

# EVALUATION OF OPTIMAL PARAMETERS IN THE CO-CULTURE OF HUMAN ANTERIOR CRUCIATE LIGAMENT FIBROBLASTS AND OSTEOBLASTS FOR INTERFACE TISSUE ENGINEERING

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## INTRODUCTION

The anterior cruciate ligament (ACL) connects the femur to the tibia, and functions as a joint stabilizer. ACL tears and ruptures are the most common knee ligament injuries, and affect over 250,000 people per year in the United States alone.[1] There is also a steady rise in reported ACL injuries due to an aging and increasingly active population. This situation is exacerbated by the high number of failures associated with existing treatment modalities for which revision surgeries are required. Current surgical procedures have not been able to fully restore knee function without associated side effects such as donor site morbidity, muscle atrophy, tendonitis, and arthritis.

The long term objective of our research program is the design of interfaces to facilitate the integration of bone with soft tissues such as ligament or tendon. Although autografts are superior to other clinical alternatives for ACL reconstruction, they are associated with a lack of initial functionality, followed by a gradual increase in strength that never reaches the original strength of native ACL.[2,3] Moreover, the grafts often fail at the attachment interface between the graft and the femoral or tibial tunnels, since the natural interface between bone and ligament is never fully restored.[6] Thus, the degree of graft integration with osseous tissue is one of the critical factors governing its clinical success. Our working hypothesis is that long term outcomes of ACL reconstructive surgeries can be improved by recreating the functional insertion site between bone and the ACL graft.

The natural interface or insertion site between ACL tissue and bone is divided into four zones of varied cellular and matrix components.[4-6] The first zone is the ACL proper comprised of fibroblasts and types I & III collagen. The next zone is a fibrocartilage region composed of chondrocytes and types II & I collagen. The third zone is mineralized fibrocartilage made up of hypertrophic chondrocytes and type X collagen.[5] The last zone is the subjacent bone composed of osteoblasts, osteoclasts, and osteocytes, and the predominant collagen is type I. In order to examine the formation of such an interface *in vitro*, and eventually replicate it on a tissue engineering scaffold, we have elected to first focus on establishing reliable co-culture systems of osteoblasts and ligament fibroblasts. To

our knowledge, *in vitro* evaluation of direct osteoblast-ACL fibroblast interactions have not been reported in the literature. In this study, we will examine the effects of media additives such as ascorbic acid (AA) and  $\beta$ -glycerophosphate (bGP) on the growth and differentiation of osteoblasts and human ligament fibroblasts (hACL). In addition, cellular response during the co-culture of osteoblasts and ligament fibroblasts under defined conditions will be determined. The objective of these studies are to first identify optimal culturing conditions under which the two cell types will maintain their phenotypes, followed by examination of cell morphology and growth during co-culture.

## MATERIALS AND METHODS

Cells and cell culture - Human osteoblast-like cells (SaOS-2, ATCC) and primary human osteoblasts isolated from explant cultures were used in this study. Monolayers of human ligament fibroblasts (hACL) were also established using explant cultures. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Life Technologies), supplemented with 10% fetal bovine serum, L-glutamine, and 1% antibiotics (Life Technologies). The cells were grown to confluence at 37°C and 5% CO<sub>2</sub> under humidified conditions.

Effects of AA concentration[AA] & bGP concentration[bGP] on hACL and osteoblast cultures - Cultures of hACL or osteoblasts were grown on tissue culture polystyrene (TCP) at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup>. The effects of AA concentration (0, 10, 30, 50, 100  $\mu$ g/ml) on cell growth and differentiation were determined as a function of culturing time (1, 3, 7, 14, 21 days). Similarly, media with bGP concentrations of 0, 1.0, 3.0, 5.0 mM were added to osteoblasts or hACL cultured on TCP at the density of  $1.5 \times 10^4$  cells/cm<sup>2</sup>. Cell growth and differentiation in these cultures were examined at 1,3,7,14,21 days.

Co-culture of human osteoblasts and ligament fibroblasts - Co-culture was established by first dividing the surfaces of each well in a multi-well plate into three parallel sections using sterile polystyrene inserts. hACL cells and osteoblasts were seeded on the left and right surfaces respectively, with the middle section left empty. Cells were seeded at 2,000 cells/section, and left to attach for 15 minutes. After the removal of inserts, the wells were rinsed with PBS. Control groups

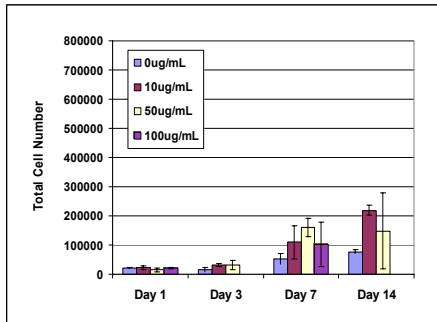
were fibroblasts alone and osteoblasts alone. Cultures were maintained in supplemented DMEM at 37°C in 5% CO<sub>2</sub>. After Day 7, media was supplemented with 3mM bGP and 10 µg/ml L-ascorbic acid. Media was changed every 2-3 days. The morphology of each cell type at the seeded sections and their growth into the middle interaction zone, were monitored at 1, 3, 7, 14, 21 days.

**Determination of cell growth and differentiation** - Cell growth was measured using a quantitative fluorometric assay (Molecular Probes). For all cultures, alkaline phosphatase (ALP) expression and mineralization were examined as differentiation markers. ALP expression was ascertained by Fast-red staining, and mineralization was visualized via Alizarin Red S (ALZ) staining. Cell growth (n=5) and differentiation (n=3) were assessed during co-culture, and as a function of AA and bGP concentrations for individual cultures.

**Statistics**— One-way and two-way analyses of variance were conducted using the JMP 2.1 (SAS) software. The Tukey Kramer test was used to test significant differences between means, with  $\alpha=0.05$ .

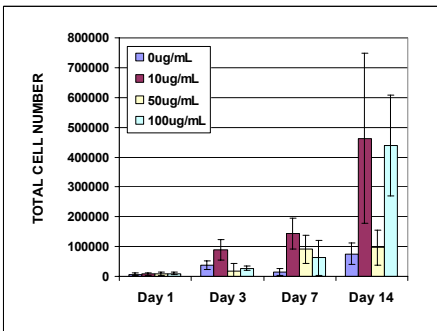
## RESULTS AND DISCUSSION

The first objective of this study was to determine the effects of media additives on the growth and mineralization of osteoblasts and ACL fibroblasts. AA is a cofactor for proline and lysine hydroxylases, which are critical in collagen synthesis. In the osteoblast cultures, a positive correlation was found between total cell number and [AA] up to 50 µg/ml. As seen in Figure 1 (mean±STD, n=5), the optimal [AA] was 10µg/ml as the highest number of cells was measured for this group after 14 days of culture. (p<0.05).



**Figure 1. Effects of [AA] on osteoblast growth**

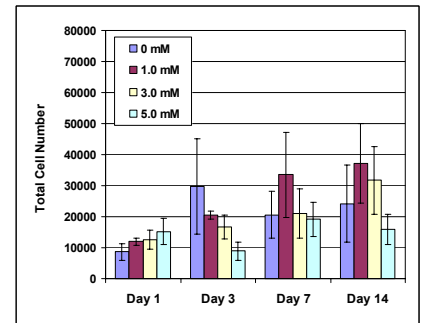
When hACL were cultured over time as a function of AA concentration, the highest cell number was observed at 10µg/ml and 100µg/ml after 14 days of culture (see Figure 2). Although not different from each other, the values were significantly higher than the 50µg/ml & control (0µg/ml) groups. Therefore, based on Figure 1 and Figure 2, the concentration of AA optimal for both osteoblast and hACL growth was found to be at 10 µg/ml.



**Figure 2. Effects of [AA] on hACL growth**

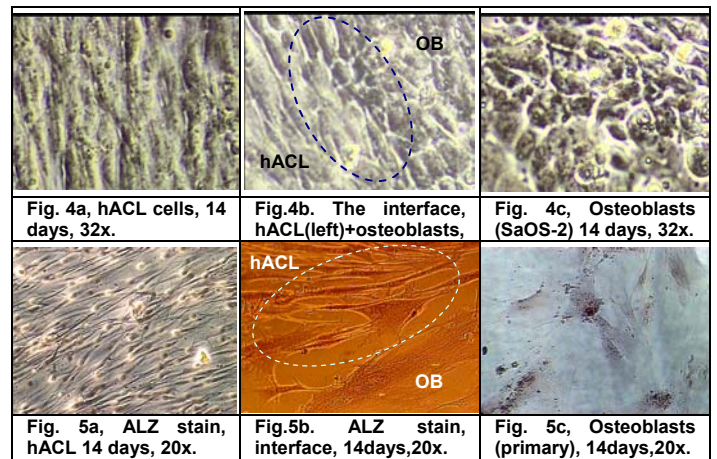
The selection criteria for optimal co-culture conditions are based on the goal of maximizing cell growth and osteoblast mineralization, while minimizing ectopic hACL mineralization. Here, both mineralization and ALP synthesis increased with increasing [AA] for individual osteoblast and ligament fibroblast cultures. These findings are consistent with those of Ganta *et.al*, where a dose-dependant increase in osteoblast mineralization was observed with increasing AA content.[7] In addition, hACL mineralization was the lowest in the 10 µg/ml [AA] group as compared to the other AA concentrations.

During mineralization, ALP reacts with bGP and the phosphate product is utilized for Ca-P formation. Based on our results, no significant change in cell number was observed for the [bGP] investigated. At 1.0mM, a significant difference between 1 day & 7 day samples (p<0.05) was observed. However, no differences were found between 1.0 mM and 3.0mM cultures. ALZ stains for the osteoblast cultures were more intense for 3.0mM



**Figure 3. Effects of [bGP] on hACL growth** than for 1.0mM. Ectopic mineralization was observed for hACL cultures at 3.0 mM, which may not be desirable. Balancing the need for maintaining phenotype of both osteoblasts and hACL, 1.0mM of bGP was selected as the optimal bGP concentration for co-culture.

With the goal of recreating the interface between bone and ACL, the second objective of this study was to begin initial examination of the interactions between osteoblasts and hACL in a co-culturing environment. After 14 days, it was observed that both hACL (left) and osteoblasts (OB, right) proliferated and expanded beyond the initial seeding areas.(Fig. 4b, 32x) These cells continue to grow into the interfacial zone, and eventually a contiguous and confluent culture was observed at the interface (dashed lines, Fig. 4b). Control groups are hACL only (Fig. 4a) and osteoblasts only (Fig. 4c).



Compared to SaOS-2 cells (Fig. 4c), primary human osteoblasts exhibited a much slower growth rate (Fig. 5c). With the addition of 10 µg/ml AA and 3mM bGP, mineralization was observed in all three zones. Stain intensity was the lowest for the hACL region (Fig. 5a), followed by the interface (Fig. 5b) and osteoblast zone. (Fig. 5c)

In summary, we report here one of the first studies to examine the interaction of human osteoblasts and ligament fibroblasts during co-culture. The optimal ascorbic acid and bGP concentration for co-culture was found to be 10µg/ml and 1.0mM respectively. Studies are underway to examine phenotypic expression at the interface, with the goal of recreating the ACL-bone insertion zone on 3-D scaffolds.

**REFERENCES** -1).AAOS Publications, 1998; 2).Jackson *et.al*, *Am.Acad. Orthop.Surg.Bull.* 40, 10-11,1992. 3).Jackson, D.W. *et.al.*; *Am.J.Sports Med.* 15:528-538; 1987; 4).Gao *et. Al.*, *J.Anat.* 188:367-373, 1996; 5).Visconti CS *et.al.*,*Arch.Biochem.Biophys.*328(1):135-142,1996;6).Weiler *et.al.* *Arthroscopy*, 18(2):124-135,2002. 7).Ganta *et al*, *Endocrinology*. 138(9):3606-12, 1997.