Comparative Analysis of Human Amnion and Adipose Derived Stem Cells for Regenerating Orthopaedic Tissues

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Introduction: Introduction: Adult mesenchymal stem cells are considered alternative cell sources for use in orthopaedic regenerative medicine. Typically derived from bone marrow or adipose tissue, these cells have the ability to self renew, differentiate towards numerous tissue lineages and exhibit immunomodulatory properties.1 Recently, scientists have begun investigating perinatal stem cell populations from the amnion harvested after the birth of term pregnancies. Developmentally, the origin of the amnion is the pluripotent epiblast, which may imply that the contained cells retain embryonic stem cell-like characteristics.2,3 There are two primary cell types within the amnion; amniotic epithelial cells (hAECs) and amniotic mesenchymal stem cells (hAMSCs). While some have attempted to validate the use of these cells in orthopaedic applications,4,6 none have conducted side-by-side comparisons of human amnion derived stem cells with that of mesenchymal stem cells with regards to their capacity to differentiate towards orthopaedic tissue cell types. We hypothesized that amnion derived stem cells would differentiate more readily into chondrocytes and osteoblasts as compared to mesenchymal stem cells. Herein we describe preliminary results from in vitro studies undertaken to illustrate these potential differences.

Methods: Materials & Methods: Human placentas were obtained via informed consent under an approved IRB protocol from healthy donors undergoing cesarean sections (Pro00031185-Greenville Health System). Amnion were harvested according to methods described by Miki et al with modification.5 Briefly, stem cells were isolated via serial digestion of the amnion as follows: Two sequential 40 minute digestions in 0.05% trypsin at 37oC were used to isolate hAECs only. Subsequently, remaining amnion was digested for 120 minutes in 2mg/mL collagenase (295U/mg) under constant agitation at 37oC. This digest dissociated the remaining hAECs as well as hAMSCs yielding a 'Mixed' population of amnion derived stem cells. Human adipose derived stem cells (hADSCs) were purchased from a commercial vendor for the comparative analysis. Passage 2 stem cells were used for all experiments. To induce osteogenic differentiation, stem cells were seeded at a density of 2.1x10^4/cm2 and cultured in monolayer for up to 28 days in differentiation media (DMEM, 10% FBS, 1%AB/AM, 0.1uM dexamethasone, 50uM ascorbate-2-phosphate, 10mM β-glycerophosphate). To induce chondrogenic differentiation, cells were seeded in micro-mass (8x10^4/10uL droplets) and cultured in differentiation media (DMEM, 1%FBS, 1%ABAM, 6.25ug/mL Insulin, 50nM Ascorbate-2-phosphate, 10ng/mL human TGF-β). Negative controls were maintained in standard culture media. Differentiation capacity was assessed via histological staining (n=2/condition) and gene transcript expression (n=4/condition). Briefly, deposition of calcium and glycosaminoglycan (GAG) was evaluated with Alizarin Red and Alcian blue staining, respectively. The percentage of the total well-plate area stained positive was quantified via color threshold analysis using NIH Image-J software by two blinded observers. Results are reported as the mean stained area ± standard deviation. For gene transcript analysis, RNA was
isolated and pooled into two samples via the Trizol procedure and expression was evaluated using target-specific primers for osteogenic (Runx2, osteocalcin) and chondrogenic (aggrecan, collagen II, Sox-9) differentiation. Real time reverse transcription PCR was performed using RETROscript and QuantiTect SYBR green PCR kits in a Rotogene 3000 thermocycler using the 2-ΔCT method. Values are reported relative to GAPDH. Statistical differences between samples were determined via a one-way analysis of variance followed by pair-wise comparison using a Tukey’s post-hoc test in JMP software.

**Results:** Results: Alizarin Red staining indicated calcium deposition in all osteogenic study groups at day 28 (hAEC: 11.32%±0.09, mixed: 20.89%±0.67, hADSC: 8.18%±1.67) (Figure 1). Notably, statistically increased positive staining for calcium was noted as early as day 14 in the hAEC and mixed groups (p<0.05). Morphological changes in the hAECs were apparent as early day 3; their classic cobblestone shape was progressively replaced with swollen cells containing multiple nucleoli indicative of an osteogenic cell. Similar trends were observed in the mixed group. Conversely, hADSCs continued to exhibit spindle morphology through 28 days. Additionally, negative controls of amnion derived stem cells also exhibited calcium deposition and changes in morphology (data not shown); no staining was apparent in hADSC controls. Alcian Blue staining revealed statistically increased GAG deposition in the hAEC and mixed groups by day 14 (hAEC: 28.41%±1.41, mixed: 30.15%±2.42, hADSC: 20.30%±0.92) (Figure 1). Morphological changes in both amnion cell groups were apparent, as cells became increasingly rounded; whereas hADSCs remained spindle-shaped. Gene transcript expression for the osteogenic marker osteocalcin (Figure 2) and Runx-2 (not shown), illustrated early and progressively increasing expression in hAECs whereas hADSCs exhibited heightened expression early which tended to decrease over time. Aggrecan (Figure 2) and collagen II (not shown) expression illustrated dramatic increases in both amnion derived stem cell groups at early time-points compared to minimal expression in hADSCs; particularly at early time-points.

**Discussion:** Discussion: Taken together, this data suggests that amnion derived stem cells appear to more readily differentiate into osteogenic and chondrogenic lineages as compared to hADSCs of the same passage number. Consistent with previous reports, histological analysis revealed all three cell types could be successfully differentiated into osteogenic and chondrogenic lineages.1,4,6,7 However, differentiation of amnion derived stem cells appeared to precede hADSCs and resulted in more robust matrix production. More specifically, histology indicated that osteogenic differentiation occurred nearly 14 days earlier in amnion derived stem cells than hADSCs. Interestingly, hAECs appear to have an innate capacity for osteogenic differentiation even in the absence of chemical induction.4 This does not appear to be true of hADSCs. Additionally, the ‘mixed’ study group appeared to exhibit stronger Alcian blue staining, indicative of improved chondrogenic differentiation, compared to hADSCs; this was also corroborated by increased aggrecan and collagen-2 transcript expression. These results also add to previous reports that indicate hAMSCs may be more efficient in chondrogenic differentiation than hAECs.4 Taken together, our data indicates that amnion derived stem cells may prove to be an optimal alternative cell source for orthopaedic regenerative medicine applications as compared to more traditional sources of mesenchymal stem cells which may be in part due to their ‘youthful’ origins. Additional studies comparing amnion derived and bone marrow derived stem cells are currently ongoing. The capability of these cell types to undergo differentiation towards a tenocyte phenotype is also underway. Funding for this work was provided by the PI’s departmental start-up.
**Significance:** Our data indicates that amnion derived stem cells may prove to be an optimal alternative cell source for orthopaedic regenerative medicine applications as compared to more traditional sources of mesenchymal stem cells.

* Denotes statistical difference compared to hADSCs at the corresponding time-point (p<0.05).