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Chondrogenic differentiation of human embryonic germ cell derived cells in hydrogels

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Abstract: Human embryonic germ (hEG) cells have the potential to self-renew over long periods of time and differentiate into various lineages. Cells derived from embryoid bodies of hEG cells express a broad spectrum of gene markers and have been induced towards cells of ecto-dermal and recently endo-dermal and mesenchymal lineages. LVEC cells express a number of surface marker proteins characteristic of mesenchymal stem cells (MSCs), indicating the potential of these cells to differentiate into mesenchymal tissues. Here we demonstrate the homogenous differentiation of LVEC cells into hyaline cartilage. Three dimensional tissue formation is achieved by encapsulating cells in synthetic hydrogels followed by incubation in chondrocyte-conditioned culture medium. Homogenous hyaline cartilage was produced, even after 63 population doublings (13 passages). The high proliferative capacity of these cells without teratoma formation, homogenous differentiation, and three-dimensional cartilage tissue formation suggests the significant potential of LVEC cells for cartilage tissue engineering applications.

I. Introduction

The intrinsic biology of cartilage tissues limits their self-regeneration potential making repair of cartilage lesions an intractable problem. Cell-based therapies such as tissue engineering utilizing cell-biomaterial composites offer great potential for cartilage regenerations. However, the use of differentiated chondrocytes as primary cell source is considerably hindered by deleterious phenotypic changes of chondrocytes upon *ex vivo* expansion [1].

Mesenchymal stem cells (MSCs) isolated from various tissues in the adult have the capability to differentiate into various mesenchymal tissues such as cartilage [2]. While MSCs are entering clinical trials for cardiac and orthopedic applications, these cells still have a limited expansion and differentiation capacity. To this end, there is a strong interest in creating alternative mesenchymal progenitor cells with greater proliferative and tissue forming capabilities [3]. Recently, Barberi *et al*. have isolated a mesenchymal stem cell population from human embryonic stem (hES) cells and demonstrated its ability to differentiate into various mesenchymal tissues [4].

Human pluripotent stem cells have been isolated from the inner cell mass of the blastocyst (embryonic stem cells) and the primordial gonadal ridge (embryonic germ cells). Both of these pluripotent cell populations have the capability to proliferate and self renew over long periods of time and the potential to differentiate towards all cell types/lineages [5, 6]. These cells have been extensively studied to understand the early embryo development as well as for cell and tissue regeneration therapies. However spontaneous differentiation of three dimensional ES/EG cell aggregates, termed as embryoid bodies (EBs), often leads to a heterogeneous population of differentiated and undifferentiated cells, thus limiting their unique role as a progenitor cell source for regenerative applications [7].

Controlled differentiation of EBs into the desired lineage is usually achieved to some extent through a "cocktail" of growth factors and other signaling molecules that provide tissue specific progenitor cell niches. For example, incubation of EB-laden hydrogels in the presence of TGF-β1 has been shown to induce chondrogenesis [8]. In addition to growth factors, the lineage commitment of stem cells is also controlled by small cell-permeable molecules such as dexamethasone, glycerol phosphate, vitamin C, retinoic acid, purmorphamine, etc [9, 10]. In this study we demonstrate that human embryonic germ derived LVEC cells with a close resemblance to mesenchymal stem cells (MSCs) can be differentiated into cartilageous tissue by providing a tissue-specific niche. The human embryonic germ-derived LVEC cell line was first generated by Shamblott *et al*., by selection and culture of cells disaggregated from hEG cell embryoid bodies [11].

II. METHODS

Cell culture: LVEC cells were expanded by culturing them as previously described [12]. EGM2MV medium (Clonetics, San Diego) included 5% fetal calf serum, hydrocortisone, human bFGF, human VEGF, R3 IGF-I, ascorbic acid, human EGF, heparin, gentamycin, and amphotericin. Tissue culture dishes were coated with bovine type I collagen (10 $\mu g/cm^2$; Collaborative Biomedical Products, Bedford, MA,). Cells were cultured at 37°C, 5% CO_2, and 95% humidity and then routinely passaged 1.0 to 10.0 using 0.025% trypsin, 0.01% EDTA (Clonetics).

FACS: LVEC cells (P13) were harvested and re-suspended to approximately 1×10^6 cells in 50 µl PBS containing 0.1% bovine serum albumin (BSA). Cell samples were separately labeled with antibodies on ice for 30 minutes, and when required, they were further incubated with secondary antibody (FITC-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) for an hour on ice after washing with PBS containing 0.1% BSA. Antibodies against the following cell surface markers were used: fluorescein isothiocyanate (FITC)-conjugated rat anti-human antigen for CD45, CD105, Stro1, PDGFR-α and HLA-ABC (Becton Dickinson; San Jose, CA), phycoerythrin (PE)-conjugated rat anti-human for CD13, CD14, CD29, CD34, CD44, CD73, CD90, CD117, CD133, CD146, CD166 and HLA-DR (Becton Dickinson, San Jose, CA). At least 15,000 events were collected from each run of flow cytometry. Data were analyzed with the FACS calibur cytometer and CellQuest software (Becton Dickinson). Bone marrow derived human mesenchymal stem cells, p5, (MSCs were procured from Cambrex, PT-2501, Lot # 4FO760) were used as a control.

Chondrogenic differentiation: Cell aggregates in the diameter range of 100-150 µm were created using an orbital shaker at 100 rpm via the rotational aggregation method as described previously [13]. The cell aggregates were dispersed in 10% (w/v) poly(ethylene glycol diacrylate) (PEGDA) solution and photo-polymerized using 0.05% irgacure 2959 to yield 3D cell-laden hydrogels (constructs). The constructs were transferred into a 24 well plate cultured using both chondrogenic medium and chondrocyte-conditioned medium with 10ng/mL TGF-β1. For chondrocyte conditioned medium, Primary bovine chondrocytes were plated at an initial cell density of 10,000 cells/ cm^2 in a tissue culture flask and cultured with chondrocyte medium [Dulbecco's Modified Eagle Medium with high glucose (gibco, Invitrogen) supplemented with 10 mM HEPES (Gibco, Invitrogen), 0.4 mM L-proline (Sigma, St. Louis, MO), 50 µg/ml ascorbic acid (sigma), 10% fetal bovine serum (FBS, Qualified), 0.1 mM non-essential amino acid (Gibco Invitrogen), and 1% penicillin streptomycin]. After the cells reached around 80% confluency, the medium was removed and the culture was replenished with fresh chondrogenic medium [high-glucose DMEM supplemented with 100 nM dexamethasone, 50 µg/ml ascorbic acid-2-phosphate, 40 µg/ml proline, 100 µg/ml sodium pyruvate, 1% penicillin streptomycin and 50 mg/ml ITS-Premix (Collaborative Biomedical: 6.25 ng/ml insulin, 6.25 mg transferiin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 mg/ml linoleic acid)]. At the end of 36-48 hours, this medium was collected and used for LVEC-laden hydrogel.

Histology and Immunostaining To evaluate chondrogenic differentiation, hydrogel constructs were fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C and transferred to 70% ethanol until processing. Constructs were embedded in paraffin, and cut into 5 µm sections that were stained with Safranin-O/fast green for chondrogenic differentiation. Immunoflourescent staining was performed according to manufacturer's protocol for collagen type II (Zymed Laboratories, San Francisco, CA). Polyclonal rabbit antibodies against mouse type II (RDI, Flanders, NJ) were used with 1:40 to 1:100 dilutions.

Biochemical analysis: To estimate GAG and collagen content, lyophilized LVEC-laden hydrogels were crushed using pellet pestle mixer (Kimble/Kontes) and digested in papainase solution (construct/1 ml papainase solution; 125 µg/mL; Worthington Biomedical, Lakewood, NJ) for 18 hrs at 60°C. The DNA content was determined using Hoechst 33258 dye [14]. The GAG content characterized by CS was measured using dimethylene blue (DMMB) spectrophotometric assay at A_{525} [15]. Chondroitin sulfate in de-ionized water was used as standard. Total collagen content was determined by measuring the hydroxyproline content of the constructs after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramine-T [16].

Gene expression analysis: Total RNA was extracted with Trizol, and reverse-transcribed into cDNA using SuperScript First-Strand Synthesis System (Invitrogen). 1 µl aliquots of the resulting cDNA were amplified at the corresponding annealing temperature for the interested geneS for 35 cycles. The PCR primers are listed in Table 1. PCR products were separated by electrophoresis at 100V on a 2% agarose gel in Tris-acetate-EDTA buffer and visualized after staining with ethidium bromide staining.

Table 1: Sequences of primers used in (RT-PCR) and their expected base pair

Gene	Primer sequence: sense/antisense	Product size (bp)
β-actin	5' TGGCACCACACCTTCTACAATGAGC 3' 5' GCACAGCTTCTCCTTAATGTCACGC 3'	396
ALP	5' ACGTGGCTAAGAATGTCATC 3' 5' CTGGTAGGCGATGTCCTTA 3'	476
Cbfa-1	5' CCACCCGGCCGAACTGGTCC 3' 5' CCTCGTCCGCTCCGGCCCACA 3'	258
Col II	5' GAAACCATCAATGGTGGCTTCC 3' 5' CGATAACAGTCTTGCCCCACTT 3'	301
AGN	5' GCCTTGAGCAGTTCACCTTC 3' 5' CTCTTCTACGGGGACAGCAG 3'	395
COMP	5' CAGGACGACTTTGATGCAGA 3' 5' AAGCTGGAGCTGTCCTGGTA 3'	314

III. RESULTS

Cell morphology of the hEG-derived population was spindle-shaped and fibroblast-like, characteristic of mesenchymal stem cells (fig. 1). Moreover, the LVEC cells expressed a number of cell surface markers

characteristic of MSCs such as CD44, CD73, CD105, HLA-ABC and Stro-1. Pellet culture of LVEC cells using chondrogenic medium resulted in up-regulation of various genes related to mesenchymal tissues. However, morphologically the pellets contained a disordered matrix that was not typical of any mesenchymal tissue, consistent with the observed heterogeneous gene expression [12].

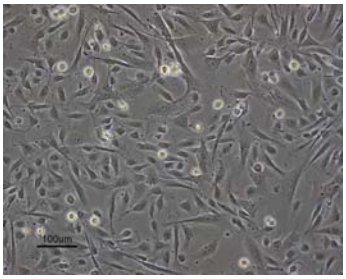

Fig.1: Phase contrast image of LVEC grown in EGM2MV medium

The LVEC cell aggregates were subsequently encapsulated in a hydrogel and cultured in chondrogenic medium with TGFβ, again yielding negative results for proteoglycan production (Fig. 2A). The cell-laden hydrogels were further cultured using chondrocyte conditioned medium. Homogenous chondrogenesis of the LVEC cells in the hydrogel was observed by Safranin-O staining when TGF-β1 was supplemented (Fig. 2B).

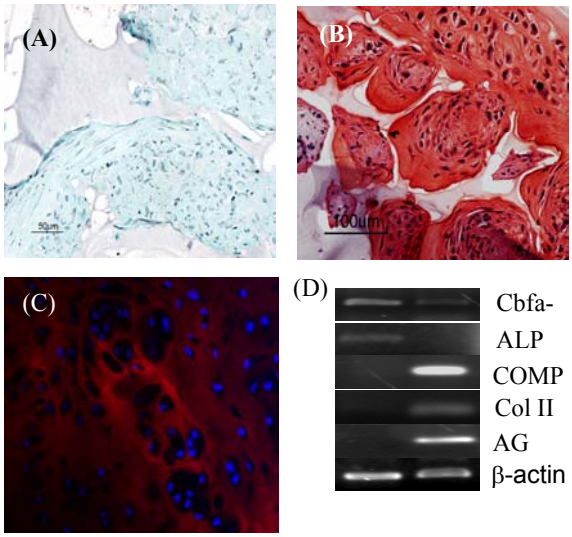

Fig 2: (A) Negative Safranin-O staining for LVEC cell-laden hydrogels after 21 days of culture in chondrogenic medium with TGF-b1 supplementation. (B) Positive Safranin-O staining when the cell-laden hydrogels were cultured in chondrocyte conditioned medium along with TGF-β1 supplementation. (C) Immunoflourescent staining for collagen type II indicating hyaline cartilage production. (D) RT-PCR for various chondrogenic markers.

The hyaline nature of the engineered cartilage was identified by immunoflourescent staining for collagen type II (Fig. 2C). Presence of cartilage oligomeric protein (COMP), type II collagen and aggrecan gene expression, which are not constitutively expressed in undifferentiated LVEC cells, further confirmed chondrogenesis (Fig. 2D). LVEC cells strongly express some of the early bone markers such as cbfa-1 and ALP. In comparison with undifferentiated LVEC cells, cell-laden hydrogels cultured using the conditioned medium significantly reduced the expression of these markers. The quantification of cartilage matrix proteins such as glycosaminoglycan and collagen further substantiates cartilage tissue production by LVEC cells (Fig. 3). Co-culturing the cell laden hydrogels in presence of chondrocytes was also able to stimulate chondrogenesis in LVEC-laden hydrogels. In summary, homogeneous three dimensional cartilage tissues can be produced by LVEC (P13) after the necessary unique biomaterial and culture conditions have been formulated.

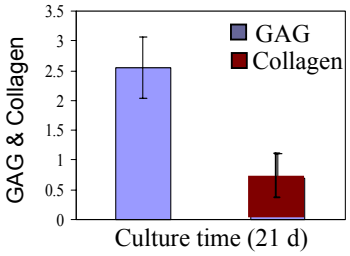

Fig. 3: Quantification of glycosaminoglycan (10gm/gm) and collagen content (gm/gm) normalized to DNA

IV. DISCUSSION

Chondrogenic differentiation of hEG-derived cells required unique conditions to produce tissue. The standard chondrogenic culture conditions used to culture the cell pellets and the cell-laden hydrogels were able to stimulate the chondrogenic differentiation of the LVEC cells as indicated by up-regulation of characteristic gene expressions. However, this did not translate into protein production and three-dimensional tissue organization characteristic of native hyaline cartilage. Both the use of a co-culture system and a chondrocyte-conditioned medium enabled chondrogenic differentiation of LVEC cells. The tissue morphology of engineered cartilage was highly indicative of hyaline cartilage. The proteoglycan and collagen staining and cell morphology was homogenous, suggesting complete differentiation to chondrocytes with minimal contamination by other cell types. Formation of homogenous cartilage tissue suggests that the primary

chondrocytes provide adequate amount of cytokines or soluble signals for the differentiation of LVEC cells towards cartilage tissue. Co-culturing method has been explored previously to achieve homogenous and lineage specific differentiation of stem cells [17, 18].

The highly homogenous tissue obtained in this study may also be a result of the hydrogel scaffold utilized for chondrogenesis. Non-adhesive hydrogels such as those based on polyethylene glycol (PEG) discourage fibroblast growth, thus selecting nonadhesion-dependant cells that produce hyaline cartilage. A combination of three-dimensional environment from a biomaterial along with appropriate soluble factors was able to provide the instructive environment to regulate the cells' commitment, and hence promote homogenous differentiation into cartilage tissue. Incorporation of further biological signals into the biomaterial may induce chondrogenesis without chondrocytes as we have demonstrated with other cell types (19). The results discussed here suggest that LVEC cells are a potentially useful stem cell population for cartilage tissue engineering applications because of their ability to undergo homogenous differentiation, ease in expansion and lack of teratoma formation when injected *in vivo*.

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