embedded in paraffin and sectioned (7 μm thick). FISH analysis was performed using directly fluorochrome-labeled probes for chromosome X and Y (CEP probes: chromosome enumeration probes; Vysis, ABBOT, Rungis, France) and hybridization according to the manufacturer's recommendations. For each probe used, reliability of the method was demonstrated in control samples examined in which the karyotype was available. Nuclei were stained with 1 μg/mL DAPI in H_2O. Microscopic images were acquired and evaluated using Nikon A1 confocal laser scanning microscope and image rendering performed using Adobe Photoshop software.

2.3. Immunoperoxidase analysis of stem cell markers

DAB staining was performed on consecutive serial AM sections as previously described [33]. The sections were incubated with the primary antibodies (Abs) (rabbit anti-c-Kit and mouse anti-Oct-4, Santa Cruz Biotechnology, Santa Cruz, USA), and then with the peroxidase labeled-secondary Abs (anti-rabbit-HRP and anti-mouse-HRP, Jackson ImmunoResearch, PA, USA) and finally stained with DAB substrate solution (Sigma–Aldrich, St. Louis, USA). The specificity of labeling was confirmed by the absence of staining by substitution of PBS for the primary Abs.

2.4. Cells isolation and culture in vitro

AM underwent a mechanical and enzymatic digestion to isolate AMCs. AM fragments were enzymatically digested in a solution of dispase and collagenase type IA (1 mg/mL, Sigma–Aldrich, St. Louis, USA) diluted in α-MEM for 90 min at 37 °C with gentle shaking. The cell solution was then passed through a 20-G needle, filtered through a 100 μm cell strainer and centrifuged 5 min at 300 g.

Pellets were resuspended in α-MEM with 10% NBCS (New Born Calf Serum), 100 U/mL penicillin, 2 mM L-glutamine, 100 μg/mL streptomycin (complete α-MEM), and cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. The medium was changed twice a week. Just before cells became confluent, adherent cells were detached with 0.05% trypsin/EDTA solution and subdivided into new flasks (all cell culture reagents and digestive enzymes are from Sigma–Aldrich, St. Louis, USA) [35].

2.5. c-Kit enrichment

AMCs were enriched for CD117 (c-Kit) positive cells using magnetic cell sorting, MACS® separation Kit (Miltenyi biotech, Bergish Gladbach, Germany), according to the manufacturer's instructions. Cells were incubated with a rabbit anti-c-Kit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA). c-Kit was detected by magnetically labeled secondary Ab (anti-rabbit IgG, Miltenyi biotech, Germany). For each selection approximately 7×10^6 cells were used [38]. c-Kit positive cells are indicated as ASC.

2.6. Western blotting

Whole cell lysates were obtained by addition of a lysis buffer as described [36]. After sonication, lysates were cleared by centrifugation for 15 min at 14,000 g in a refrigerated centrifuge. The protein lysates were then added to SDS sample buffer and boiled. 70 μg of protein extract from each sample, quantified by a Bradford Protein Assay (Biorad; Bradford, 1976), underwent 8% SDS-polyacrylamide gel electrophoresis and then blotted onto a PVDF membrane as previously described [37]. The membranes were blocked with 3% dry milk and 2% bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, USA) in tris-buffered saline–Tween 20 (0.1%) (TBS-T). Blots were incubated overnight with the primary Abs (diluted in blocking buffer: TBS-T + 2% BSA and 3% milk): anti Oct-4 and anti c-Kit (Santa Cruz Biotechnology, Santa Cruz, USA), anti Slug and anti β-actin Abs (all from Sigma–Aldrich, St. Louis, USA). Membranes were further incubated with peroxidase-labelled anti-rabbit, anti-mouse secondary Abs (Jackson ImmunoResearch, West Grove, PA, USA). All membranes were detected using ECL (**E**nhanced **C**hemio**L**uminescence, Amersham). Anti β-actin Ab was used as control of protein loading in timing experiments. Quantitative optical density analysis of c-Kit, Slug and Oct-4 bands was quantified by densitometric scanning using Image J Data Analyzer software. Relative content was normalized to β-actin and is presented as the marker/β-actin ratio.