Increased cell density in alginate bead suspension inhibits dedifferentiation of bovine chondrocytes

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May 8, 2009

Introduction

Tissue engineering, as described by Dr. Robert Langer, is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ." (Langer et al., 1993) One emerging field within tissue engineering is cartilage engineering. Cartilage is a dense connective tissue composed of chondrocytes, collagen, elastin and proteoglycans, and it lacks blood vessels; because of the lack of blood supply, the wound-healing response of cartilage is limited, and as such, cartilage degeneration and damage have grave consequences. In response to this issue, cartilage engineering holds great potential to restore function using cartilage tissue substitutes. Current approaches to cartilage engineering include the development of natural and synthetic scaffolds, sources of mature chondrocytes and chondroprogenitor cells, and chondroinductive growth factors (Tuli et al., 2003).

Most important to the overall success of engineered cartilage is maintenance of cell viability as well as active metabolism and synthesis of a functional extracellular matrix (Heywood et al, 2004). In order to enhance these factors, cells must be well distributed within the scaffold while maintaining cell-to-cell stimuli. Previous research has shown that cell viability decreases as cell density increases (Heywood et al, 2004). Consequently, in this study cell-seeding density was investigated, comparing densities at a 1:5 ratio and the associated effects on chondrogenesis. However, in higher density cell suspensions, cells have a greater opportunity for autocrine signaling as well as cell-cell signaling, thus promoting maintenance of the chondrocyte phenotype. At the same time, however, if the cell density is exceedingly high, competition for nutrients could create a stressful environment, leading to dedifferentiation of the cells. It was hypothesized that the positive effects of a higher cell-seeding density would override the negative effects, such that a higher cell density would more effectively maintain the chondrocyte phenotype.

In order to test this hypothesis, chondrocytes were cultured in standard culture media and seeded at approximately 2 (1x) and 10 (5x) million cells/mL in a low viscosity alginate (250 cps, 2% Sigma Aldrich). Chondrocytes normally produce large amounts of collagen II ECM fibers, whereas de-differentiated chondrocytes produce collagen I. Therefore, measured levels of collagen I and II in the two samples was used as an indicator of de-differentiation within the alginate construct. Cell viability was assessed using a Live/Dead ® Fluorescence Assay, and collagen I and II gene transcript and protein levels were determined using RT-PCR and ELISA, respectively.

Results

Protocols were according to the 20.109 Spring 2009 lab wiki: http://openwetware.org/wiki/20.109(S09) accessed on April 15, 2009, unless otherwise noted.

Live/Dead ® Fluorescence Assay to evaluate cell viability

Cells were seeded at 2 and 10 million cells/mL in a 2% Sigma Aldrich alginate suspension and cultured for 8 days. A Live/Dead ® Fluorescence Assay was performed in order to evaluate cell viability. Cells seeded at 1x and 5x cell densities demonstrated equally high cell viability as well as a round morphology, indicative of the chondrocyte phenotype (Figure 1).

Comparison of Collagen I and II gene transcript levels by RT-PCR and gel analysis

Following 5 more days of culture, approximately 450,000 live cells/mL were recovered from the 1x sample and 100,000 live cells/mL from the 5x sample. RNA was then isolated from these samples at 80μg/mL and 156μg/mL, respectively. These concentrations were determined based on 260/280nm spectroscopy absorbance scans; 1x RNA had an absorbance reading of 2.0 and a 260/280 ratio of 1.4, and 5x RNA had an absorbance reading of 3.9 and a 260/280 ratio of 1.6. RT-PCR was performed using 100 ng of RNA in order to generate cDNA for collagen I and II. In order to determine gene transcript levels, these samples were run on a 1.2% agarose gel (Figure 2). ImageJ Analysis and normalization to GAPDH showed that the normalized ratio of collagen type II: collagen type I was increased in the 5x sample compared to the 1x sample, with ratios of 1.44 and 0.74, respectively.

It was previously hypothesized that cells seeded at 1x cell density would have a higher de-differentiation rate than those seeded at 5x. As such, these results confirm our hypothesis, but, at the same time, are not necessarily dependable results. The 260/280 absorbance ratios of the RNA, for example, indicate that the RNA isolated was impure, and furthermore that the 1x samples, with a 260/280 ratio of 1.4, had more protein contamination than the 5x samples, with a 260/280 ratio of 1.6. Because the amount of RNA loaded for RT-PCR is not necessarily accurate, it is possible that the values and ratios determined by ImageJ gel analysis are skewed. Additionally it should be noted that perhaps a higher cell density leads to low cell viability; whereas 450,000 cells/mL were recovered of the 1x sample, only 100,000 cells/mL were recovered of the 5x sample.

Alternatively, it is possible that the results are accurate, and in fact cells seeded at 10 million cells/mL is in fact the optimal cell density for maintaining chondrogenesis, and seeding at a lower cell density leads to more rapid de-differentiation. Further testing using a wider variety of cell densities should be conducted to confirm what the optimal cell density is.

Comparison of Collagen I and II protein levels by ELISA

In order to measure relative protein levels of collagen I and II in the two samples, protein was extracted from the cultured cells on day 13 in order to perform an indirect enzyme-linked immunosorbent assay (ELISA). Collagen I and II proteins at known concentrations were used to generate standard reference curves. Using the results from BPEC absorbance readings and the standard curves, the collagen II/I ratio was calculated to be 0.81 for the 1x sample and 0.77 for the 5x sample. The 1x sample had .16μg/mL of collagen I and .13μg/mL of collagen II; the 5x sample had .18μg/mL of collagen I and .14μg/mL of collagen II.

These results suggest that in fact cells seeded at a 1x density, or approximately 2 million cells/mL, maintained chondrogenesis more effectively than cells seeded at 5 times that density. This is a surprising result, and furthermore, it does not match the results of RT-PCR gene transcript level analysis. Additionally, because the ratio of collagen II to I is in fact quite close between the 1x and 5x sample, it is possible that in fact these values are statistically equivalent. In order to make further conclusions from this data, statistical analysis, such as a T-test, of the results should be done.

The discrepancy between gene transcript and protein levels could be due to errors in the ELISA procedure. The nature of ELISA is such that signals may be inaccurate due to ineffective blocking or antibody binding. For example, ineffective blocking can lead to non-specific binding of antibodies, thus artificially boosting protein signals. Because collagen I and II proteins were analyzed in different sets of microwells, it is possible that one plate was better blocked than another, and perhaps the 1x signal is close to the 5x signal because of non-specific antibody binding.

In regards to the fluorescence signals themselves, although calculated concentration levels were low, they were still well within the range of standard concentrations, and plotted against the standard curves, the experimental data fit quite well. As such, it seems reasonable to conclude that signal discrepancies are not due to low overall protein levels compared to the standard curve. Furthermore, duplicate standards and samples were also used and showed comparable results, thus confirming that there were no technical errors between wells.

In addition to technical failures, however, the difference in gene transcript and protein level results could also be due to biological factors which effect RNA and protein levels differently. For example, at a higher cell density, chondrocytes might be producing a higher concentration of enzymes which breakdown proteins such as collagen I or II. It is possible that although the 5x sample had a higher collagen II/I gene transcript ratio, the collagen II proteins themselves were broken down by collagenase at a faster rate than in the 1x sample, thus leading to similar collagen II/I protein ratios. As such, the ELISA results would in fact be accurate and reliable, and further testing could be done to determine collagenase levels.

Discussion

Comparison of cell seeding at 2 and 10 million cells/mL yielded interesting, if conflicting, results. RT-PCR and gel analysis of gene transcript levels of collagen I and II suggested that seeding at 10 million cells/mL (5x) drastically improved maintenance of chondrogenesis. Meanwhile, ELISA of cells harvested at the same time suggested that cells seeded at 2 million cells/mL were just as effective as cells seeded at 10 million cells/mL, if not slightly more effective. Taking into consideration technical errors, it seems more likely that RT-PCR yielded credible results, and higher cell-seeding density does, in fact, inhibit de-differentiation. However, because of the biological differences between analyzing gene transcript levels versus protein levels, it also possible that in fact these results are not conflicting. In order to confirm that the protein level analysis does not conflict gene transcript level analysis, the experiment should be repeated. Additionally, however, it would be interesting to measure levels of enzymes such as

collagenase to determine whether enzyme levels play a significant role in the protein levels at higher cell-seeding densities.

It would also be interesting to consider seeding cells at other cell densities in order to determine the optimal cell density for maintenance of the chondrocyte phenotype. Additionally, it is possible that the 1:5 seeding ratio was not drastic enough, thus explaining the results of ELISA analysis. In order to take into account these possibilities, further experimentation should be done with a variety of cell densities, ranging from even less than 2 million cells/mL to densities far beyond 10 million cells/mL. By doing this, it would be possible to generate an experimental curve of the collagen II/I ratio as a function of cell seeding density. From this data, an optimal seeding density could be determined, thus enhancing the functionality of cartilage tissue substitutes and promoting long-term success of cartilage tissue engineering.

References

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2. Langer R, Vacanti JP. Tissue engineering. *Science*. 1993; 260: 920-6.
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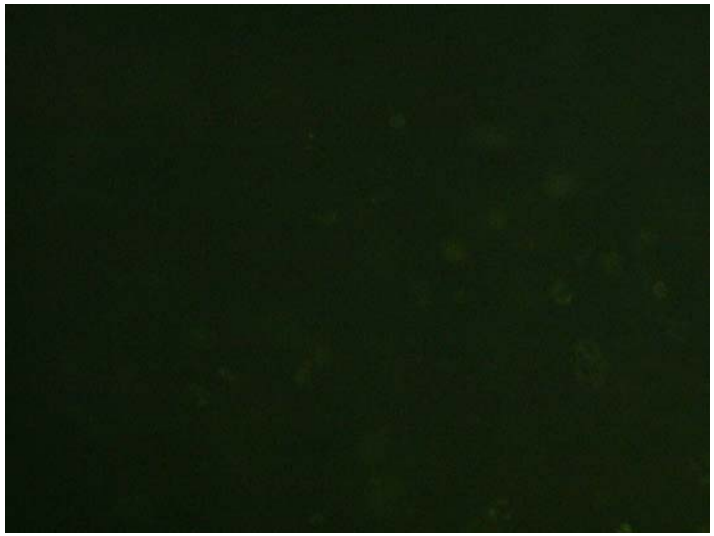
(a)



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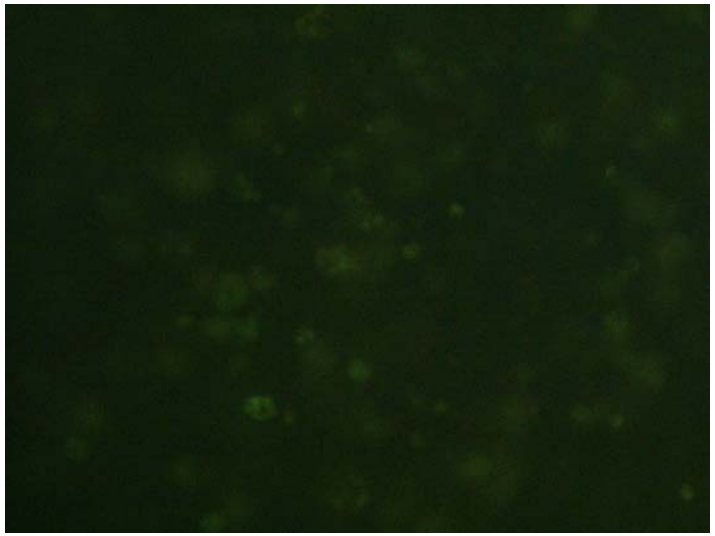

Figure 1 **Live/Dead® Fluorescence Assay of 3D Chondrocyte Culture in Alginate Beads** Cells from alginate beads were dyed with ethidium homodimer-2 variant and SYTO 10, and viewed under FITC excitation. (a) Cells seeded at a 1x density show high viability, and single-cell suspension. (b) Cells seeded at 5x show similar viability and appear evenly distributed throughout the bead. All cells demonstrate chondrocyte-like phenotype, in particular a round morphology.

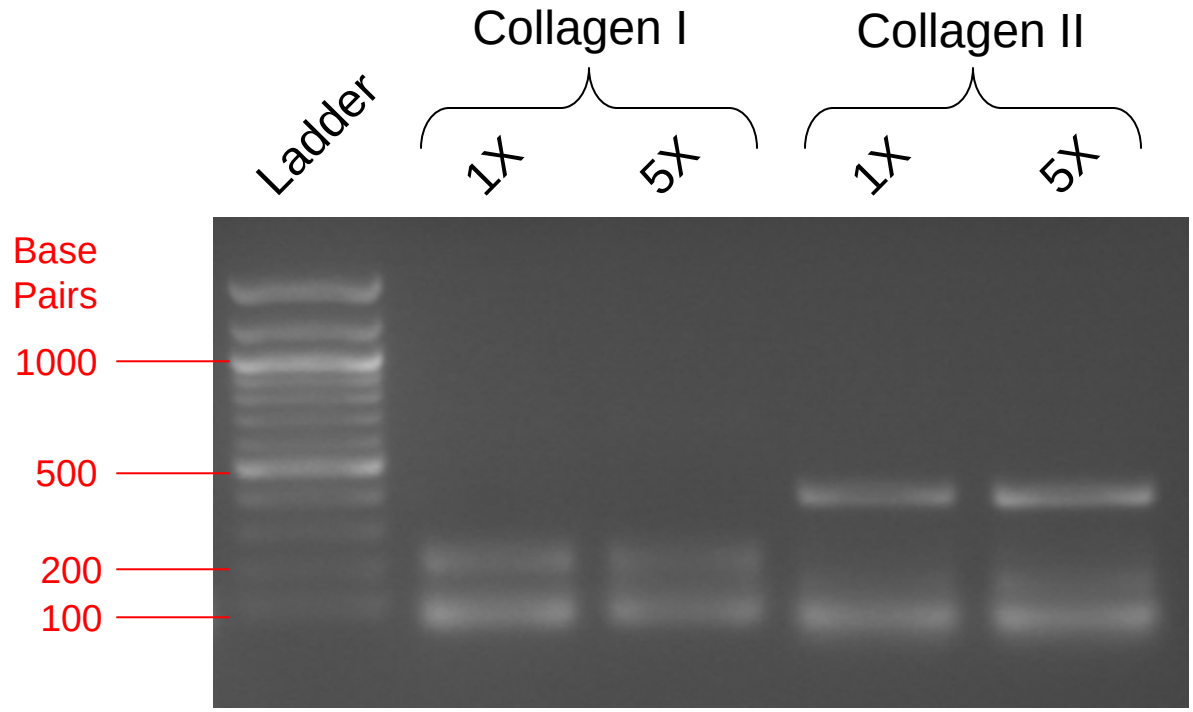

Figure 2 **RT-PCR and Agarose Gel Analysis yields Greater Collagen II levels in 5x Seeded Cells compared to 1X** RNA, isolated from the chondrocyte 3D cultures in alginate beads, underwent RT-PCR and the resulting cDNA was run on a 1.2% agarose gel. The GAPDH transcripts are represented by the bands at 100bp in lanes 2-5. In Lanes 2 and 3, the collagen I transcripts of the 1x and 5x samples are present around the 200bp marker. In Lanes 4 and 5, the collagen II transcripts of the 1x and 5x samples can be found around the 500 bp marker.