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Epiphyseal Chondroprogenitors Provide a Stable Cell Source for Cartilage Cell Therapy

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Articular cartilage regeneration poses particularly tough challenges for implementing cell-based therapies. Many cell types have been investigated looking for a balanced combination of responsiveness and stability, yet techniques are still far from defining a gold standard. The work presented focuses on the reliable expansion and characterization of a clinical grade human epiphyseal chondroprogenitor (ECP) cell bank from a single tissue donation. A parental human ECP cell bank was established, which provides the seed material for master and working cell banks. ECPs were investigated at both low and high cumulative population doublings looking at morphology, monolayer expansion kinetics, resistance to cryogenic shock, colony-forming efficiency, and cell surface markers. Three-dimensional micropellet assays were used to determine spontaneous extracellular matrix deposition at varying population doublings and monolayer 2D differentiation studies were undertaken to assess the propensity for commitment into other lineages and their stability. ECPs exhibited remarkable homogeneity in expansion with a steady proliferative potential averaging three population doublings over 8 days. Surface marker analysis revealed no detectable contaminating subpopulations or population enrichment during prolonged culture periods. Despite a slight reduction in Sox9 expression levels at higher population doublings in monolayer, nuclear localization was equivalent both in monolayer and in micropellet format. Equally, ECPs were capable of depositing glycosaminoglycans and producing aggrecan, collagen I, and collagen II in 3D pellets both at low and high population doublings indicating a stable spontaneous chondrogenic potential. Osteogenic induction was differentially restricted in low and high population doublings as observed by Von Kossa staining of calcified matrix, with a notable collagen X, MMP13, and ADAMTS5 downregulation. Rare adipogenic induction was seen as evidenced by cytoplasmic lipid accumulation detectable by Oil Red O staining. These findings highlight the reliability, stability, and responsiveness of ECPs over prolonged culture, making them ideal candidates in defining novel strategies for cartilage regeneration.

Key words: Chondrocytes; Progenitor cells; Articular cartilage regeneration; Cell banking

INTRODUCTION

The avascular and alymphatic nature of articular cartilage coupled with its notably low cell to tissue volume ratio are at the core of its limited inherent regenerative capacity. Traditional tissue engineering strategies have therefore encountered many hurdles but have evolved dramatically in the past few decades (1). The latest strategies seem to concentrate more on chaperoning regeneration rather than tissue replacement (9). However, we are still far from determining a gold standard for cell-based cartilage therapeutic strategies with the choice of cell source remaining a central and controversial issue. Bone marrow-derived adult mesenchymal stromal cells (MSCs) have lately become the most popular choice due to their multipotency and reported immune-modulatory capabilities, especially in cases of allogeneic transplantations (8). Concerns of homogeneity, reliability, and stability still predominate current investigations (17). For instance, MSCs may present markers of chondrogenesis when stimulated by potent recombinant morphogens, particularly from the transforming growth factor (TGF)-β superfamily. The forced differentiation process, however, often evolves into a phenotypic overshoot by recapitulating endochondral ossification (11). These often observed instabilities and heterogeneous cellular responses have motivated some groups to propose extensive cell sorting and population enrichment protocols in an effort to normalize results and reduce donor variability (13,18,21). These hurdles have made adult MSCs very difficult to control for applications in cartilage tissue regeneration.

In a microenvironment as damaged and unstable as that of osteoarthritic cartilage, the need for a responsive yet stable therapeutic cell type is a key element. Fetal cartilage

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has been shown to exhibit a remarkable ability for self-repair (15), and much like other progenitor and stem cell types, fetal cells appear to exhibit immune-modulatory activity (14,20). Human epiphyseal chondroprogenitors (ECPs) therefore present distinct advantages by having both a prededicated tissue-specific repertoire of differentiation potential and fine-tuned sensitivity to rapidly changing environmental cues during the course of natural tissue growth and maturation. Compared with isolated quasi-quiescent adult chondrocytes normally found embedded in the adult tissue, ECPs would theoretically exhibit superior stability in withstanding environmental variability. ECPs would also maintain a commitment to their differentiation program, one that necessitates an inherent mild plasticity, allowing them to modulate their response to varying microenvironments.

The work presented here focuses on the characterization of a human ECP cell bank from a single tissue donation produced within a federally registered transplantation program in Switzerland. These results reported herein mark the first step in defining an ECP-based cellular therapy strategy for cartilage regeneration, which could be used in early degenerative joint indications or cell therapy-assisted surgeries such as microsurgeries of the hand.

MATERIALS AND METHODS

Tissue Processing and Cell Banking

Human ECPs were isolated from the proximal ulnar epiphysis of a 14-week gestation donor (Centre Hospitalier Universitaire Vaudois Ethics Committee Protocol #62/07). The procedure mirrored that used by our group for fetal skin fibroblasts (19). Briefly, the tissue biopsy was microdissected and mechanically dispersed. ECP outgrowth was observed at 1 week (Fig. 1A) and expansion was accomplished at 2 weeks in Dulbecco's modified Eagle medium (DMEM) with 25 mM dextrose, 1 mM sodium pyruvate, 5.97 mM L-glutamine, and 10% clinical grade fetal bovine serum (Invitrogen, Glasgow, Scotland), free of antibiotic supplementation. The parental cell bank was established with 100 vials of $5–10\times10^6$ cells and stored in the vapor phase of liquid nitrogen, providing cells for a working cell bank dedicated to in vitro investigations as well as cells for in vivo validations down the line. All cell culture media and supplements were purchased from Invitrogen, Life Technologies Ltd. (Paisley, UK), unless otherwise noted.

Cell Expansion and Culture

Monolayer expansions were performed in standard tissue culture polystyrene flasks in basal media containing DMEM with 25 mM dextrose, 1 mM sodium pyruvate, 5.97 mM L-glutamine, and 10% fetal bovine serum (Sigma, St. Louis, MO), free of antibiotic supplementation. ECPs were seeded at 4,000 cells/cm^2, placed in standard humidified tissue culture incubators at 37°C with 5% CO_2, and passed after reaching 90% confluence following two media changes over an average of 9 days per passage.

Proliferation, Viability, and Recovery

The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI) was used to monitor proliferation kinetics in basal media on standard tissue culture polystyrene at 2, 4, 6, 8, 10, 12, and 14 days. This was performed on ECPs after 9 and 18 cumulative population doublings (PDs) to evaluate potential losses in proliferative potential. The 490-nm absorbance values, which correlate with the amount of metabolically active cells, were read using the Wallac VICTOR^{2TM} 1420 multilabel plate reader (PerkinElmer, Waltham, MA). The stability of cells was assessed after freezing and stocking of ECPs. Vials from the working cell bank containing 2 million cells/ml, previously cryopreserved in an isopropanol bath at –80°C overnight then transferred and stocked in liquid nitrogen for 3 years, were thawed in a 37°C water bath. The LIVE/DEAD® viability/cytotoxicity staining kit (Invitrogen, Life Technologies Ltd., Paisley, UK) was used to quantify live and dead cells immediately after thawing by tracking green fluorescent calcein-AM-positive live cells and red fluorescent ethidium homodimer-1-positive dead cells using the BD Accuri® C6 flow cytometer and CFlow® software for analysis (Accuri® Cytometers, Ann Arbor, MI). To evaluate the plating efficiency following thawing, cells were seeded onto standard tissue culture polystyrene for 24 h in basal media. The remaining floating cells, both live and dead, were quantified in a standard counting chamber using Trypan blue exclusion.

Surface Marker Profiling

Surface marker detection was performed on ECPs after 9 and 24 cumulative PDs to assess population stability and homogeneity. ECP surface markers were detected using monoclonal mouse antibodies specific for human CD14, CD26, CD34, CD44, CD90, CD105, and CD166 (Abd Serotec, Dusseldorf, Germany) in combination with an AlexaFluor® 488 goat anti-mouse IgG polyclonal antibody (Invitrogen, Life Technologies Ltd., Paisley, UK). Equally, phycoerythrin-tagged monoclonal CD45, CD73, and human leukocyte antigen (HLA)-DP,DQ,DR antibodies as well as fluorescein-tagged HLA-A,B,C antibodies were also used (R&D Systems, Minneapolis, MN). Appropriate isotype controls with equivalent concentrations were used to determine nonspecific staining. Ethidium bromide monoazide (Sigma, St. Louis, MO) was used to detect and exclude cells with damaged membranes. The CyAN™ ADP analyzer (Beckman Coulter, Indianapolis, IN) was

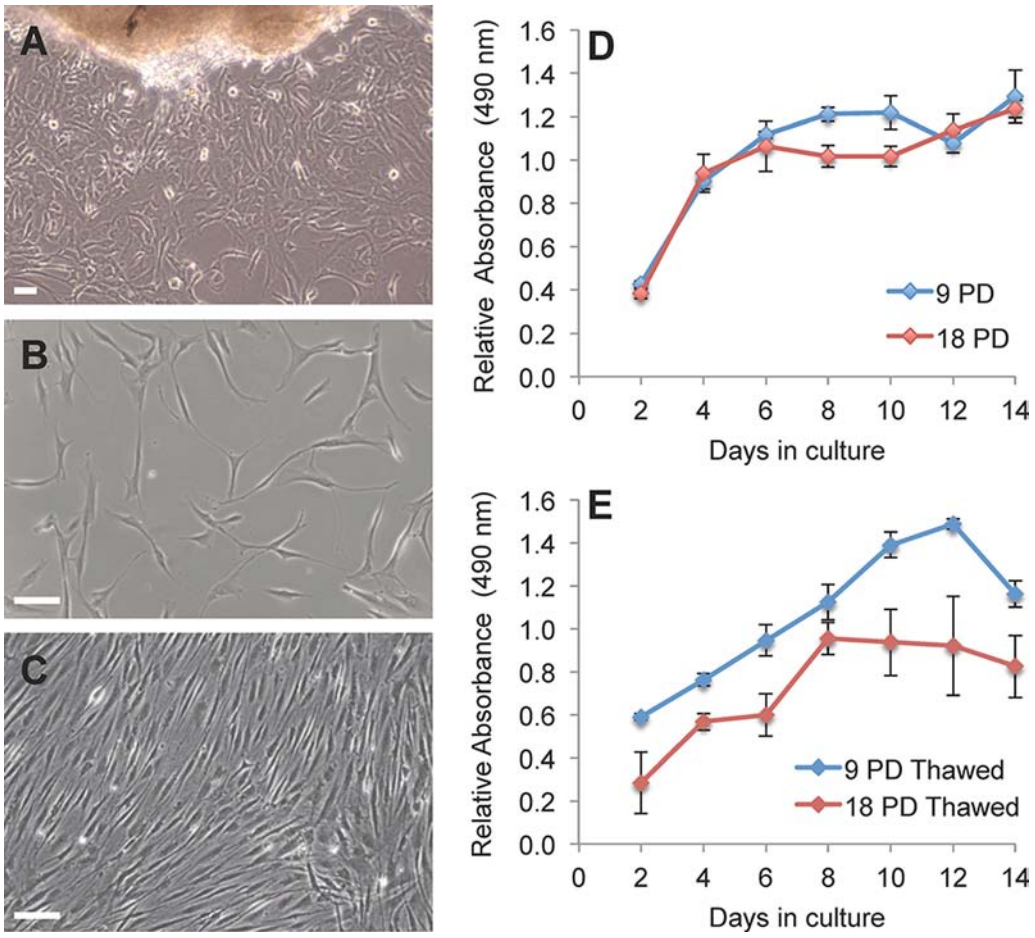

Figure 1. Epiphyseal chondroprogenitor (ECP) outgrowth from the original tissue biopsy after 1 week in culture (A). ECPs exhibiting homogenous spindle like morphology in subconfluent (B) and confluent (C) culture conditions. Proliferation kinetics seeded after 9 and 18 PDs following passage (D) or freshly thawed after long-term cryopreservation in liquid nitrogen (E). Scale bars: 100 µm.

used to perform flow cytometry assays, and FlowJo software (Ashland, OR) was used for analysis.

Spontaneous Chondrogenesis

After 9 and 18 cumulative PDs, micropellets were formed by centrifuging 5×10^5 cells in Nunc U96 MicroWell™ plates (Thermo Fisher Scientific, Waltham, MA) at $500\times g$ for 5 min and maintained in 200 µl serum-free media containing DMEM with 25 mM dextrose, 1 mM sodium pyruvate, 5.97 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% insulin-transferrin-selenium (ITS), and 50 µg/ml L-ascorbate (Sigma, St. Louis, MO), with three medium changes per week. ECPs micropellets formed after 9 PDs were harvested for analysis after 17 days in culture to reveal the presence of spontaneously expressed chondrogenic markers. ECP micropellets formed after 18 PDs were harvested after 27 days to allow for more matrix deposition as the pellets had not reached the equivalent size of their 9 PD counterparts by 17 days.

Micropellets were fixed in 4% paraformaldehyde for 2 h at room temperature, washed thoroughly with phosphate-buffered saline (PBS), dehydrated, and embedded in paraffin (Merck, Whitehouse Station, NJ). Sections of 5 µm in thickness were then rehydrated and stained with Alcian blue (Merck, Whitehouse Station, NJ) to detect glycosaminoglycan deposits and a nuclear fast red counterstain (Bio-Optica, Milan, Italy) to highlight cells. Micropellet sections were also processed for immunohistochemistry, screening for chondrogenic markers. Samples underwent heat-mediated antigen retrieval for 20 min at 95°C in 10 mM trisodium citrate buffer (pH 6). Intracellular sex-determining region Y box 9 (Sox9) staining required 20-min incubation in 0.25% Triton X in PBS prior to primary antibody tagging overnight at 4°C with a mouse anti-human Sox9 monoclonal

antibody (0.70 µg/ml, ab76997, Abcam, Cambridge, UK). Secondary tagging was performed at room temperature with AlexaFluor® 488 goat anti-mouse IgG polyclonal antibody (1:1,000, Invitrogen, Life Technologies Ltd., Paisley, UK) and mounted using Vectashield® mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Aggrecan staining required a 30-min hyaluronidase (2 mg/ml, Sigma, St. Louis, MO) treatment at 37°C before applying the primary mouse anti-human aggrecan monoclonal antibody (1:100, AHP0022, Invitrogen, Life Technologies Ltd., Paisley, UK). Collagen II staining required a treatment with chondroitinase-ABC (0.25 U/ml, Sigma, St. Louis, MO) at 37°C for 1 h before applying the primary mouse anti-human collagen II monoclonal antibody (1:100, ab3092, Abcam, Cambridge, UK). Collagen I was stained using a primary rabbit anti-human collagen I polyclonal antibody (1:200, ab292, Abcam, Cambridge, UK). After an incubation overnight at 4°C, the ImmPRESS anti-mouse Ig or anti-rabbit Ig system (Vector Laboratories, Burlingame, CA) was used followed by peroxidase revelation with DAB (Vector Laboratories, Burlingame, CA).

Multilineage Differentiation

After 9 and 18 cumulative PDs, ECPs were plated onto standard tissue culture 12-well plates, grown in basal media until reaching 50% confluence and then kept in differentiation media with changes three times per week.

ECPs were subjected to adipogenic culture conditions over 27 days in DMEM with 25 mM dextrose, 1 mM sodium pyruvate, 5.97 mM L-glutamine, 1% ITS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 µM dexamethasone, 100 µM 3-isobutyl-1-methyl-xanthine (Sigma, St. Louis, MO), and 100 µM indomethacine (Sigma, St. Louis, MO) to evaluate potential adipogenesis. Cells were then fixed in 4% paraformaldehyde for 15 min at room temperature, washed in PBS, and stained using Oil Red O to reveal cytoplasmic lipid deposits.

Cells were also monitored over 21 days in terminally differentiating osteogenic media consisting of DMEM with 25 mM dextrose, 1 mM sodium pyruvate, 5.97 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml L-ascorbate, 10 mM β-glycerophosphate (Sigma, St. Louis, MO), and 10 nM dexamethasone (Sigma, St. Louis, MO). Cells subjected to osteogenic induction were fixed in 4% paraformaldehyde for 15 min at room temperature, washed in PBS, and processed following standard Von Kossa staining procedure to reveal calcified nodules. Total RNA isolation was also performed using the Nucleospin® RNA/protein extraction kit (Macherey-Nagel, Dueren, Germany). Total RNA was primed with random hexamers, and cDNA was produced using Taqman® reverse transcription reagents from Applied Biosystems, Life Technologies Ltd. (Paisley, UK). Quantitative PCR was performed using the Fast SYBR® Green PCR Master Mix from Applied Biosystems, Life Technologies Ltd. (Paisley, UK) as well as primers synthesized by Microsynth (Balgach, Switzerland) for collagen type X alpha 1 (Col10a1; F: 5′-TGCTAGTATCCTTGAACTTGGTTCAT-3′; R: 5′- CTGTGTCTTGGTGTTGGGTAGTG-3′) (16), matrix metalloproteinase 13 (MMP13; F: 5′-CCTCCAGTTT GCAGAGCGCTACC-3′; R: 5′-TCTCCTTCAGGATTC CCGCGAGA-3′), A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS5; F: 5′-ACG ATGCCACCCAGCAGTGC-3′; R: 5′-ACAGCACACC ACAGGCGAGC-3GCCACCCAGCAGTG), and the housekeeping gene β2 microglobulin (B2M) (F: 5′-GCATTCGGGCCGAGATG-3′; R: 5′-AATCTTTGG AGTACGCTGGATAGC-3′). Gene expression data were processed following the comparative Ct method, normalizing gene expression levels by that of the housekeeping gene and using as a biological reference the normalized gene expression levels from cells grown after 9 PDs in basal media until confluence.

Statistical Analyses

Proliferation and gene expression data are represented as means ($n = 3$), with error bars showing standard deviations. Gene expression levels were analyzed statistically using Student's t test where $p < 0.05$ indicated statistically detectable differences.

RESULTS

Growth Kinetics and Behavior

Isolated ECPs exhibited a remarkable morphological homogeneity in monolayer culture (Fig. 1B, C). Cells retained the same morphology and homogeneity after 18 PDs. ECP growth kinetics were equivalent at relatively low (9) and high (18) PDs (Fig. 1D), with a notably stable proliferative potential averaging 2.9 ± 0.3 (mean ± SD) PDs every 9 days up to 24 PDs. The same stability is observed after cryogenic preservation and thawing, albeit with an observed slower onset of proliferation at later passages or PDs (Fig. 1E). The plating efficiency of freshly thawed cells after prolonged storage in liquid nitrogen was maintained at 86% with an initial viability between 82% and 90% for cells frozen after 6 and 18 PDs.

Homogeneous and Stable Surface Marker Profiles

Surface marker detection revealed stable population homogeneity for all tested markers at 9 PDs (Fig. 2A) and 24 PDs (Fig. 2B) with no discernible subpopulation enrichment. The same pattern was observed at 18 PDs (data not shown). The ECP population does not contain potentially contaminating cell types as seen by

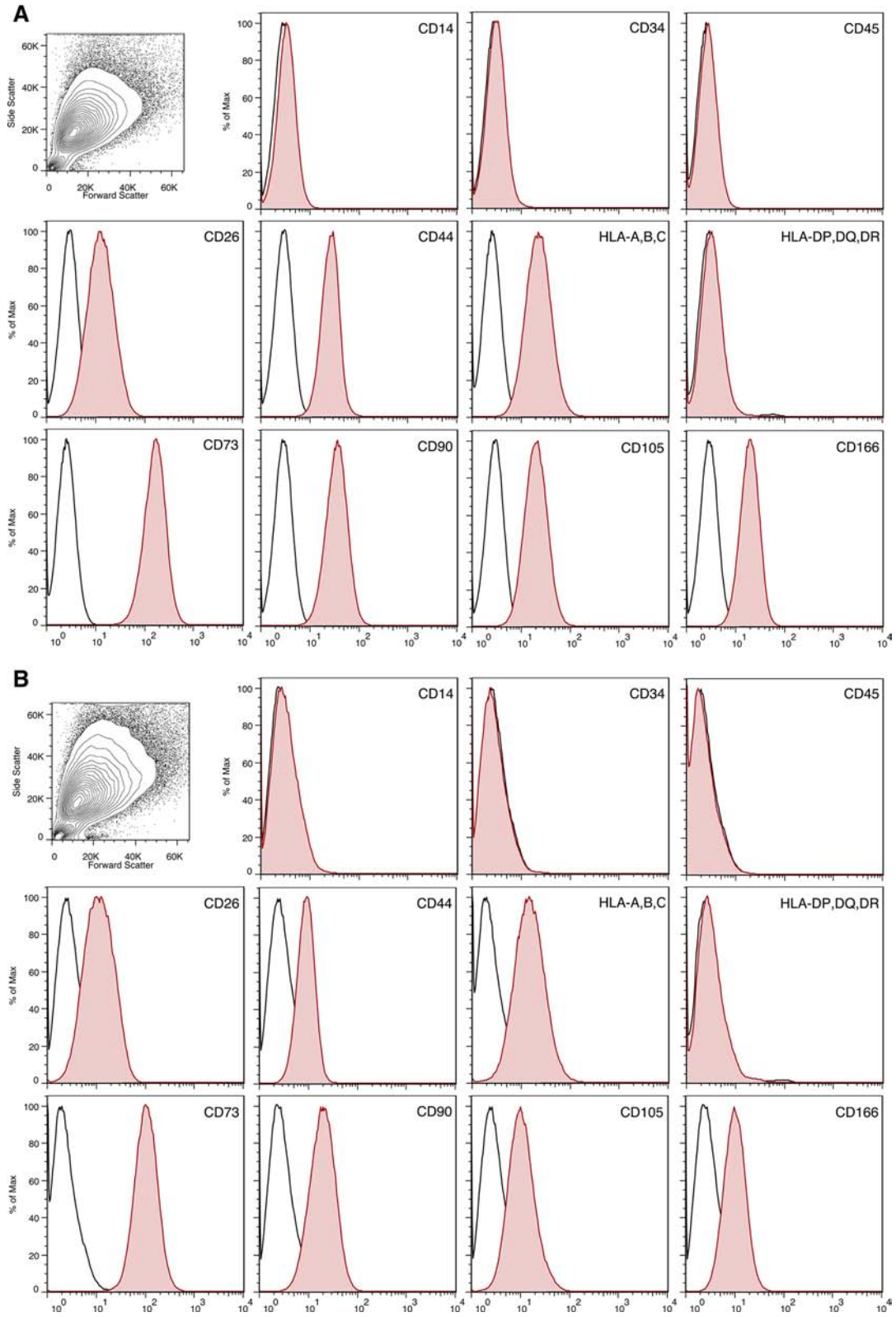

Figure 2. Surface marker profiles reveal homogenous populations for ECPs throughout growth to 9 PDs (A) or 24 PDs (B). Tinted red histograms are indicative of the tagged population, and empty black histograms represent isotype controls for each cell surface marker. Scatter plots show 5% population density contours with dots representing outliers. CD, cluster of differentiation; HLA, human leukocyte antigen.

the negative markers CD14, CD34, CD45, and HLA-DP,DQ,DR. ECPs appear to be distinctly positive for CD44, CD73, CD90, CD105, and CD166 and mildly positive for CD26 and HLA-A,B,C. The latter may be indicative of an immune privilege such as that present in adult MSCs. Since chondrocytes do not present a specific surface marker and in fact share many positive markers with adult bone marrow-derived MSCs, analyzing the relative surface marker profiles consistent of ECPs yielded a chondrogenic (CD90 bright, CD105 dim) rather than an undifferentiated stem-like phenotype (CD90 dim, CD105 bright) (6). Equally, CD105 levels were maintained throughout PDs, possibly indicating a delay in dedifferentiation, a particularly problematic aspect of adult chondrocyte monolayer expansions.

Spontaneous Chondrogenic Potential

ECPs were cultured in three-dimensional micropellets in a medium devoid of growth factors (free of serum-bound or recombinant growth factors) to assess their spontaneous chondrogenic potential. The nuclear localization pattern of the transcription factor Sox9, a known master regulator of chondrogenesis, was equivalent in 3D micropellet cultures for cells after 9 and 18 PDs (Fig. 3). The 18 PD pellets were left for an additional 10 days in culture to allow more matrix deposition. Both low and high PD micropellets showed a distinctive glycosaminoglycan deposition with aggrecan deposits concentrated in clusters. Collagen I deposition was seen throughout the pellet agglomeration while collagen II deposition was only pericellular, an expected pattern for fetal cartilage

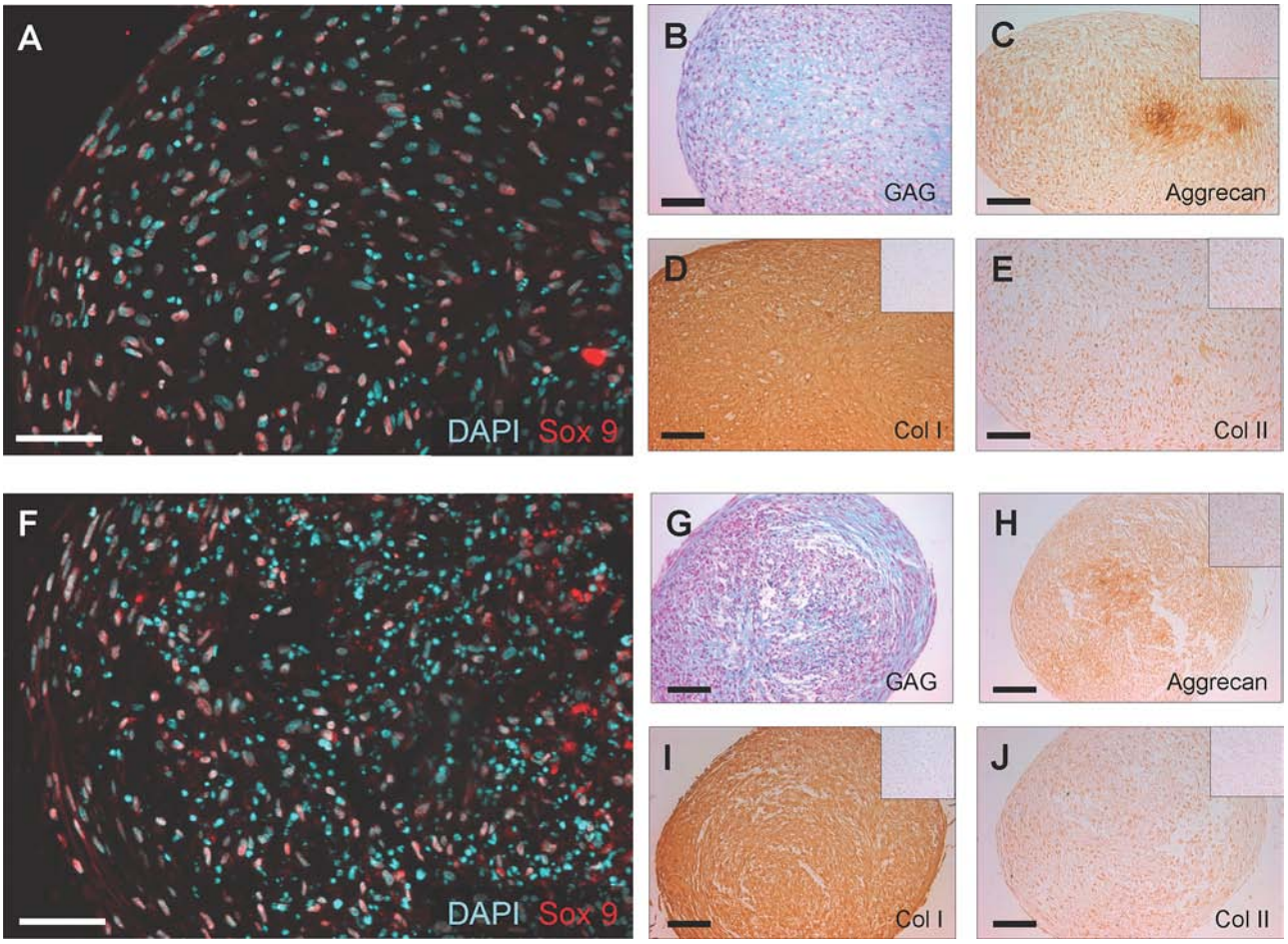

Figure 3. Spontaneous chondrogenesis in micropellets performed following 9 PDs and grown in serum-free media for 17 days (A–E) as well as pellets following 18 PDs and grown in serum-free media for 27 days (F–J). Sox9 nuclear localization is visualized in red overlapped with DAPI stained nuclei in blue (A, F). Glycosaminoglycan (GAG) deposition is visualized in blue after Alcian blue staining with a nuclear fast red counterstain to reveal cells (B, G). Immunohistochemical staining of human aggrecan (C, H), collagen I (D, I), and collagen II (E, J) is revealed using a DAB peroxidase substrate in brown with the respective background staining represented in smaller cropped panels at the same scale. Scale bars: 100 μm.

cells and tissues that are loosely cross-linked to allow for rapid development and remodeling during morphogenesis (3). This type of primary matrix produced by ECPs may therefore help harbor the cells within a cartilage defect during the early stages of regeneration before de novo tissue maturation occurs.

Restricted Multilineage Plasticity

A relatively aggressive adipogenic differentiation protocol over 27 days generated very few cells with cytoplasmic lipid accumulation (an average of 1 cell per 34,000) (Fig. 4A). The same behavior was observed both after 9 and 18 PDs. Terminally differentiating osteogenic induction over 21 days was able to induce minor matrix calcification at 9 PDs (Fig. 4B). Induced cells at 18 PDs exhibited a delay in differentiation with no detectable matrix calcification after 21 days (Fig. 4C). The differentiation towards this path was, however, restricted at 9 and 18 PDs as observed by the downward trend in expression for collagen X (a short chain collagen produced by hypertrophic chondrocytes) (Fig. 4D) and ADAMTS5 (a potent disintegrin and metalloproteinase with thrombospondin motifs that cleaves aggrecan, a fundamental proteoglycan in cartilage) (Fig. 4E), as well as an unchanged basal expression of MMP13 (matrix metalloproteinase 13 or collagenase 3) (Fig. 4F). Stronger downregulation trends were observed after 18 PDs for collagen X and ADAMTS5 along with an inherent baseline decrease in MMP13. These three markers, often present in osteoarthritic cartilage, are strong indicators of chondrocyte terminal differentiation

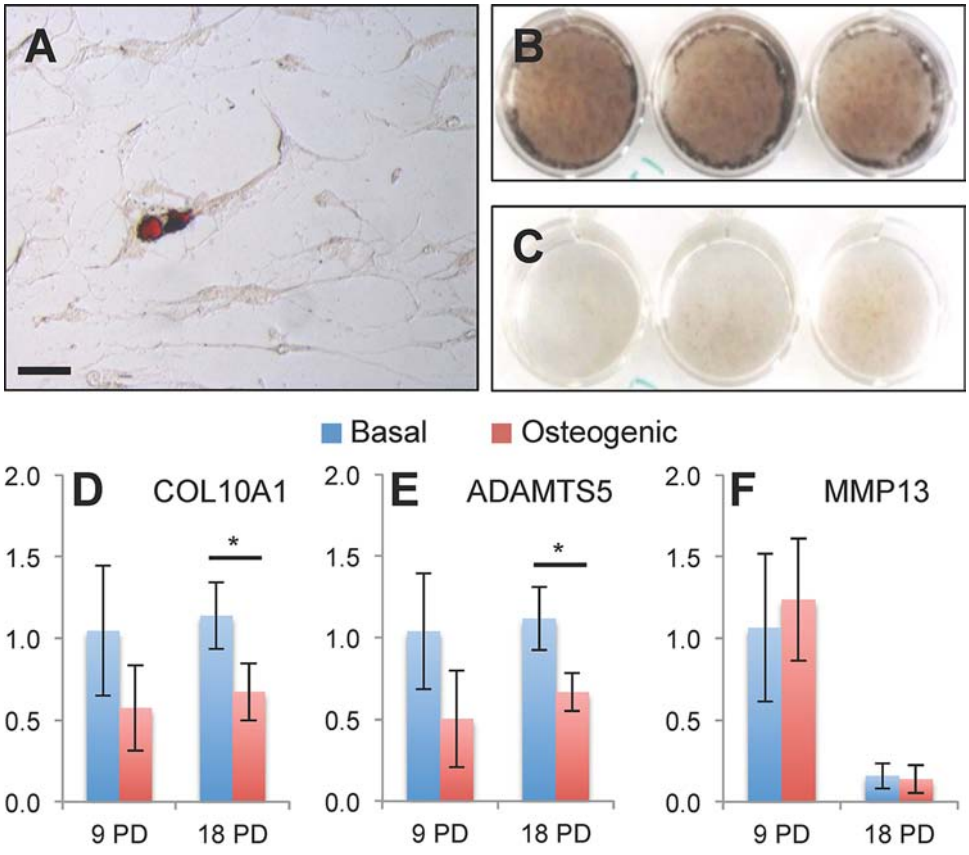

Figure 4. Adipogenic differentiation yields 1 in 34,000 cells with distinctive cytoplasmic lipid deposition visualized in red using the Oil Red O stain. Scale bar: 100 μm. (A) Terminally differentiation osteogenic induction yields calcified nodules visualized in brown–black using Von Kossa staining in cells after 9 PDs (B) but not after 18 PDs (C). Gene expression levels for COL10A1 (D), ADAMTS5 (E), and matrix metalloproteinase 13 (MMP13; F) are represented for cells grown in basal media (blue) as well as those subjected to 21 days of osteogenic induction (red). Gene expression levels in cells with low and high PDs are represented with the data normalized to the housekeeping gene, β-2 microglobulin, and represented as a fold difference relative to normalized gene expression levels in 9 PD cells grown in basal media as a biological basal reference. *Statistically detectable differences ($p < 0.05$).

and hypertrophy. Their upregulation could indicate the beginning stages of cartilage calcification or endochondral ossification, both tightly regulated processes present in the growth plate and the deep zone in mature articular cartilage. A cell type that readily displays these markers would not be able to maintain a stable chondrogenic phenotype and would inadvertently produce a hypertrophic, calcified tissue (10,24,25). As expected, collagen II expression was undetected in monolayer cultures (data not shown). The differentiation cocktail used to induce these reported effects did not contain any recombinant morphogen supplementation to better assess the inherent osteochondral plasticity of ECPs, which was found to be restricted. This was also the case using higher levels of dexamethasone resulting in low collagen X expression levels as well (data not shown).

DISCUSSION

A common limitation in cartilage cell therapy has always been cell number and the expansion potential of therapeutic cells, all the while maintaining a stable, reliable, and homogenous cell population. Adult cell dedifferentiation or early onset of senescence may occur at early passages, a behavior difficult to estimate in PDs with the heterogeneity observed in adult stem cells and adult chondrocytes primary culture processing. Indeed, as adult chondrocytes possess a very slow natural turnover rate in vivo, the ex vivo expansion of these adult cells has been shown to cause an accelerated aging process equivalent to 30 years after merely 3 PDs. The drastic variability observed between donors is also a fundamental hurdle to guaranteeing a positive clinical outcome since the quality of harvested cells has been reportedly difficult to determine (4,12,22,26). The observed behavior of ECPs, a combination of stable and predictable growth kinetics as well as resilience to the shock often associated with cryogenic preservation, makes it possible to scale out the production of such cells without compromising quality. We have observed similar advantages with fetal skin fibroblasts processing and have shown that the therapeutic potential from a 1 cm^2 skin biopsy can produce at least 35×10^9 biological constructs of 102 cm^2 for the treatment of burns and wounds. This type of management of cell banks can thus provide clinical material for hundreds of thousands of patients (2). This aspect becomes especially important when the cell banking process focuses on maximizing potential therapeutic yields from one biopsy versus alternative strategies such as multiple-donor pooling.

At 14 weeks of gestation, human long-bone epiphyses are well defined anatomically from the underlying and calcifying growth plate, with a notably homogenous outlook as secondary ossification has not yet occurred. This aspect, along with meticulous biopsy processing, is apparent through the unimodal, narrow population surface marker profiles of ECPs, indicating homogeneity with cells maintaining steady surface marker profiles throughout PDs with no apparent subpopulation enrichment.

This phenotypic stability is further supported by a spontaneous chondrogenic potential in ECPs, stable up to 18 PDs. This comes as a striking and essential advantage over undifferentiated, uncommitted stem cells, which require a plethora of growth factors to generate a tissue-specific phenotype. With stem cell differentiation protocols often generating unpredictable phenotypic instability (7), working with a prededicated cell type such as ECPs avoids the need for cell priming and preconditioning prior to implantation. The resistance of ECPs to multilineage differentiation such as adipogenesis for instance further supports the need for a predictable outcome in vivo rather than a situation in which implanted cells may affect off-target host tissues.

ECPs were also capable of modulating the quality of deposited matrix, forming small, calcified nodules in the presence of subtle changes in surrounding environment, much like those occurring in full thickness cartilage defects. The observed restricted osteogenic or terminally differentiating potential is a major advantage when considering cartilage cell therapy as it indicates ECPs capable of responding to cues from various osteochondral regions. A better understanding of their differentiation repertoire will best be investigated in vivo where microenvironments are finely tuned and difficult to replicate in vitro.

Implanting ECPs in vivo would undoubtedly allow novel interactions to occur between the implanted cells and the host tissue, which will inform the way the implantation must occur to deliver the best outcome and best interaction with the degenerated tissue. Previous studies on fetal bone and skin cells suggest an immune privilege or immunoregulatory functions (14,20). Fetal cells have in fact been described to reside in maternal tissues for decades after pregnancy and potentially participate in repair of maternal injuries (23). Furthermore, some fetal tissues have been found to produce HLA-G, which has been shown to possess tolerogenic effects (5). ECPs may indeed employ various mechanisms to redefine the immune microenvironment in the implanted site, which may help regulate repair or reduce inflammation, especially in the days following implantation. Such interactions will have to be thoroughly investigated in vivo to best predict the efficiency and regenerative potential of ECPs.

In conclusion, this work investigates the reliable expansion of epiphyseal chondroprogenitors for developing a cartilage repair strategy using cells with marked advantages

over adult chondrocytes or adult stem cells. The core ECP properties shown, such as their stability, homogeneity, spontaneous chondrogenic potential combined with a restricted plasticity for terminally differentiating osteogenic induction as well as adipogenic induction, are some of the major advantages with this cell type.

The estimation of therapeutic applications for cell banking of ECPs from one single organ donation, based on the characterization to date, would be approximately 10^8 treatments when compared with similar clinical use and cell numbers from cell therapies based on autologous chondrocytes or bone marrow-derived MSCs in current cartilage regeneration regimens. Furthermore, ECPs exhibiting such phenotypic stability would not require in vitro cell priming or conditioning prior to implantation in vivo, which drastically facilitates the transition to preclinical studies.

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