ABSTRACT

Title of Document:	CO-CULTURE OF BONE MARROW STROMAL CELLS AND CHONDROCYTES FOR BONE TISSUE ENGINEERING: MICROARRAY STUDY OF CHONDROCYTE SECRETED FACTORS
	Sathyanarayana Janardhanan, M.S., 2011
Directed By:	Associate Professor and Associate Chair John P Fisher, Fischell Department of Bioengineering

Tissue engineering refers to the assembly of biomaterials, cells and signaling molecules to develop functional tissues based on strategies derived from developmental processes. Cells play a crucial role, in that they can secrete a library of molecules, not entirely characterized in the laboratory, and yet provide repeatable results during *in vitro* experiments. Under conditions of co-culture with mesenchymal stem cells, the underlying biology of chondrocytes can elucidate the signal expression during the early bone development process called endochondral ossification. This interaction is tightly regulated in chondrocytes and results in the recruitment and differentiation of mesenchymal stem cells (MSCs) into osteoblasts. We executed a co-culture system, to observe the potential of alginate encapsulated bovine articular cartilage chondrocytes to induce osteogenic differentiation of bovine bone marrow stromal cells and to observe the interaction on a global scale by making use of the microarray platform. We identified certain genes expressed by chondrocytes that show substantial activity in co-culture systems such as versican (*VCAN*), secreted frizzled related protein 1 (*SFRP1*), matrix metallopeptidase 13 (*MMP13*), extracellular matrix protein 1 (*ECM1*) and collagen type 1 (*Col1A1, Col1A2*).

CO-CULTURE OF BONE MARROW STROMAL CELLS AND CHONDROCYTES FOR BONE TISSUE ENGINEERING: MICROARRAY STUDY OF CHONDROCYTE SECRETED FACTORS

By

Sathyanarayana Janardhanan.

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Advisory Committee:
Associate Professor John P. Fisher, Chair
Professor Sheryl Ehrman
Associate Professor Srinivasa R. Raghavan

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Dedication

Dedicated in memory of Sudeep Kandasamy

We continue to treasure every memory you left behind.

Acknowledgements

This thesis work happened over a period of time when I had the fortune of company and support by some of the best people I have known. I would like to thank Dr John P Fisher, my advisor and guide at the University of Maryland, who accommodated me into the Tissue Engineering and Biomaterials laboratory at a very early stage and guided my program with an intense sense of directionality and purpose. I owe a lot of my sense of accomplishment from this work to Dr Fisher's support and encouragement.

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Chapter 1: Introduction

The simultaneous development of biomaterials of complex properties and understanding of developmental biology spurred interest in establishing a platform for *in vitro* tissue development. Tissue engineering encompasses the range of techniques used to address tissue development challenges by utilizing a combination of biomaterials, cells and signaling factors. The central tenet of tissue engineering utilizes the advantages of each of these components to create living tissue that can be incorporated to defective or injured sites in a clinical setting.

The properties of biomaterials that are of importance to this ensemble are mechanical strength, biocompatibility and internal architecture. These properties can simultaneously provide the physical attributes of the target tissue, allow growth and transformation of supported cell types and allow for uniform growth throughout the volume of the material. Cells constitute another integral part of tissue engineering systems. The ability of certain cells to function outside its source tissue and maintain its gene and protein expression, also known as its phenotype, can be exploited to induce their growth on biomaterials outside a living system. The creation of a suitable microenvironment surrounding these cells involves developing surface characteristics for optimum adhesion and developing an appropriate media formulated to support growth and differentiation, or transformation in phenotype. Given these conditions, cells respond to the microenvironment by secreting matrix molecules that are required to sustain a regulated growth of the tissue and successful integration to a destination site if implanted. Signaling molecules play the role of key molecular players in the

growth, differentiation and regulation of tissue development. The presence of signaling molecules, naturally secreted or exogenously introduced, influences the growth and differentiation of cells and this influence is often exploited to stimulate cells grown *in vitro* and hence forms an integral part of tissue engineering systems.

The importance of signaling factors is especially critical since the developmental process *in vivo* takes place largely by signaling cascades involving a series of predictable responses given by cells to signaling molecules relayed by other neighboring cell populations. Tissue engineering systems attempt to recreate local microenvironments found in such cases, not only to study the underlying biology but also to study to identify optimum conditions to maximize growth of cells. Co-culture systems present unique *in vitro* methods to culture groups of cells in suitable orientation in order to allow for effective exchange of such signaling molecules. This provides scope for answering questions related to the particular role of each individual cell population in the overall signaling cascade. Furthermore, the introduction of biomaterials creates more realistic microenvironments, allowing for more accurate determination of such pathways.

In the present context, we study an aspect of the signaling pathway called endochondral ossification. Bone tissue development occurs by this process and it is one of two processes by which all bone formation occurs in mammalian systems. It involves the secretion of a cartilage template by chondrocytes. Osteoblast-precursor cells from the layer of tissue surrounding this cartilage template called perichondrium, invade the cartilage matrix and differentiate to osteoblasts. Simultaneously, chondrocytes grow in size and alter their matrix content. Secretion of

matrix metallopeptidases (MMPs) by chondrocytes facilitates replacement of articular cartilage extracellular matrix (ECM). This is accompanied by vascularization of the cartilage space and further invasion of osteoblasts into the template. By a series of steps, hypertrophic cartilage matrix (collagen type X) is resorbed and replaced with collagen type 1, (bone matrix). The sequential steps adopted by each cell population during this process are regulated by the signaling molecules secreted at each stage by the chondrocytes and osteoblasts. Understanding the mechanism of bone formation at a cellular level involves identifying the key signaling factors involved.

In this work, a co-culture system that allows for a paracrine interaction between chondrocytes and undifferentiated bone marrow mesenchymal stromal cells is set up to identify the factors secreted by the chondrocytes that drives the differentiation of BMSCs to osteoblasts. Using a combination of an indirect co-culture system that retains chondrocytes in alginate hydrogels at a high concentration suspended over a monolayer of BMSCs, the gene expression of chondrocytes and the osteogenic differentiation of BMSCs were studied simultaneously.

Chapter 2: Co-culture strategies in bone tissue engineering: on the impact of culture conditions on pluripotent cell populations

Introduction

Traditional molecular biology methods have been constantly applied to study interactions between cell populations of different phenotypes involved in bone tissue development. The surge in the application of pluripotent mesenchymal stem cell populations in this field has renewed interest in understanding their interactions with other cell types involved in the different schemes of tissue development. Early studies placed related cell types in direct contact to evoke the exchange of signaling molecules by the formation of gap junctions. With the development of tools in *in vitro* biology, these populations were cultured in close proximity but at a finite distance that eliminates the chance of cell-to-cell contact and encourages all communication by means of soluble protein expression into the surrounding media in an indirect co-culture setting. The introduction of biomaterials in the area of bone tissue development introduced a third dimension to these *in vitro* experiments and enabled more accurate reproduction of microenvironments for the study of mesenchymal stem cells and cells belonging to related differentiated forms. Most preliminary biomaterial based co-culture experiments were focused on demonstrating the targeted differentiation that was achieved as a result of pre-meditated juxtaposition of known cell types and previously established capacity of such materials to support the differentiation. and the advancements in areas such as gene expression quantification (microarrays, microRNA assays), biomaterial design (stereolithography, microfluidics) and antibody research. In more recent studies, these

developments have been applied to study the intercellular signaling and the key factors involved at much higher resolutions[1-4]. Blitterswijk et al[5], point out the various forms of intercellular signaling that can occur in any given coculture system. Cells are known to interact in a number of manners including cell-to-cell contact and soluble factor secretion. These modalities of intercellular communication impact the regulatory control of these processes and the uniqueness of each interaction is often exploited while developing tissue engineering strategies[6]. Research in the field of bone tissue engineering provides us information identifying specific factors, pathways and mechanisms involved in formation of bone precursor cells, mineralized matrix and the physiological characteristics of these cells in a tissue engineered environment[7-11]. Of particular focus in this review is the body of tissue engineering that deals with applying mesenchymal stem cells to differentiate and mature into osteoblasts and eventually deposit mineralized matrix. Molecular interactions play an important role in this process and it is widely acknowledged that the intercellular signaling that occurs during the course of the processes of endochondral and intramembranous ossification should be considered while comprehending processes such as wound healing or incorporation of engineered biomaterials into the host tissue. This is especially true with cell-seeded implants that can interact with the surrounding cells in the host tissue[12-15]. To understand the implications of such signaling pathways, *in vitro* strategies are often employed to identify and establish relationships between different cell types and how closely their interactions resemble known theories of bone tissue formation. Co-culture techniques provide this opportunity to place cells in suitable proximity in order to induce differentiation or

growth in a fashion that closely correlates to the biological phenomena we aim to understand[4,16].

The two main contributing factors to the success of any co-culture system are the physical form in which the individual cell populations are presented and the properties of the individual morphogens that they secrete. A biomaterial may be used to augment the nature of cellular interaction permitted by the host environment and subsequently influencing the physiological characteristics such as proliferation, differentiation and protein expression. Secreted proteins play a major role in the intercellular communication. Proteins involved in such signaling can be intracellular, matrix bound or secreted into the adjoining media volume. The accessibility and local concentration of these proteins is influenced by the spatial distribution of these cells and their related matrices. In addition to the choice of biomaterial and secreted protein content by the cell population type, the relationship between the form of co-culture and the signaling modality thus made possible, provides the additional degree of freedom that can be exploited to understand and apply these interactions. For instance, one of the earliest co-culture experiments by Tsuchiya et al[6] established that mesenchymal stem cells and chondrocytes co-cultured on the same pellet can deposit a pronounced cartilaginous matrix. The indirect co-culture of similar cells has been shown to yield different results in a number of other studies[17-19], raising the need for a consensus on the role of factors in the engineering of mineralized bone tissue and their occurrence as a function of the method used as outlined in Figure 1A. Through the course of this review we attempt to present a synthesis of outcomes of co-culture strategies in bone tissue engineering and how recent advancements can be applied to

the advantage of co-culture studies to further our understanding of molecular interactions and their manifestations in the development of viable tissue engineering constructs.

Common Platforms in Co-culture tissue engineering

Two enabling platforms have contributed to studying relationships in co-culture systems : micromass culture and indirect co-culture.

Micromass culture

Micromass culture involves high density in vitro culture of cell populations[20]. The interest in this technique of cell culture started with studies attempting differentiation of embryonic stem cells into chondrogenic nodes[21]. The micromass system has certain key advantages that make them ideal

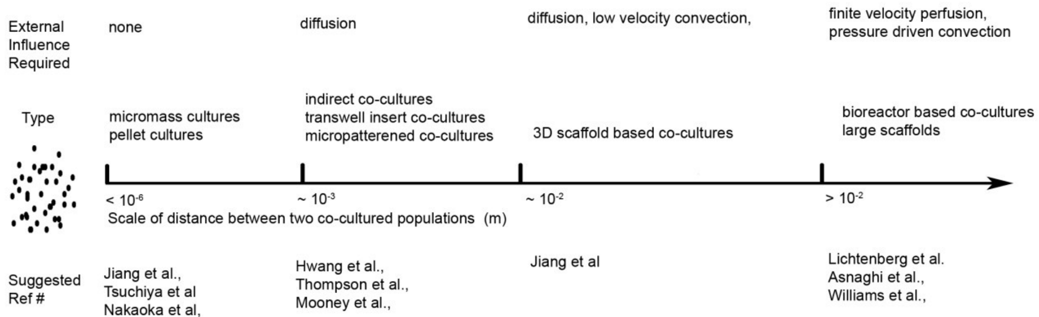

Figure 1. **Scales of length involved in co-culture paracrine interaction.**
Scale of lengths involved in co-culture interactions can be very important in designing strategies for mesenchymal stem cell differentiation. Modes of interaction between cell populations influence the growth and differentiation of MSCs by determining the local microenvironment.

candidates for certain tissue engineering studies. The high density of cell populations that can be packed into a small volume enable the creation of a three dimensional construct that comprises strictly of cells and cell based matrix products, thus

eliminating the need for a biomaterial to host these cells[22,23]. This “scaffold-free” culture can be especially important in certain types of studies that aim at understanding only the signal transduction cascades involved and avoid any influences exerted by the presence of a biomaterial. In these studies, close tracking of gene, protein, and matrix expression is necessary to understand the underlying mechanisms that control processes like differentiation and growth[24,25]. The micromass system provides a platform to track short term expression trends that can yield large sample populations for immunohistochemical, gene expression and protein studies. The close apposition of cells allows for intercellular communication channels by way of gap junctions or tight junctions that can be established at a higher degree in these cultures. This platform has been extensively used to study developmental biology and arthritis, where a number of studies have demonstrated relative ease in comparing interactions between cell populations in events where matrix immobilized factors play an important role in the signal transduction, thereby providing a larger concentration of these molecules.

The micromass platform is used extensively for growth factor delivery[26]. With regard to bone tissue engineering, this can be especially important while understanding the effects of exogenously delivered factors that constitute one of the central tenets of the field. As discussed in a different context below, the high density cell populations enable a greater efficiency of delivery of these factors and the biomaterial-free construct ensures lesser loss during transport as compared to traditional delivery methods. One can argue that this mode of delivery brings about a kinetic disadvantage to the cells at the core of the micromass. But with suitable optimization,

a scaffold-free delivery system can be advantageous in the delivery of small molecules in tissue engineering systems.

A third application of micromass cultures is in transfection studies. The reasons why transfecting micromass cultures can be more effective as compared to biomaterial or monolayer cultures are similar to the reasons mentioned with regards to growth factor delivery[27,28]. The tightly packed volume of cells enables effective delivery of vehicles in a small region of space, which can otherwise be compromised by absorptive factors such as materials or media in other culture methods. The core strength of micromass cultures is the enhanced cell densities and confined volumes that create a sharp advantage while preparing cells for tissue culture applications. The capacity of this platform to be used in co-cultures flows immediately from these advantages in enhancing intercellular interactions. In a number of co-culture mesenchymal stem cell-chondrocyte culture, mesenchymal stem cell-epithelial cell culture or mesenchymal-hematopoietic stem cell culture, a number of added growth factors are used to influence differentiation and growth in addition to the secreted factors. The combination of traditional co-culture techniques with the unique advantages offered by high density micromass cultures creates a powerful platform for high throughput preparation of cells for tissue culture. While employing micromass techniques in part or entirely in direct in vivo applications is yet to be demonstrated, the platform provides opportunity for studying interactions and directing differentiation.[12,14,29,30]

Indirect co-cultures

The indirect co-culture system is typically used to study paracrine interactions between distinct cell populations. It differs from the micromass system in that it

allows for various physical geometries within which individual cell population can be hosted before, during and after the co-culture. Cells intended to be placed in co-culture can be cultured in various forms including monolayer, in three-dimensional scaffolds or even in micromass culture. The physically distinct locations of the cell populations help track individual phenotype changes and gene expression characteristics[17,18,23,31,32]. The shared media volume also aids in accounting for factors that may be constitutively expressed by one or more of the cell populations and also for any temporal changes in the secretory protein expression profile that is typical of many primary cell populations involved in tissue regeneration. In the context of bone tissue engineering, this form of co-cultures has been exploited for the study of differentiation processes of mesenchymal stem cells. For instance the endochondral ossification process involves a spatio-temporal gene expression gradation in chondrocytes, mesenchymal cells and eventually osteoblasts, which progressively causes it to change its matrix composition and also secrete factors that trigger pathways causing switches in the phenotype and characteristics. The temporal changes during this process can be studied effectively through an indirect co-culture system. Paracrine signals between the distinct populations are exchanged via soluble media at all times while the individual cell populations can still be isolated at any point. The second advantage of this system is that the cell populations can be in different physical forms. Recent research has cast light on the phenotype changes especially in chondrocytes with changes in morphology. While attempting to understand processes such as endochondral ossification, it is important to be able to carry out studies without introducing an added effect of morphological changes

owing to culture of cells in vitro. Some specific examples of indirect co-culture techniques are discussed below.

In-vitro Cocultures

The first *in vitro* co-cultures explaining the effects of factors secreted by osteoblasts and chondrocytes were aimed at understanding the effects of certain known molecules that were believed to play a role in the process of bone development[16]. Most of these early studies focused on the effects of such factors on growth conditions such as proliferation and secretion of other known factors. Lacombe-Gleize et al. established that proteins secreted by osteoblasts stimulated the proliferation of chondrocytes and they concluded that the ratio of the active to latent forms of TGF-b1 had a role to play in the phenomena[33].

Later studies that looked at differentiation of these cell types identified a number of factors that could be playing a role in the communication between chondrocytes and osteoblasts in their different extents of differentiation. Gerstenfeld et al established that paracrine signaling played a role in the selective osteogenic differentiation of mesenchymal stem cells exposed to soluble factors secreted by non-hypertrophic chondrocytes cultured in a transwell membrane system[18,19]. These results are different from the work of Tsuchiya et al where a similar selection of cells resulted in a higher expression of cartilaginous matrix or a chondrogenic differentiation. In yet another experiment, Grassel et al utilized a transwell system to study the role of cartilage explant secreted factors on the chondrogenic differentiation of MSCs[34]. Although both studies utilized chondrogenic media, the modes of paracrine signaling in each of

these studies bear a unique significance for future studies. Thompson et al demonstrated a similar osteogenic inductive effect on bone marrow stromal cells by primary articular cartilage chondrocytes in three-dimensional scaffolds. As noted earlier, the culture of chondrocytes in three-dimensional scaffolds retains their phenotype and permits any change that could potentially be brought about on the chondrocytes in co-culture to be effected as a result of a biological response to the morphogens involved without introducing a bias caused by the de-differentiation by the monolayer culture[35,36]. It is yet to be established whether a micromass culture has any such de-differentiating effect on primary cell populations commonly used in bone tissue engineering. A three dimensional morphology is critical to the retention of the chondrogenic phenotype as noted in some studies[37]. For example, embryonic bodies derived from embryonic stem cells differentiate better in chondrogenic medium when cultured in a three-dimensional scaffold as compared to a monolayer culture when subjected to an identical induction[21]. Grellier M et al raise a similar point in their review of endothelial cell-osteoblast interactions, alluding to the necessity of a suitable three-dimensional environment to enhance cell survival and cell-cell interactions[38,39]. This can be especially important in the context of bone tissue engineering, owing to the fact that since chondrocyte involvement precedes osteoblast involvement in the endochondral ossification process, maintaining the phenotype of these cells can be essential in the application of co-cultured constructs in bone tissue engineering[40].

While chemical supplements can effect a desired differentiation of MSCs, exerting a paracrine signaling effect using differentiated cell types can be an effective technique to induce differentiation in an implantable co-culture system. The extent and nature of differentiation using chemical stimulants and/or exogenous factors is significantly different from that achieved via continuous co-culture methods without using them.[41] While supplements certainly improve *in vitro* differentiation as measured by protein and gene expression studies, in the context of implantable cultures, further work is required to ascertain the optimal stage of *in vitro* osteogenic differentiation that needs to be achieved in order to ensure appropriate bone formation and integration into the host tissue. The roles played by a host of factors such as Indian Hedgehog (Ihh), Parathyroid hormone related peptide (PTHrP), Matrix Metalloproteinase 9 (MMP9), Matrix Metalloprotease 13 (MMP13), Vascular Endothelial Growth Factor (VEGF) and certain members of the Collagen family including Col1A1,Col1A2, ColX and other matrix protein such as fibromodulin, aggrecan and versican have been speculated in the differentiation of MSCs and established in recent literature[13,14,42,43]. The paradigm of secretion of these molecules varies largely. Some growth factors are secreted constitutively and the expression rates are not influenced by the ongoing differentiation state of the cells they are in co-culture with. There are other growth factors that show significant shift in expression as a response to the activity during the co-culture, predominantly demonstrating the role of these species in the chemical cross-talk that cause differentiation and subsequent production of important matrix related factors. Although it is difficult to ascertain the extent of differentiation between cell-based scaffolds pre-cultured in chemical stimulants and their co-

cultured counterparts, it is evident from literature that paracrine signaling between progenitor cells and differentiated cell types provides an excellent starting point for developing therapeutic applications in injury and defect repair.

Ex vivo co-culture

Tissue explants are often used as a source of morphogenetic factors to influence the differentiation of cells. An intact tissue sample or a decellularized mass of a well defined section of a tissue can yield a large quantity of signaling factors that can be applied in *in vitro* studies. The role played by the microenvironment on the physiological characteristics and molecular behavior has been stressed on earlier[32,34,44]. Although well reproduced in a number of *in vitro* experiments, there is still need for the expression of certain molecules that can play an important role as molecular switches to elicit or aid a number of differentiation cascades. Particularly, the roles played by the components of the extracellular matrix (ECM) and the proteins that bind to the ECM, in the differentiation and regulation of bone precursor cells are yet to be characterized. The regulation of these matrix related proteins is known to be carried out in an autocrine fashion and hence the development of the signaling cascades to effect their biosynthesis can happen over long periods of time. Using primary cells in short term co-culture experiments often does not provide enough temporal flexibility to enable the synthesis of these factors, often leading to an insufficient reproduction of *in vivo* microenvironment conditions. Differentiated primary cells placed in co-culture with precursor cells aid in expressing and recreating a number of these factors. *Ex vivo* experiments that culture native tissue explants with target MSCs provide a means to enable the solubilization of such

factors that can otherwise be hard to synthesize *in vitro* or introduce exogenously. Wei et al. pointed out another important aspect of *ex vivo* co-cultures, that cellular components introduced into the co-culture system by the explants can play a role in the differentiation of MSCs. While it is not well documented if contact-interactions between cell processes originating from explanted tissues could regulate differentiation or proliferation of MSCs, one could hypothesize that under conditions that occur *in vivo,* these processes could act in regulating differentiation, along with gap junctions and other paracrine interactions during the cell signaling cascades that constitute the process of endochondral ossification. Of special interest in this scheme of signaling is the demonstrated chemotactic effect exerted by the explants on monolayer MSCs that are grown in co-culture. This phenomenon can be exploited to distribute MSCs across the target areas of growth or within a macroporous scaffold.

Ex vivo systems pose one drawback in their application to co-culture experiments. Since the explants are acquired primarily from harvested tissue, the biological factors, such as distribution of cells or cellular components in the donor site, can add a degree of uncertainty to the experimental design. While the overall signaling effects can be attained in the right media, the ability to optimize the level of induction can be limited owing to the fixed densities of cells and factors in the explants. Nonetheless, such systems can be excellent platforms to understand the bridge between *in vitro* tissue development and *in vivo* implant integration.

<u>In vivo cocultures</u>

The concept of *in vivo* co-cultures applies to techniques used to implant heterogenous cell mixtures, usually after encapsulation in a scaffold or *in vitro* expansions into

spheroids. Implanting a cell mixture ectopically can utilize the microenvironment of the implantation site and also the regulatory effect by the paracrine signaling effecting by the co-cultured cell populations. This concept has been utilized in embryonic stem cell research for a long time in order to commit the differentiation to a pre-determined lineage[22]. In other experiments, *in vitro* pre-treatments of progenitor cells were followed by encapsulation and ectopic implantation *in vivo* to elicit differentiation. Such combination of techniques opens doors to a number of possible strategies that can be employed to meet needs pertaining to defect repair. Materials that can be used as pre-vascularized scaffolds, cancellous scaffolds or layered constructs form ideal candidates for *in vivo* bone tissue engineering applications. The biological significance of such close appositions of cell populations should be completely understood in order to accurately predict their capacity to calcify and subsequent form bone. The stability of the individual phenotypes can be easily ascertained and optimized *in vitro* to balance influencing parameters.

Conditioned media culture

A number of differentiation pathways in the osteogenic signal expression cascade are triggered by proteins that are expressed by cells from the mesenchymal lineage without the requirement of any pre-treatment[45]. A volume of media used to incubate such cells can accrue a reasonable concentration of these proteins can be potent to elicit differentiation of MSCs when added exogenously, independent of the presence of the secreting cells. This method of pre-conditioning of media has been used to differentiate MSCs. The potential of this technique has been realized to ascertain the existence of cross-talk between co-cultured cell types and also identify any influence

played by such inter-cellular signaling in modulating of the signal expression. The time frame of incubation and volume of conditioned media required to differentiate a population of MSCs can be controlled and the results of the related extents of differentiation compared to correlate secreted factor delivery with its consequence. This can be especially important while scaling up such operations to bioreactors, where this method of transferring soluble factors from one culture to another can be translated into a continuous process to ensure constant supply of signaling molecules as opposed to the pulsed delivery in a static conditioned media co-culture[46].

Co-culture Bioreactors

Recent interest in bioreactors has enabled researchers to scale up traditional *in vitro* culture systems. Co-culture bioreactor systems have been attempted in the past as extensions of *in vitro* cultures with mixed success. Since bioreactors involve mechanical aspects to their designs as well as sterile growth environments, this compartmentalization typically provides a wide scope to design multiple population co-cultures that are otherwise difficult to establish *in vitro.* The additional degree of freedom introduced in bioreactor cultures is achieved by utilizing a wider range of material geometries and sizes to host the cells, most of which constitute the limitations of traditional *in vitro* systems[47,48]. Although a number of hydrogels currently used are not immediately compatible for bioreactor usage due to their mechanical instability in a flow system, there is vast scope for optimization of parameters such as the mechanical strength, pore size, and introduction of multiple layers of reinforcement to provide the required properties, flow rates and reactor geometry to circumvent these difficulties. The importance of bioreactors to bone

tissue engineering is especially prominent because of the need to form biocompatible tissue that possesses not only the mechanical characteristics but also the biochemical identity of bone tissues in order to ensure complete integration when brought to a clinical setting. The limiting challenges that *in vitro* cultures face can be met by bioreactor cultures especially with respect to creating a microenvironment over large volumes.

Beyond the obvious purpose of scale up, bioreactors have been employed for multiple purposes. The integrity of different biomaterials in a bioreactor, in terms of their capacity to support growth and differentiation can be viewed as a good measure of their performance and efficacy when used in vivo[49]. A number of studies have explored different parameters that affect flow contributing to the interest in areas such as scaffold architecture and adhesion characteristics within biomaterials when used in a bioreactor. This can be especially important in bone tissue development owing to the strong influence played by oxygen levels on the expression and regulation of osteogenic markers such as Osterix and Osteonectin[50]. As mentioned earlier, indirect co-culture systems provide efficient means of studying growth factor involvement and delivery. With the introduction of convection, the biomaterials and cells used in these systems can be effectively dubbed into various forms and orientations to yield a number of meaningful combinations that mimic in vivo tissue development more closely. Studies have shown that convection aids in better distribution and growth of mesenchymal stem cells in bioreactors and the added shear stress promotes greater differentiation.[51-56] Other studies have successfully demonstrated the ability of two or more distinct populations to be co-cultured in a flow system to yield an implantable

tissue engineering construct with well defined and controllable properties. The ability to delineate and quantify limiting factors and culture outcomes in scaffold-based bioreactor cultures has enabled the extension of these reactors to play a role in areas that fall at the interface of developing bone tissues and endothelial, hematopoietic, cartilaginous and other such peripheral cellular systems[57,58]. MSCs in scaffold culture in bioreactors were demonstrated to play a role in effectively expand and enriching a CD34+ hematopoietic stem cell population[59,60]. MSC-endothelial cell co-culture is a widely studied field targeted at developing bone tissue engineering strategies for creating simultaneous calcification and vasculature of scaffolds. As mentioned earlier the capacity to compartmentalize different populations while using a common flow medium enables a whole range of bioreactor configurations to be used. [61-63]

Recent Advances

New techniques in molecular biology have constantly been adopted to improve the scope of findings in the field of tissue engineering. Platforms that enable enhanced systematic evaluation of influencing parameters are now applied to study co-culture systems. The relationships between the different factors discussed above have been studied with these newer platforms with enhanced efficiencies made possible by adopting techniques in high resolution imaging, micro-patterning, protein assembly, high-throughput gene expression methods and advanced bioreactor techniques. While scaling up a co-culture system, the influencing factors pose interesting challenges. With increase in the size of the cell populations in culture, it is important to understand the short scales of interaction between key signaling molecules. This

raises enhanced flow and resolution issues where large scale rectors compromise the detail of interaction, thus not realizing the full potential of intercellular signaling. Developments in biomaterial lithography techniques have enabled the culture of multiple populations of cells in well defined micro-architectures at high geometric resolutions. Recent work by Takashi et al where they have adopted capillary force lithography to yield a wide range of co-culture patterns to understand aspects of hepatocyte-fibroblast co-cultures such as existence of gap junctions more sophisticatedly.[6] This is an example of how parameters such as spatial distribution and orientation of cells, that are generally kept random during culture conditions, can now be well defined in order to carry out high resolution optical analyses. A similar platform was developed much earlier by Bhatia et al. to study hepatocyte and non-parenchymal cell interactions.[64] This platform in conjunction with laser-mediated cell population recovery techniques pose excellent avenues in bone tissue engineering especially in the context of studying transient phenotype changes like those suggested earlier. With the growing knowledge of bioreactors and biomaterial-cell interactions, the need to visualize cells and more importantly the crosstalk between distinct populations is gaining increasing relevance. Micro-patterning of biomaterials to accommodate cells in very specific spatial configurations provides co-culture tissue engineering a large scope to study interactions and quantify local phenomena such as matrix production, creation of gap junctions and transport of signaling molecules that can be crucial while designing systems of increasing complexity.[57,65,66]. In the context of bone tissue engineering, there is increasing attention being given to controlled micro-architecture of scaffolds and its possible influence on differentiation[67]. The

ability of cells to adhere better and form interconnected colonies in controlled architectures influences the growth and differentiation and the ultimate suitability of these biomaterials. At a macroscopic level, this is encouraging to the field of tissue engineering owing to the fact that higher predictability of flow parameters in controlled architecture scaffolds results in better scale up and compartmentalization. In order to use these micropatterned biomaterials for better imaging cell-cell interactions, often the seeding densities have to be much smaller and on occasions the conditions under which the cells are grown are less conducive for growth and adhesion.[6] Some interesting works in the area of high resolution imaging of co-cultured populations demonstrate the scope of this platform such as localizing enzymes and proteins secreted as part of a molecular crosstalk or visualizing the existence of gap junctions and distribution of secreted matrix.

Conclusion

Co-culture strategies provide excellent platforms for understanding biological interactions between cell types known to play a concerted role in many key developmental processes that lead to tissue repair and regeneration. Although the concepts of co-culture are relatively new to the field of tissue engineering, they have been applied in a number of other fields such as cancer research, tumor biology and a developmental biology. However, there is a need in bone tissue engineering to gain understanding and control over both mechanical and biological properties of biomaterials in order to achieve clinically successful outcomes. The important challenge in utilizing co-cultures is ensuring that the entire span of the biomaterial used is exposed to the same microenvironment. In a number of cases where the cells

are not directly in contact, this problem could occur where the peripheral cells are exposed to a different microenvironment as compared to cells at the core of the material. Suitable treatment methods can ensure that this uncertainty can be circumvented by ensuring an active convective flow of factors around the material or by applying such differential microenvironments to the experiments advantage. There are a few questions that need to be addressed in the context of bone tissue engineering. Characterization of the effectiveness of secreted growth factors as a function of the paracrine communication length for different families of factors can be useful especially with the arrival of many new strategies. Although this can vary significantly between different combination of cell types and conditions, a good grasp of the upper and lower bounds of this length of interaction can aid in streamlining the design of future platforms. The progress made over the years has brought us to a sound understanding of the influence of co-culture of various cell populations on the growth and differentiation of pluripotent cells. The real challenge ahead of utilizing these co-cultures in aiding effective clinical tissue preparations is the ability to optimize physical characteristics of the system such as diffusive length, spatial distribution of factors, time scale of diffusion and the biological significance of secreted factors. A good starting point would be to model an *in vivo* developmental process as a co-culture system and derive these parameters to emulate similar results in a laboratory setting.

Chapter 3: Osteogenic induction of bone marrow stromal cells by co-culture with articular cartilage chondrocytes: a microarray study of chondrocyte gene expression

Introduction

Bone development in mammals is a sequential process that initiates with the formation of a pre-cursor cartilage template. Surrounding cells of mesenchymal origin invade the matrix and differentiate to osteoblasts, inducing calcification of the cartilage matrix and eventually forming an immature bone matrix. These sites of early bone development are resorbed by osteoclasts and eventually replaced by a fully functional bone matrix secreted by the osteoblasts[68]. The timed progression of these processes is made possible by the predictable response of cells of various phenotypes involved. The presence of a continuous matrix allows passage of the various families of signaling molecules that tightly regulate and guide differentiation, protein expression and growth[14,29,40,69-71]. Frequently during this process, families of proteases are secreted to resorb the matrix, which make room for fresh cell phenotypes to invade the region. The expression of these factors is controlled positively and negatively by a number of pathways. Sox9 delays chondrocyte maturation and sustains phenotype retention and elevated proliferation. Proteins belonging to the Ihh family also contribute to this delayed maturation[72]. Constitutive expression of proteins during the proliferative phase activates growth factors belonging to the TGF beta (TGF- β) superfamily. Significant members of this family include BMPs and their associated proteins. Downstream cytokines participate in the osteogenic differentiation of MSCs and in the terminal differentiation of

chondrocytes[1]. The transcription factors associated with these cytokines impose a balance of positive and negative regulation on the phenotypic state of the chondrocytes and cells in the osteogenic lineage[40]. Thus, the cartilage template forms the basis of future bone growth by mediating the diffusion and differentiation of perichondrial MSCs resulting in the invasion of blood vessels, resorbtion of cartilaginous matrix and substitution by bone matrix secreted by the osteoblasts. The timed differentiation of chondrocytes and changes in their secretory protein expression is critical in controlling the differentiated state of the MSCs[73]. *In vitro* tissue engineering applications utilize biomaterial templates, enabling cell phenotypes and signaling molecules to reconstruct the events that occur during endochondral ossification with the aim of creating and facilitating bone regeneration[7,18,31,74,75]. Local microenvironments typically comprise of collagen based matrix components along with growth factors belonging to families such as Indian hedgehog (Ihh), Parathyroid hormone related protein (PTHrP), bone morphogenetic protein (BMP) and proteases belonging to the matrix metallopeptidase (MMP) family. Intracellular and secretory protein expression influences the collagen content surrounding the cells[76,77]

To study the interaction between chondrocytes and MSCs during early stage bone development, co-culture systems are often used in varying conformations[18,22,31,78,79]. The restrictions imposed by the physiology of cells outside their native tissue can be accommodated by creating suitable microenvironments *In vitro* that assist or sustain normal phenotypic behavior[36]. Our primary goal is to elucidate the role of scaffold suspended primary chondrocytes on the osteogenic differentiation of bone marrow

stromal cells (BMSCs). Earlier studies demonstrated the ability of secretory signals from chondrocytes to induce osteogenic signal expression of BMSCs in monolayer[17]. These studies explore the different pathways of cell signaling by which chondrocytes effect the transformation of a primary MSC population into a differentiated aggregation of cells that can surround itself with a mineralized matrix. While some studies have attempted to study the differentiation cascades in the BMSCs, few have elucidated the changes in chondrocytes that drive this differentiation. A systematic study can help identify the simultaneous changes in signal expression of chondrocytes linked to the intercellular communication. The main challenge in identifying new factors in these signaling cascades using protein methods is the low extent of their expression. Some of these secreted factors are believed to be intracellular or linked to the matrix; both circumstances pose significant challenges in quantifying them. Although low levels of expressed proteins can be identified accurately in exploratory 2D gels[80], gene expression analysis is more easily achievable on a global scale when localized RNA is carefully isolated from a certain population of cells[81]. The large database of gene expression data corresponding to carefully calibrated culture conditions, provides an opportunity to study the differentiation at great detail and the accuracy can be backed up by subsequent validation methods. *In vitro* experiments in tissue engineering carried out with very specific microenvironments can be studied under a high resolution of clarity for exploratory studies that can potentially involve multiple genes across many different families[13,82].

In this work, we studied the gene expression changes in bovine articular cartilage chondrocytes with the aim of identifying upregulated factors within this population

that can play a role in the osteogenic induction of BMSCs. The chondrocytes were encapsulated in spherical alginate beads and were placed at a finite distance of 1 mm from BMSCs. This method of co-culture prevented direct cell-cell contact while allowing soluble factors secreted by either population to diffuse freely through the shared medium. These culture conditions permitted retention of a spherical morphology in the chondrocytes. The monolayer culture in BMSCs allows the creation and subsequent inspection of a matrix surrounding the adherent population formed in response to chondrocytes co-culture without any other contributing factors. This methodology allows for the unbiased detection of upregulated genes during the BMSC-Chondrocyte co-culture. The specific objectives of this study were (1) to evaluate the potential of chondrocytes-secreted signaling factors to induce the osteogenic differentiation, (2) secure global gene expression data of chondrocytes under conditions of co-culture and develop a comparison with the corresponding expression under control conditions and, (3) identify and accurately quantify the expression of genes by the chondrocytes coding for secretory proteins that demonstrate greater than 5-fold upregulation during the co-culture study period.

Methods

Bovine bone marrow and articular cartilage harvest

All tissue samples for this study were obtained from 2-4 week bovine meta-tarsal bones. The tissue was sourced and the bone marrow was isolated within 24 hours of tissue harvest. After initial preparation of the meta-tarsal bones to expose the lamellar core at mid shaft, bone marrow was aspirated using a syringe containing Minimal

Essential Medium, Alpha Medium (α-MEM) + 10% Fetal Bovine Serum. The tissue fragments and bone dust were removed using a 70 μm filter. The resultant cell suspension was centrifuged at 1000 xg for 5 mins to pellet the cells and remove any immiscible components remaining in the media. The cells were resuspended in α-MEM + 10% FBS and plated at a density of 4000 cells/cm^2 flasks. The flasks were incubated for 48 hours after which the plastic-adherent bone marrow stromal cells were isolated and the supernatant media was removed. The adherent cells were washed with PBS and cultured further in media for about 4 days constituting the first passage of the cell culture. Once the flask reached 90% confluence the cells were isolated for the first sample in the study. These cells were typically passaged 3 times after the initial plastic-adherence selection, constituting the three passages before their use in the study.

Bovine articular cartilage was harvested from the crown of the exposed cartilage in the metatarsal-phalangeal joints. The isolated cartilage shavings were digested in collagenase P and the digested solution was filtered and washed with media four times, undergoing centrifugation and resuspension during each wash. The isolated cells were then encapsulated in alginate beads to retain their spherical morphology and cultured in Dulbecco's modified eagle medium (DMEM) + 10% FBS. The chondrocytes were retained in the beads throughout the course of the study with regular media changes. At each time point, 15 beads were dissolved using ethylenediamine-tetraacetic acid (EDTA) to release the cells for further analyses.

Cell Culture

For all cell culture assays, BMSCs from the third passage were used. In order to establish osteogenic differentiation of the bone marrow stromal cells in the co-culture system and compare the extents of differentiation under different culture conditions, four distinct groups were established. The groups contain BMSCs (1) in co-culture with chondrocytes suspended in alginate beads, (2) in control media, (3) in osteogenic media and, (4) in media pre-conditioned by primary chondrocytes. The conditions under which the individual groups are maintained are tabulated in Table 1. Co-culture was carried out in Corning transwell ™ plates (Corning Incorporated, Lowell, MA). The BMSCs are cultured in monolayer by seeding them on the tissue culture plastic. The chondrocytes are encapsulated in alginate beads and are suspended in the transwell inserts above the monolayer. Inserts with a pore size of 0.4uM were chosen to prevent any cell migration between the BMSC monolayer and the beads. The osteogenic control group consists of BMSCs in monolayer cultured in media with osteogenic supplements, 10 nM dexamethasone and 2.16 g/L sodium beta-glycerolphosphate. The conditioned media group consisted of BMSCs cultured in media that was pre-conditioned by alginate bead encapsulated chondrocytes. In order to supplement the media with fresh FBS, the media from the pre-conditioning well is mixed with fresh α-MEM supplemented with 10% fetal bovine serum in a 1:1 ratio. In order to maintain the concentration of any constitutively secreted factors by the chondrocytes, the cell number of chondrocytes in the pre-conditioning wells was kept twice that in the co-culture wells. The study was carried out for 21 days. The control group consisted of BMSCs in monolayer cultured in α-MEM supplemented with 10%

fetal bovine serum. The respective media was changed every two days and the cells were not lifted from monolayer until the time point for each assay. The conditions of cell culture are listed in Table 1.

Group	Cell composition at seeding	Seeding Density (cells/well)	Media	Volume of media (mL)
Control	Monolayer : BMSC	40,000	α-MEM supplemented with 10% Fetal Bovine Serum	2
Co-culture	Monolayer : BMSC Transwell Chamber : Chondrocytes in alginate beads	BMSC : 40,000 Chondrocytes : 1.5 x 10^6	1 : 1 v/v mixture of α-MEM & D-MEM supplemented with 10% Fetal Bovine Serum	3
Osteogenic control	Monolayer : BMSC	40,000	α-MEM supplemented with 10% Fetal Bovine Serum , ascorbic acid, 10nM dexamethasone, Na-beta-glycerolphosphate	2
Conditioned Media	Monolayer : BMSC	BMSC : 40,000 Chondrocytes preconditioning media : 1.5 x 10^6	1 : 1 v/v mixture of α-MEM & D-MEM preconditioned by alginate encapsulated chondrocytes supplemented with 10% Fetal Bovine Serum	2

Table 1. **Conditions of cell culture.** To compare groups under different circumstances while eliminating any external influences on the study

Osteogenic signal expression of bone marrow stromal cells (BMSCs)

RNA isolation and quantitative real time polymerase chain reaction

Osteogenic gene expression in BMSCs was evaluated by measuring the relative expression of marker genes between the different experimental groups. *Alkaline phosphatase(ALP)* and *osteocalcin (OC)* are early markers of osteogenic gene expression respectively and their relative expression was compared between the different experimental groups over 5 times points spanning the 21 day study. The time points were chosen to detect early, middle stage and late stage differentiation. Gene expression was assessed using SYBR Green Dye in all groups on Days 1, 4, 8, 14 and 21. At each time point, cells from 3 wells of a 6 well-plate were independently isolated from each group using Trypsin in ethylenediaminetetracetic acid (EDTA). The yield ranged from 5×10^4 cells per well in earlier time points to about 8×10^5 cells at the later time points. RNA was isolated from these cells using RNeasy Mini Plus kits (Qiagen, Gaithersburg, MD) per manufacturers protocol. Total RNA harvested from this method was stored at -20^0C and was transcribed with a high capacity cDNA archive kit prior to its use in PCR. Cells were harvested from well plates in triplicate to create three samples of transcripts per experimental group per time point.

The SYBR green dye technique of PCR makes use of a forward and reverse primer pair for each gene to amplify its expression in the sample. Since the relative gene expression is relevant in this study, we made use of GAPDH as an endogenous control. We utilized the $\Delta\Delta C_t$ technique to interpret data from the study. The GAPDH gene in the Control group from Day 1 was used as a calibrator. Gene expression was

recorded as fold change over the calibrator gene. Three biological replicates were utilized in the study.

Alkaline Phosphatase in BMSCs

The intracellular alkaline phosphatase protein level was assayed using a standard immunological technique. Chromophoric substrate, p-nitrophenyl phosphate (pNPP) is used to quantify phosphatase enzymes. Its activity can be detected by using a standard spectrophotometer by the distinct color shift to yellow shade following enzyme action. Cells were harvested from well plates in triplicate using trypsin, followed by centrifugation and resuspension in PBS to exclude any trace amounts of media. The resuspended cells were centrifuged again and resuspended in 75uL of M-PER reagent and incubated for 10 minutes on a shaking platform. M-PER reagent is part of a mammalian protein extraction kit, used to lyse cells and release the intracellular components. The lysate was diluted and assayed in triplicate with pNPP and incubated for 30 minutes in the dark. The reaction was stopped using sodium hydroxide before detecting the reacted substrate at 405 nm. A standard curve using 4-nitrophenol was used as a reference to quantify the concentration of the consumed pNPP substrate. The calculated alkaline phosphatase (ALP) protein levels were normalized to DNA content to account for variable proliferation between groups and between time points.

Mineralization

The deposition of calcium by BMSCs was assayed using the method of Gregory et al. by directly visualizing and subsequently quantify the extent of calcium deposition by the cells at each time point[83]. Cells were fixed with 10% w/v paraformaldehyde for 15

min, followed by multiple washes with PBS. Staining is carried out by adding 1 mL Alizarin Red S dye solution (40 mM ARS in acetic acid) to the cell surface and incubated for 15 minutes. Following a series of washes with PBS to remove any unused dye, phase contrast images of the cell surface were taken to visualize the formation of mineralization nodules. Quantification of calcium deposition was carried out by dissolving the mineral layer in approximately 750 µL of 10% v/v acetic acid. The contents of the well plate were transferred to microcentrifuge tubes, vortexed for about 30 sec and wrapped in before being incubated at 85 degree C for 15 mins. The tubes were then centrifuged at 20000 xg for 20 min. In a microcentrifuge tube, 500 µL of the supernatant was combined with 200 µL of ammonium hydroxide. The assay was carried out in triplicate using 150 µL of the mixture and read under a spectrophotometer at 405 nm. A standard curve was constructed based on predetermined ARS concentrations.

Gene expression evaluation in chondrocytes

Microarray

A genome-wide expression analysis was conducted on chondrocytes to study their gene expression during the osteogenic induction of bone marrow stromal cells. Two groups were utilized in this study to identify chondrocytes gene expression patterns, the co-culture group and the control group. The co-culture group comprised of BMSCs in the monolayer with alginate encapsulated chondrocytes suspended in membrane inserts as described earlier. The media consisted of a 50%-50% mix of α-MEM and DMEM, supplemented with 10% FBS. The control group consisted of alginate encapsulated chondrocytes loosely placed in DMEM + 10% FBS with no

additional supplements. The control group served as a frame of reference to identify genes that showed specific over-expression due to the presence of BMSCs in the co-culture group. An additional sample was prepared to serve as an absolute control against which the raw expression data of all other groups could be normalized. This was built into the experimental design to avoid any false positives while comparing the data between experimental groups since the Day 0 group corresponds to the phenotypic expression of the chondrocytes at the start of the experiment prior to any influence from the growth media or soluble cytokines, and is hence designated as the 'absolute control'. The fold change for each gene over its value in the Day 0 control was utilized to compare and contrast the gene expression between co-culture and control groups in Days 4 and 14.

RNA Isolation

RNA was isolated from chondrocytes on Days 1, 4, 8, 14, and 21 as described earlier. Briefly, alginate beads from the co-culture and control groups were washed with PBS to remove any trace media. Alginate beads were transferred to individually labeled well plates and approx. In order to initiate the bead dissolution process, 4 mL of a calcium chelating agent, EDTA was added to the beads. Following 20 min incubation, 4 mL of DMEM + 10% FBS was added to the suspension. The samples were then transferred to 15 mL Falcon tubes and centrifuged at 1000 xg for 10 min to pellet the chondrocytes. Qiagen RNeasy Mini Kit was used to isolate total RNA from the pelleted cells per manufacturer's protocol. In addition to the above samples, chondrocytes were isolated from bovine cartilage as described above with bovine

cartilage harvest and RNA was isolated from the freshly harvested cells to serve as the absolute control.

	Sample Identity	RNA Concentration (ng/uL)	rRNA ratio (28s/18s)	RNA Integrity Number (RIN)
Sample 1	Day 0 Control	19	2	9.9
Sample 2	Day 0 Control	31	1.8	10
Sample 3	Day 4	62	2.1	10
Sample 4	Day 4 Control	44	2.4	10
Sample 5	Day 14	78	2.1	10
Sample 6	Day 14 Control	252	2.2	10

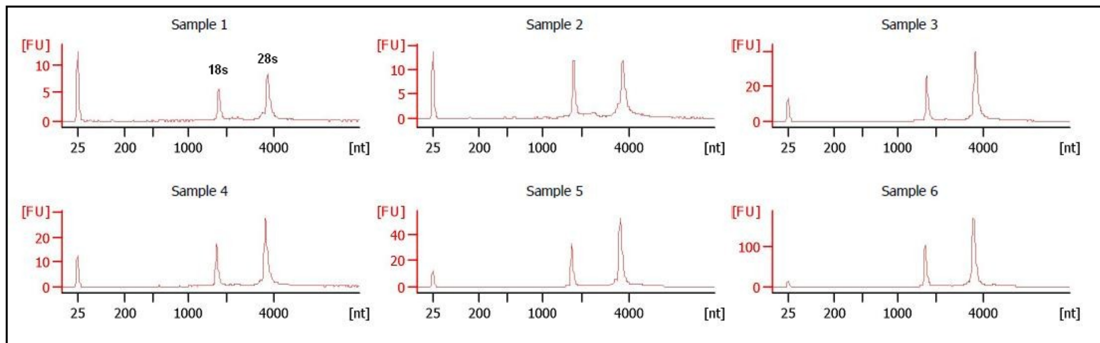

Table 2 : **Quality Control of RNA harvested from Chondrocytes.** RNA samples prepared for the microarray experiment were first analyzed for quality using the Agilent Systems Bioanalyser 2100 for integrity, prior to running them through a chip. The sharp 18s and 28s peaks represent high purity of the RNA and the 28s/18s ratio.

Quality Control and Microarray Chip Run

The GeneChip Bovine Genome Array consists of 24,128 bovine transcripts scaling the entire bovine genome. Six bovine microarray chips were used for the purpose of the study as charted in Table 3. Since earlier studies showed significant osteogenic gene expression in BMSCs in two weeks, the experimental groups at Day 4 and Day 14 were designated as early stage and a late stage samples respectively in the differentiation study. Also, since the results of this study were subsequently validated using PCR in triplicate, we limited the one the use of Genechips to one array per experimental group as listed in Table 3. The raw data was saved for analysis and acquisition of heat maps.

Sample ID	**Sample Name**	**Population Description**
1	Day 0	Freshly harvested articular cartilage chondrocytes
2	Day 0	Freshly harvested articular cartilage chondrocytes
3	Day 4	Alginate encapsulated chondrocytes cocultured with BMSCs for 4 days
4	Day 4 Control	Alginate encapsulated chondrocytes cultured for 4 days
5	Day 14	Alginate encapsulated chondrocytes cocultured with BMSCs for 14 days
6	Day 14 Control	Alginate encapsulated chondrocytes cultured for 14 days

Table 3. **Samples analyzed using bovine GeneChip microarray chips**

Data mining and expression evaluation

Raw data from the microarray data analysis from each chip is reported as a data set containing two values for each of the 24,128 genes, viz., Cell Call, indicating the presence, marginal presence or absence of expression of the gene and a present Value Call (pValCall). The Cell Call is used to ensure that the raw data used for gene expression is above a certain threshold set at the background noise across all samples, in order to avoid erroneous calculations using values below a baseline signal quality. The value of the signal indicates the untransformed raw data. This data is normalized and log transformed using the MAS 5.0 algorithm developed by Affymetrix using standard protocols. The Day 0 calibrator sample served as a baseline expression sample to which all expression data was normalized prior to computing fold change comparisons. These fold changes were compared between the co-culture and control groups on Days 4 and 14 to obtain the list of differentially expressed genes. Table 6 shows the list of genes that show substantial expression (fold change > 5) in one or more time points.

Fold change comparisons

Fold change represented by microarray chip data provides an indication of expression patterns that may be used to identify genes involved in biological processes. This study aims at evaluating the factors expressed by the chondrocytes that could have potential influence on the MSCs. Since chondrocytes are suspended in three-dimensional scaffolds and maintained at a finite distance from the MSC population, factors involved in the intercellular signaling originating from either cell population are expected to be secretory or matrix linked. The Gene Ontology(GO) terms

assigned to each gene provides a brief annotation of the consensus on the cellular localization, function, and biological process available for the respective gene. The GO terms for the genes were used to earmark those with high fold changes that are present in the extracellular or matrix linked components and are thus potentially linked to the signal transduction during co-culture of MSCs and chondrocytes.

Genes of interest were selected based on their enhanced expression in one or more time points during the co-culture. The criteria for selection of genes were as follows :

The fold change of the gene in the Day 4 or Day 14 co-culture group should be greater than or equal to 5 compared to the Day 0 sample.(eg. FC Day 4 / Day 0 >= 5)

The GO Terms indicate presence of the corresponding proteins encoded by these genes in the extracellular domain or as membrane bound.

Validation of chondrocyte expression data from microarray analysis

Validation of chondrocytes gene expression was performed using quantitative rt-PCR. The reaction volume consisted of SYBR Green Dye, cDNA, forward primer, reverse primer and DNAase free water. The primers designed for this purpose using Primer Express 3.0 (Affymetrix, Carlsbad, CA). Briefly, the custom made primers were analyzed for consistency by creating a standard curve. Following this, RNA from the respective samples was reverse-transcribed to cDNA using High Capacity cDNA Archive Kit sourced from Applied Biosystems (Foster City, CA). GAPDH was used as an endogenous control. The control group on Day 0 was used as the calibrator and expression for all other groups was calculated based on the Delta-Delta Ct method. The Applied Biosystems ABI Prism 7000 Sequence Detector was utilized to study the gene expression levels.

Statistical Analysis

Unless mentioned otherwise, all experiments were carried out with three biological replicates corresponding to each sample. In addition, three technical replicates were performed during all assays for each biological replicate. Experimental values were reported as means with the corresponding standard deviation in the figures. One-way analysis of variance (ANOVA) was performed and results were reported as statistically significant as found using Tukey's Multiple Comparison test within a 95% confidence interval ($p < 0.05$).

Results

Bone Marrow Harvest and Cell Culture

Bone marrow stromal cells harvested by the technique described earlier, yields a largely heterogeneous population. To separate TCPS adherent BMSCs from non-adherent hematopoetic cells, the mixture was incubated for 48 hours in a tissue culture flask. Following a change in media and continued culture, these cells proliferated at a steady rate. BMSC harvest from bovine meta-tarsal bones yielded approximately $2X10^5$ cells per pair of bone samples. A preliminary study was conducted to evaluate their osteogenic differentiation potential. This study showed a significant alkaline phosphatase protein expression by the BMSCs, encouraging their use in the subsequent studies.

All groups of BMSCs exhibited growth throughout the course of the study. The cells were plated at a low density of 4×10^4 cells / well in a 6-well plate so that the surface area was about 80% - 90% confluent by Day 21 as seen in the phase contrast images

(Figure 2A). Data from all end-point studies conducted were normalized to a proliferation dependant factor (such as DNA content or GAPDH mRNA content) to account for this growth.

FIGURE 2A

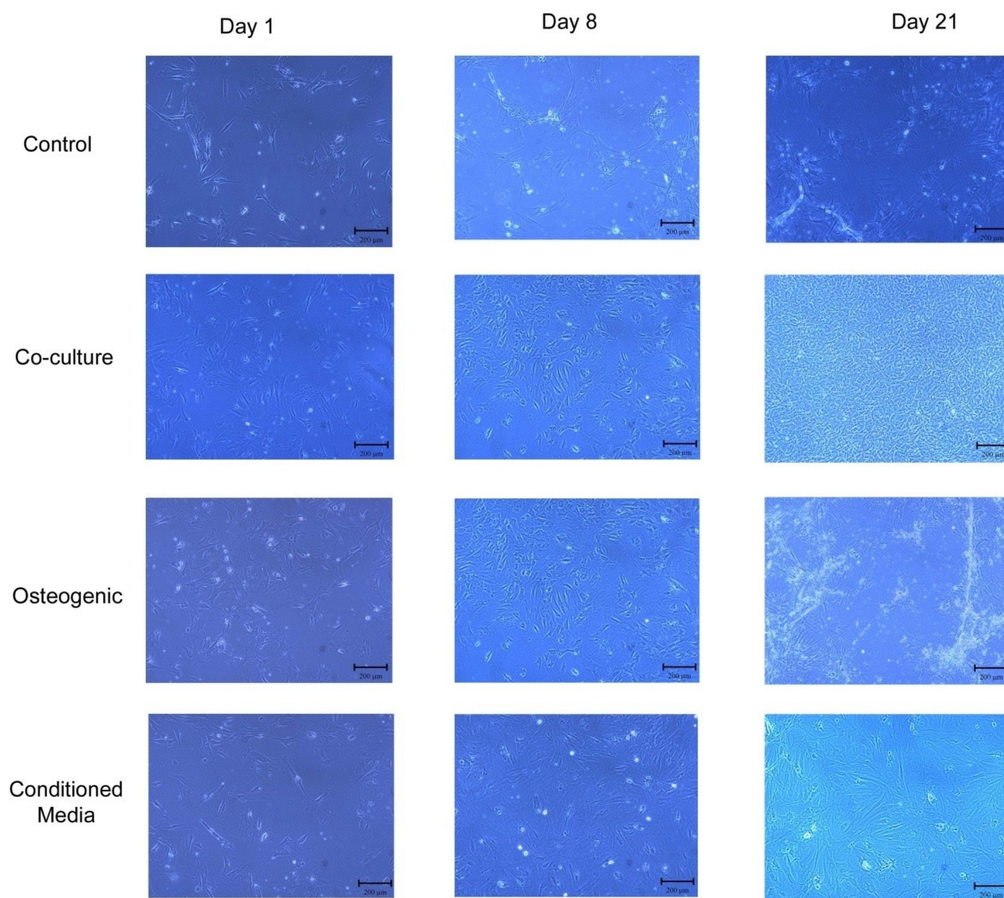

Figure 2A. **BMSC Proliferation.** BMSCs proliferate throughout the course of the study in all groups.

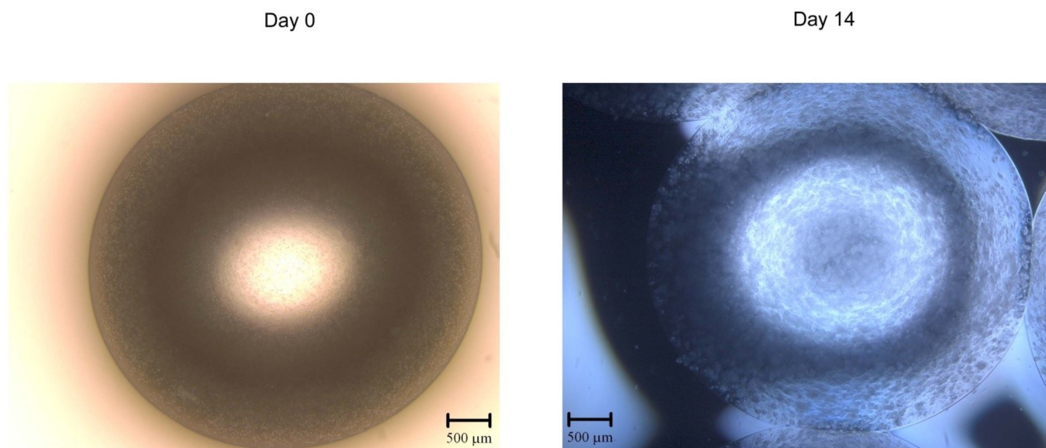

Figure 2B. **Chondrocyte proliferation (2X).** Chondrocytes proliferate during the course of the study. Cell migration within the bead and to the exterior is rare but chondrocytes proliferate to form colonies of cells within the bead over along with the expansion in bead size.

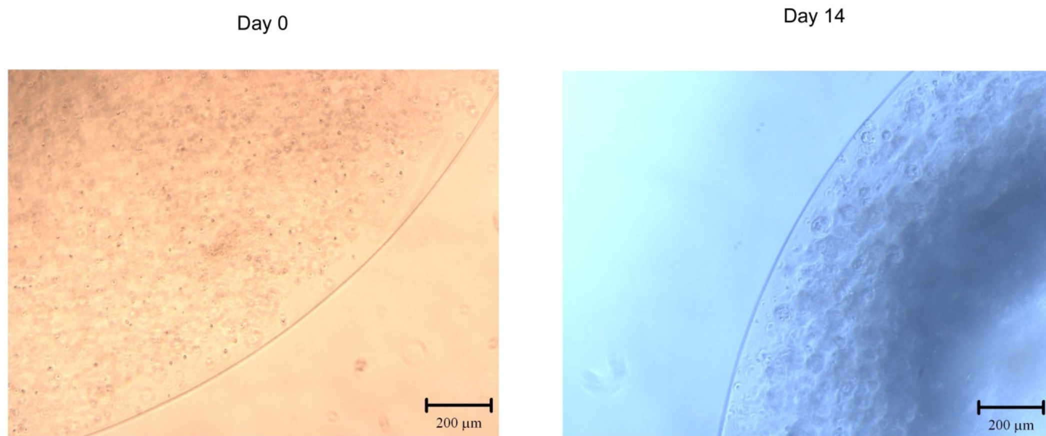

Figure 2C. **Chondrocyte proliferation (10X).** Close observation of the chondrocytes bead reveals formation of colonies in spaces formerly occupied by isolated cells (denoted by black spots on Day 0).

Osteogenic signal expression of BMSCs

Quantitative real time polymerase chain reaction

As mentioned earlier, the expression level of the control group on Day 0 served as a calibrator to evaluate fold changes for all other groups in the study. *ALP* gene expression was detected in all groups from Day 4 through Day 21. A significant expression of the *ALP* gene was observed on Day 8 in the co-culture and osteogenic group. The co-culture group expressed a fold change of 4 while the osteogenic control expressed a fold change of 6. These differences were observed to be more pronounced with time. The osteogenic control group and the co-culture group registered significantly higher ALP expression as compared to the control and conditioned media groups ($p<0.05$). On Day 14, the co-culture group demonstrated a 13-fold increase over the calibrator and this represents the peak expression for this group. The osteogenic control group showed the highest expression on Day 14 with a fold change of 170. This expression level was maintained throughout the rest of the study for the osteogenic control group but the co-culture group returned to a baseline level expression by Day 21 of the study (Figure 3).

Osteocalcin (*OC*) expression was expected to be pronounced in the induced groups (co-culture, osteogenic control and conditioned media groups) on Days 8 through 21. On Days 1 and 4, no significant *OC* expression was noticed. In the co-culture group, the osteocalcin expression level on Day 21 was significantly higher than on Day 14. Towards the later time points all groups registered a rise in the level of *OC* expression but discernable pattern was noted to compare the expression between individual groups.

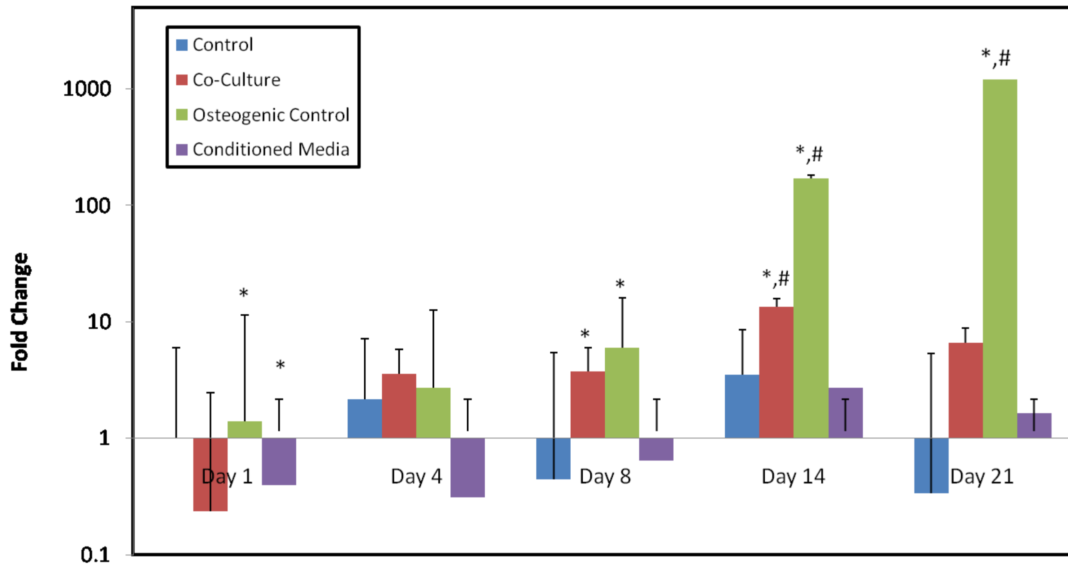

Figure 3. **Alkaline phosphatase gene expression in BMSCs.** Gene expression for the Alkaline Phosphatase (ALP) gene was carried out using recently detached BMSCs on Days 1,4,8,14, and 21. The osteogenic control group exhibits significantly higher expression even at the early time points. By Day 8, the co-culture group expresses ALP above the control and conditioned media groups but lower than the osteogenic control. This trend continues on Day 14 following which the co-culture group registers a sharp drop in ALP expression while the osteogenic group sustains the expression level. (All values are represented as mean ± standard deviation, n=3. * represents significant difference between groups on a given time point. # represents significant differences between values of the same group on different time points, $p < 0.05$.)

Alkaline Phosphatase protein expression

On Day 1, no significant difference in expression of ALP was noticed between the groups. On Day 4, the co-culture group and the osteogenic control registered a 2-fold increase in expression. By Day 8, the co-culture and the osteogenic control group registered a significantly higher expression of the protein than the other groups and also registered a marked increase from their levels on Days 1 and 4. ALP expression for the co-culture group reaches a maximum on Day 8, reaching an extent of 4-fold increase over the extent on Day 1. The Day 8 expression level is higher than the co-culture and marks the peak expression for the group. This increased expression level

was sustained throughout the rest of the study as seen with the ALP gene expression of the osteogenic control group. The conditioned media group, although initially expected to show some expression, registered no significant expression over the control group at any point of the study (Figure 4).

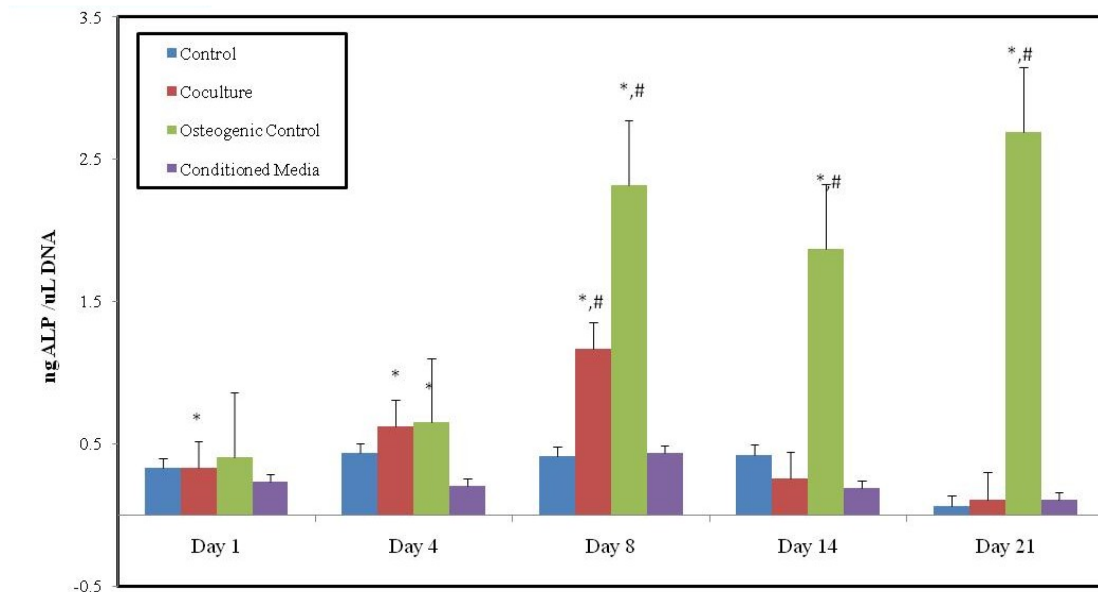

Figure 4. **Alkaline phosphatase protein expression in BMSCs.** Intracellular ALP protein levels are detected by spectrophotometric detection of the reacted chromophoric substrate pNPP on Days 1,4,8,14, and 21. The co-culture group registers significant early rise in the ALP protein expression in the first half of the study, expressing more ALP than other groups on Days 1 and 4 and showing comparable levels of ALP expression with the osteogenic control group on Day 8. While the osteogenic control group sustains this expression, the co-culture group registers a sharp loss of intracellular ALP protein content, falling to the level expressed by the control group. The conditioned media group showed no demonstrable ALP expression. '*' signifies expression above all other groups on a given time point and '#' refers to expression of a given group significantly varying from its level at other time points. (All values are represented as mean ± standard deviation, n=3. '*' represents significant difference between groups on a given time point. '#' represents significant differences between values of the same group on different time points, $p < 0.05$.)

<u>Mineralization of the BMSC monolayer</u>

Alizarin Red S allows for the staining of confluent monolayers of cells that form mineral nodules. No visible staining was noticed until Day 21. After several washes

intended to remove any unincorporated dye, all monolayer samples exhibited a background staining, procuring a similar stain pattern for all experimental groups. However upon inspection at a larger magnification, the osteogenic control group exhibited larger number of stained nodules. These nodules also stained with a higher intensity than those found in other groups (Figure 5).

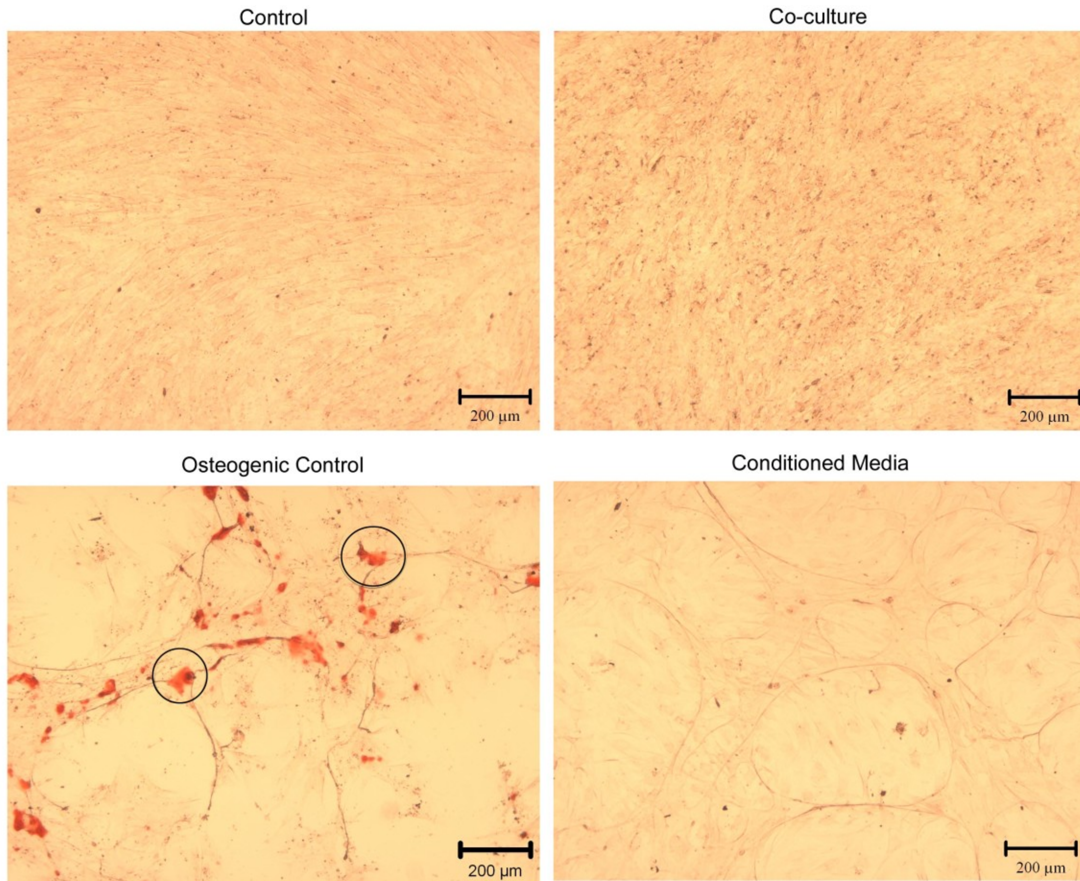

Figure 5. **Mineralization of BMSC monolayer.** Alizarin Red S staining of fixed BMSC monolayers on Day 21 of the study reveals levels of mineral nodule formation in the different groups. The co-culture group possessed spots of mineralization of varying sizes. The osteogenic group exhibits well defined zones (as indicated) of mineralization that can be compared with a background stain picked up by the cell body.

Microarray Data Analysis

Secretory factor expression in chondrocytes was assessed to deduce the genes involved in the signal transduction between primary chondrocytes and BMSCs. When

the co-culture conditions between chondrocytes and BMSCs were kept constant throughout the course of the study, the osteogenic induction of BMSCs was found to be transient rather than sustained. Noticeable changes occur in the chondrocyte phenotype, and subsequently in its gene expression during the co-culture with BMSCs. Since chondrocytes are known to maintain their phenotype in three-dimensional scaffolds, we believe that any change in signal expression of these cells must owe its origin to a change in the phenotypic state induced by the local microenvironment. Therefore a comparative analysis of chondrocytes with and without co-culture influence by MSCs is surmised to yield a short list of genes specifically over-expressed as a result of this co-culture interaction. These differentially expressed genes could provide a pool of potential candidates secreted by chondrocytes that direct and sustain the differentiation of induced MSCs past their early osteogenic signal expression.

The analysis revealed 239 genes that registered a fold change of 5-fold or more over the absolute control. Table 4 provides an account of the number of genes that register high fold changes and the number of different gene annotations to which these genes are assigned. This gives an idea of the distribution of upregulated genes across the time periods of the study and the large of number of unique cellular location/function these genes are assigned to. Table 5 contains the genes shortlisted based on the criteria mentioned in the methods. Supplementary Table 1 (at the end of the document) contains the list of all genes that show significant expression on one or more time points of the study. The distribution is also seen in the hierarchial

clustering image in Figure 6. Upon validation of these genes, the following results were obtained.

		Fold change > 5	**Fold change > 6**	**Fold change > 8**
DAY 4	No of genes	202	80	17
	No of annotations	79	34	10
DAY 14	No of genes	75	17	0
	No of annotations	30	10	0
DAY 4 AND 14	No of genes	38	11	0
	No of annotations	14	6	0

Table 4. **Distribution of upregulated genes.** This table notes the distribution of genes across the time points. Most upregulation occurs at the earlier time point, Day 4. The wide range of annotations to which these genes belong denotes a systemic gene expression response across different cell functions and location of the corresponding genes within the local environment of the cell.

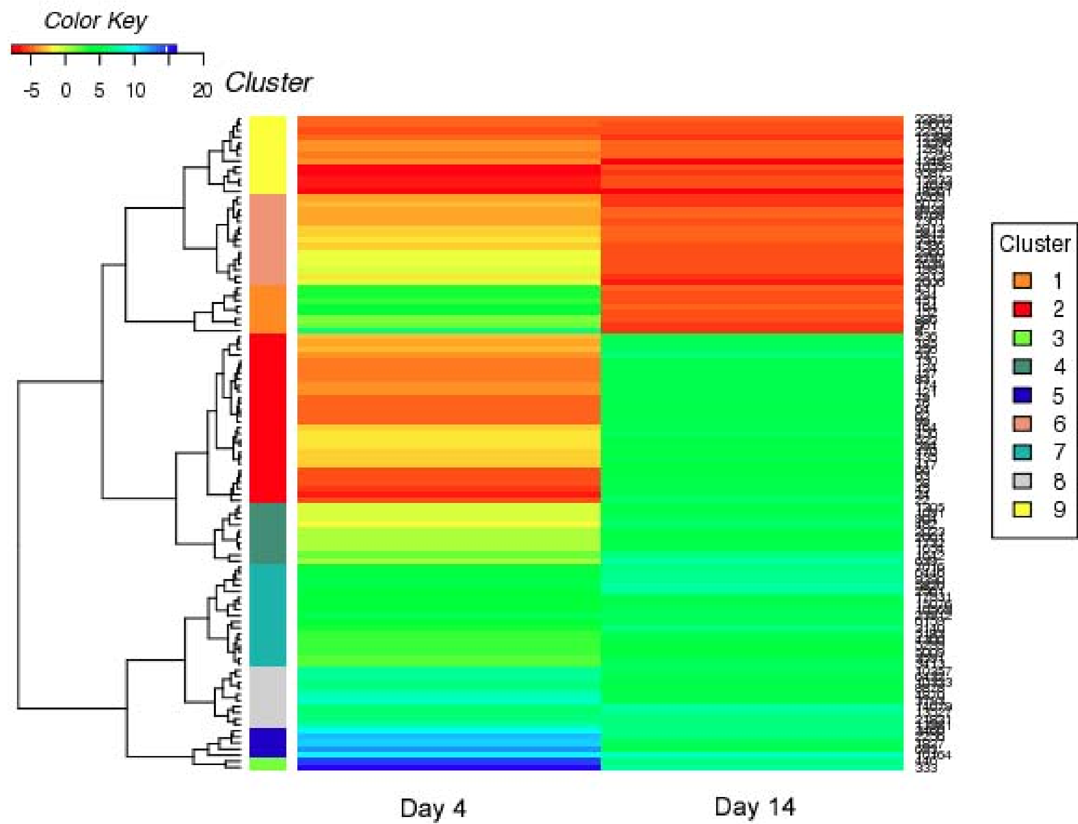

Figure 6. **Complete hierarchical clustering.** Shown here is the complete hierarchical clustering of 24,129 genes on Days 4 and 14. The samples represented in this heat map denote the expression levels of chondrocytes in co-culture with BMSCs on the two time points. The down-regulation in expression levels denotes a global change in gene expression profile.

	Gene Title	Gene Symbol	GO Process	GO Component
1	secreted frizzled-related protein 1	SFRP1	extracellular region	protein binding
2	versican	VCAN	extracellular region / proteinaceous extracellular matrix	calcium ion binding / sugar binding / hyaluronic acid binding
3	extracellular matrix protein 1	ECM1	extracellular region / extracellular space	extracellular matrix constituent
4	matrix metallopeptidase 13 (collagenase 3)	MMP13	extracellular region/ proteinaceous extracellular matrix	metalloendopeptidase activity/ calcium ion binding / peptidase activity / metallopeptidase activity / hydrolase activity /metal ion binding
5	collagen, type I, alpha 1	COL1A1	extracellular region / proteinaceous extracellular matrix	extracellular matrix structural constituent identical protein binding
6	collagen, type I, alpha 2	COL1A2	extracellular region / proteinaceous extracellular matrix	extracellular matrix structural constituent/ protein binding, bridging identical protein binding / SMAD binding

Table 5. **High fold change genes in the extracellular domain.** The genes shortlisted for further analysis, were referenced in the Gene Ontology database.

Extracellular matrix protein 1 (*ECM1*) registered an 18-fold rise on Day 14 over its value on Day 4 in the co-culture group. On Day 14, this value was significantly higher than the control group. Matrix metallopeptidase 13 (*MMP13*) showed a similar trend with a 20-fold over-expression on Day 14 in the co-culture group as compared to its level on Day 4. Matrix remodeling associated protein 8 (*MXRA8*) registered an increase in the expression levels over the time points. However upon validation, the elevated expression was noticed to be characteristic of both groups, and hence no discernable trend was observed. (Figure 7A-B)

Nidogen 1 (*NID1*) was observed to express significantly higher on Day 4 in the co-culture group. By the later time point, both groups registered elevated expression of the gene. Secreted frizzled related protein 1 (*SFRP1*) expressed a negative correlation in the expression of the two groups across the time points of the study. *SFRP1* registered a 3-fold higher expression in the control group as compared to the co-culture group on Day 4; however the trend reversed on Day 14. Also across time points, the co-culture group registered a 2-fold higher expression on Day 14 as compared to its level on Day 4, while the opposite trend was documented in the control group. Versican (*VCAN*) was noticed to have clear trend. On Day 4, the co-culture group reported nearly a 10-fold higher expression than the control group. By Day 14, the control group matched the expression levels of the co-culture group (Figure 7A-B).

Gene Title	fc.day 4 / day 0	fc.day14 / day 0
collagen, type I, alpha 1	**5.99**	**0.74**
collagen, type I, alpha 2	**10.82**	**6.84**
collagen, type II, alpha 1	**1.22**	**0.39**
collagen, type III, alpha 1	2.73	2.07
collagen, type IV, alpha 6	2.48	3.08
collagen, type VI, alpha 1	2.14	1.58
collagen, type IX, alpha 1	3.16	2.90
collagen, type X, alpha 1	**-3.52**	**-1.50**
collagen, type XI, alpha 1	3.54	3.32
collagen, type XXII, alpha 1	1.14	1.07
collagen, type XIII, alpha 1	0.11	0.31
collagen, type XVI, alpha 1	0.54	0.03
collagen, type XVIII, alpha 1	3.62	2.97
collagen, type XXVII, alpha 1	0.56	1.15

Table 6 : **Collagen expression profile for co-cultured chondrocytes.** Collagen expression levels changed across the time points indicating a possible shift in the nature and composition of the matrix surrounding these chondrocytes. All numbers represent the fold change of expression of the gene over its expression on Day 0 in the control group.

Gene Title	fc.day 4 / day 0	fc.day14 / day 0
matrix metallopeptidase 1 (interstitial collagenase)	3.61	1.00
matrix metallopeptidase 2 (gelatinase A)	3.87	1.72
matrix metallopeptidase 3 (progelatinase)	1.10	-0.13
matrix metallopeptidase 7 (matrilysin,)	-3.17	0.04
matrix metallopeptidase 9 (gelatinase B)	**4.24**	**-2.70**
matrix metallopeptidase 11 (stromelysin 3)	3.28	1.93
matrix metallopeptidase 13 (collagenase 3)	**5.96**	**-1.13**
matrix metallopeptidase 14 (membrane-inserted)	-2.16	-3.24
matrix metallopeptidase 15 (membrane-inserted)	0.36	-0.43
matrix metallopeptidase 16 (membrane-inserted)	0.54	0.55
Matrix metallopeptidase 17 (membrane-inserted)	-2.43	-0.55
Matrix metallopeptidase 19	-0.40	-0.63

Table 7. **Matrix metalloprotease(MMP) expression profile for co-cultured chondrocytes.** Some members of the MMP family are important cytokines involved in the osteogenic signal transduction and play a critical role in regulating osteogenic signal transduction and endochondral ossification. All numbers represent the fold change of expression of the gene over its expression on Day 0 in the control group.

Discussion

Bone marrow stromal cells contain a heterogeneous population comprising of a small pluripotent fraction[84,85]. The capacity of the subpopulation to proliferate when maintained under the culture conditions is evident as observed from the data (Fig 2A-C). We demonstrated that chondrocytes suspended in three dimensional alginate beads were able to induce osteogenic differentiation of BMSCs in monolayer via

secreted soluble proteins. As observed by evaluation of osteogenic gene expression of BMSCs, the extent of differentiation is comparable to the levels achieved by using the chemical concoction of dexamethasone and the phosphate substrate, Na-beta-glycerolphosphate[86]. A similar trend was noticed on evaluation of the secreted protein ALP that marks an early onset of osteogenic differentiation.

To further elucidate gene expression trends in chondrocytes and prospect information about specific genes that show selective expression in co-culture, we carried out a global gene expression analysis of chondrocytes cultures in three dimensional alginate beads, both in co-culture with BMSCs and in isolation. When comparing the relative expression of the experimental and control groups on a global scale on Days 4 and 14, there is a strong negative correlation suggesting a marked change in the gene expression profile across the time points. On closer examination, a number of genes register significant differential expression in one or more time points. Gene Ontology terms available in the public domain remain one of the strongest tools in bioinformatics to sort through large data sets such as the one in this case[87]. We were able to shortlist 8 genes of interest for our analysis by choosing genes that registered a positive five over-expression and subsequently using their GO terms to narrow down those genes that fall extracellular region or membrane region. Validation of microarray data is an important aspect of the study. It provides an opportunity to verify the results using an alternate method, where the parameters used (primer design, reaction time) are set by the user. Our validation of the genes of interest listed in Table 3 provided quantitative information that clearly delineates a marked phenotypic behavior of chondrocytes that are in co-culture with BMSCs. The specific

roles played by each of the 8 secreted factors identified in this study during the osteogenic differentiation of BMSCs fit into a larger regulatory apparatus that simultaneously modulates chondrocyte and osteoblasts maturation during endochondral ossification. Most differentiation processes are controlled by a combination of tightly regulated factors.

This mode of quantification however does not shed much light on the genes that are intrinsically expressed at high levels in both groups. Two such genes demonstrated significant fold changes in the microarray chip data and also exhibited high levels of expression during validation. Collagen Type 1, alpha 1 (*Col1A1*) and Collagen Type 1, alpha 2, (*Col1A2*) showed high levels of expression across all groups in both time points. Due to their biological relevance and their specific performance among other collagens, their role in the osteogenic differentiation process should be explored further to complete our understanding of the signal transduction.

In addition to mining for differential expression, the data yielded other trends that were interesting. We were able to generate reports on the Collagens and MMPs that are typically indicative of the stage of differentiation of mesenchymal cells (Table4, Table 5). On observing the collagen gene expression, we were able to document the general reduction in the expression of chondrocyte-specific genes such as Collagen II and Collagen X (respectively for pre-hypertrophic and hypertrophic chondrocytes) and the loss of expression of majority of the MMPs. This reduction in expression levels alludes to a marked change in chondrocytes phenotype[10,88,89].

During endochondral ossification, the cartilage template, comprising of chondrocytes at various stages of differentiation, calcifies under the influence of the expanding

osseous front and the recruited osteoblasts[12]. The enhanced expression of *MMP13* and type 1 collagen (*Col1A1*, *Col1A2*), suggests an osteoblastic phenotype adopted by the chondrocytes[75,90-92]. To further confirm this, we carried out a short PCR study to verify the expression of an early stage marker, *ALP,* and late stage marker, *OC*. Although *ALP* was not observed in the co-culture groups to any significant extent, osteocalcin was expressed significantly in co-cultured chondrocytes after 14 days of indirect contact with BMSCs. The expression of *OC* in the co-culture group was also found to be increasing throughout the time period of study (Figure 8). Although not discussed extensively in literature, the osteoblastic phenotype adopted by co-cultured chondrocytes can account for a shift in the secretory protein expression. The modified gene expression profile could in turn be the cause of the transient nature of its capacity to induce osteogenic differentiation of BMSCs. Expression of osteogenic genes in hypertrophic chondrocytes has been alluded to earlier in as resulting from regulatory effects exerted by retinoids in hypertrophic chondrocytes that results in expression of cartilage and osteoblasts specific genes. The sustained expression of *Col1A1* however is not typical of hypertrophic chondrocytes but rather osteoblasts in the late stage of differentiation. These trends suggest that co-culture of chondrocytes and BMSCs can trigger more than one pathway in the cell populations. By studying the expression of a more specific array of genes and their corresponding proteins can shed more light on the pathways that are upregulated. By further calibrating the

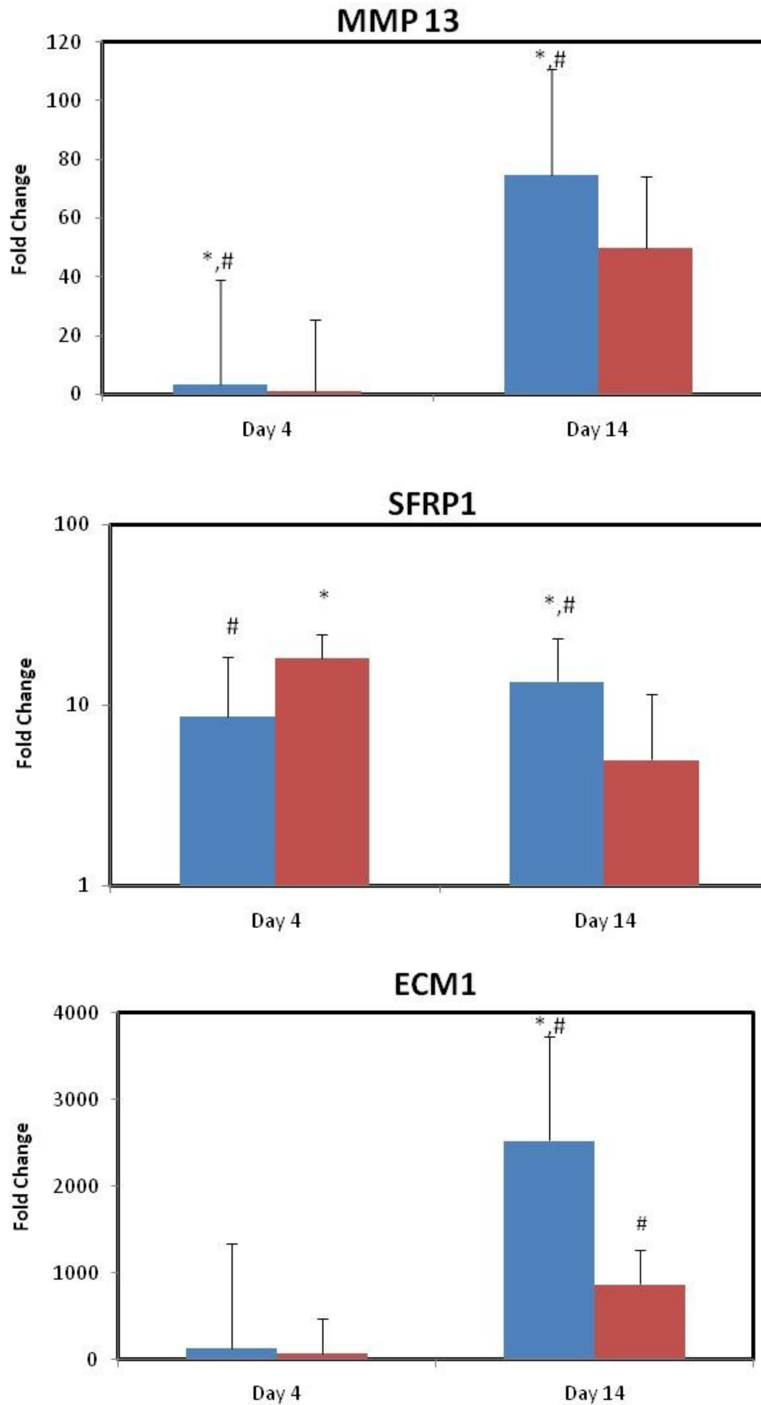

Figure 7A. **Validation of chondrocyte gene expression :** ***MMP13, SFRP1, ECM1*****.** All figures are represented as Mean ± Standard Deviation. * refers to significant different between two experimental groups within the same time point ($p<0.05$). # refers to significant difference in the expression value of a given group across time points.

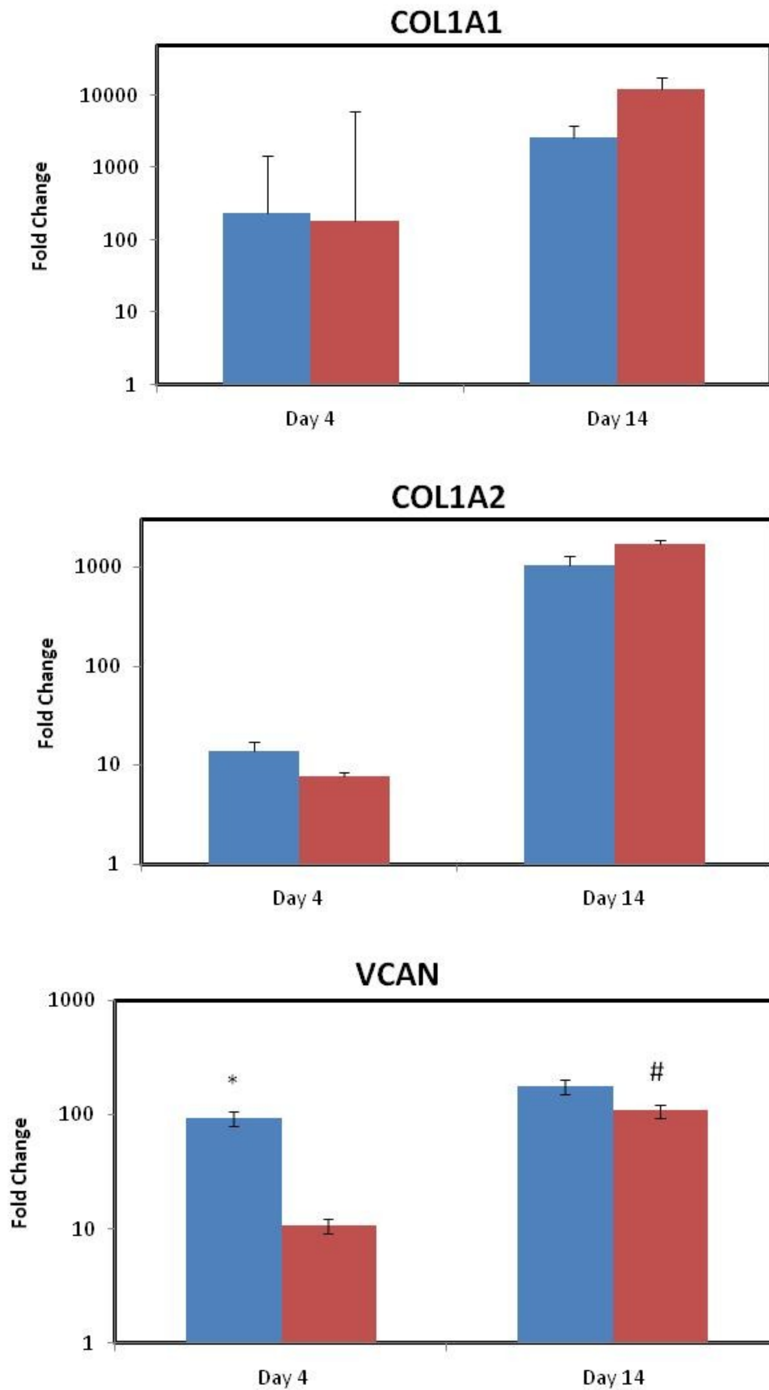

Figure 7B. **Validation of chondrocyte gene expression : Col1A1, Col1A2, VCAN.** All figures are represented as Mean ± Standard Deviation. * refers to significant different between two experimental groups within the same time point ($p<0.05$). # refers to significant difference in the expression value of a given group across time points.

culture conditions, these effects can be adopted to develop a strategy to drive differentiation of BMSCs in a more predictable manner.

Involvement of individual pathways in a certain differentiation system can be identified by tracking specific families of proteins. In the context of endochondral ossification, the expression of collagens and MMPs is significant and also aid in understanding the current stage of differentiation the chondrocytes population. The collagen and *MMP* profiles of chondrocytes suggest an elevated expression of *Col1*, *MMP9* and *MMP 13* on Day 4 of the co-culture and a sustained expression of *Col1* on Day 14. Earlier studies have suggested that hypertrophic chondrocytes exhibit an osteblastic gene expression. Further investigation into the downstream proteins of SOX9 and RUNX2 can suggest the dominance of one pathway over another or provide evidence of some alternative expression system in place. This is an interesting advantage of global expression assays, in that they reveal a wealth of information that strengthens our understanding of widely accepted and adopted differentiation protocols.

While this study focused on identifying differentially expressed factors, it must also be noted that a number of proteins are potent at small concentrations and the extent of expression of the corresponding gene can be low enough to be missed in the scheme of this study. Alternate approaches have been carried out to detect such factors by other groups involving proteomic and mass spectrometric analyses.

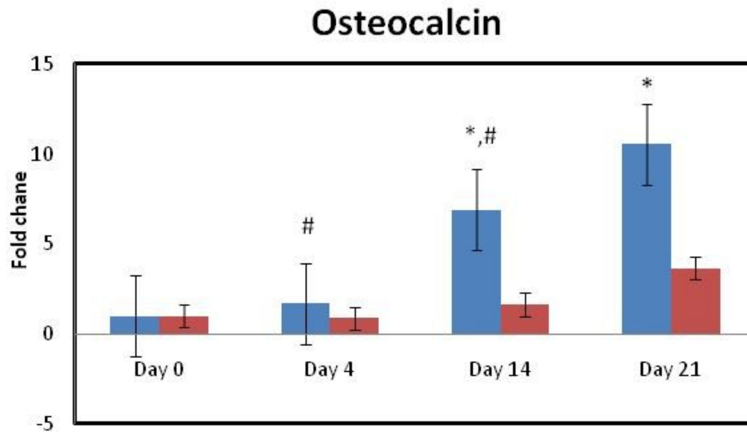

Figure 8. **Expression of osteogenic genes in chondrocytes.** All figures are represented as Mean ± Standard Deviation. * refers to significant different between two experimental groups within the same time point ($p<0.05$). # refers to significant difference in the expression value of a given group across time points.

Conclusion

This study confirms the ability of chondrocytes suspended in three dimensional scaffolds to induce osteogenic differentiation in bone marrow stromal cells. Furthermore, gene expression trends in chondrocytes can be clearly delineated by using microarray chips and the adopting a suitable technique of analyzing the data. This has yield useful insights into understanding the underlying secretory expression. We identified 6 genes of interest, *Col1A1, Col1A2, ECM1, MMP13, SFRP1, and VCAN,* that could potentially play an important role in the osteogenic differentiation of BMSCs as influenced by chondrocytes. Closer observation yields substantial insight into the transforming nature of chondrocytes as a result of co-culture, emulating expression trends exhibited during endochondral ossification. Further evaluation of these trends can aid in understanding the closely orchestrated developmental process from a molecular standpoint.

Chapter 4: Conclusion

A clear understanding of endochondral ossification provides the basis for efficient development of bone tissue engineering strategies. In order to emulate the sequence of events in a predictable manner a clear understanding of the limitations of each individual co-culture setup is important. The individual aspects of signaling such as population density, population size, and nature of signaling factors determine the type of co-culture adopted to study these schemes of communication. Indirect co-cultures between chondrocytes and bone marrow stromal cells cultured in distinct zones, provides a platform for studying secretory factor involvement in the process of endochondral ossification. Superimposing the physical attributes of this system with gene expression data generated by the microarray experiments, provides an analysis of the gene expression that codes for secretory proteins in chondrocytes. The timeline of osteogenic differentiation of BMSCs and the timeline of chondrocyte gene expression suggests a constant communication between the two populations that is necessary for the progression of the endochondral ossification. This work provides the primary basis of understanding chondrocyte gene expression change in a soluble co-culture system with mesenchymal stem cells. The results of the experiments provide a global scale understanding of chondrocyte function during this differentiation cascade and also provide specific information pertaining to select families of secretory molecules. Further validation of these results increases our understanding of the individual genes that code for secretory proteins.

The data also contains a depth of information that permits further evaluation of differentiation pathways under similar co-culture conditions. One of the advantages

of using the microarray platform is that the expression data is available across the entire genome that permits the verification of regulatory molecules that are generally more complicated to estimate by other immunological methods. Meta-analysis of microarray data in the context of tissue engineering can cast light on the regulation of entire pathways, aiding the current understanding of differentiation. These techniques are widely applied in cancer research to study the involvement of regulatory molecules in metastasis. Also, histological analysis of sections can provide data on spatial and temporal shift in the matrix composition of molecules during the differentiation. Although histological studies have been carried out in many contexts, there is still a need to correlate the molecular changes in the cells with the changes in the matrix to understand the timeframes involved in *in vitro* bone tissue formation. A clear understanding of the progression of molecular differentiation and matrix remodeling can help not only strategy development but also evaluation of new techniques in when adopted in clinical trials. A majority of current strategies rely on tracking changes in certain key markers that may provide a snapshot of the current differentiation state of the entire population. However, these correlations between molecular drivers and matrix components can be more relevant and platform-independent in determining the state of the developing tissue and the process of understanding regulatory networks can aid in developing such molecular scoring schemes that define and demonstrate tissue formation.

Appendices

Supplementary Table 1. **GO Terms of genes with > 5 fold change**. Based on the method of interpretation of data, the genes that encode matrix factors or secretory proteins significantly in one or more experimental group are listed in this table. NCBI index refers to the unique number designated by the National Center for Biotechnology Information to each gene. The primer sequences were designed using the Applied Biosystems SDS software by utilizing the gene sequences available in literature against the NCBI indices mentioned. *: GAPDH was used as an endogenous control and is not considered as a gene exhibiting differential expression.

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
1	serpin peptidase inhibitor, clade	SERPINF1	extracellular region	serine-type endopeptidase inhibitor activity	11.82	4.22
2	collagen, type I, alpha 2	COL1A2	extracellular region	extracellular matrix structural constituent	10.82	6.84
3	S100 calcium binding protein A9	S100A9	cytoplasm	calcium ion binding	10.52	3.98
4	collagen, type I, alpha 2	COL1A2	extracellular region	extracellular matrix structural constituent	9.6	6.74
5	complement component 3	C3	extracellular region	endopeptidase inhibitor activity	9.5	0.75
6	butyrylcholinesterase	BCHE	nuclear envelope lumen	carboxylesterase activity	9.19	7.33
7	asporin	ASPN	extracellular region	protein binding	9.18	7.82
8	speckle-type POZ protein	SPOP	nucleus	protein binding	9.08	3.59
9	haptoglobin	HP	extracellular region	catalytic activity	9.03	2.29
10	Apolipoprotein D	APOD	extracellular region	transporter activity	9.01	6.62
11	similar to TLH29 protein precursor	ISG12	---	---	8.61	4.5

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
12	histamine N-methyltransferase	HNMT	cytoplasm	methyltransferase activity	8.53	1.79
13	ependymin related protein 1 (zebrafish)	EPDR1	extracellular region	calcium ion binding	8.48	5.83
14	complement component 2	C2	extracellular region	catalytic activity	8.36	4.2
15	collagen, type I, alpha 1	COL1A1	extracellular region	extracellular matrix structural constituent	8.36	2.56
16	lumican	LUM	extracellular region	protein binding	8.2	6.07
17	chloride channel accessory 2	CLCA2	---	---	7.97	5.85
18	Sterile alpha motif domain containing 9	SAMD9	---	---	7.91	5.18
19	phospholipase A1 member A	PLA1A	extracellular region	catalytic activity	7.6	3.76
20	transmembrane protein 176A	TMEM176A	membrane	---	7.31	-0.49
21	chemokine (C-X-C motif) ligand 12	CXCL12	extracellular region	cytokine activity	7.17	1.69
22	alanine-glyoxylate aminotransferase 2	AGXT2L1	mitochondrion	catalytic activity	7.14	3.54
23	secreted frizzled-related protein 4	SFRP4	extracellular region	protein binding	7.06	0.49
24	periostin, osteoblast specific factor	POSTN	proteinaceous extracellular matrix	heparin binding	7.04	4.88
25	lysozyme C-2	LYZ2	---	lysozyme activity	7.02	4.4
26	complement factor I	CFI	membrane	catalytic activity	6.97	3.38

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
27	aspartoacylase (Canavan disease)	ASPA	nucleus	zinc ion binding	6.96	1.41
28	transcription elongation factor A (SII), 3	TCEA3	nucleus	nucleic acid binding	6.84	5.66
29	nidogen 1	NID1	proteinaceous extracellular matrix	calcium ion binding	6.8	2.71
30	extracellular matrix protein 1	ECM1	extracellular region	---	6.71	2.29
31	secreted frizzled-related protein 2	SFRP2	extracellular region	protein binding	6.7	0.1
32	keratocan	KERA	extracellular region	protein binding	6.68	2.45
33	claudin 11	CLDN11	plasma membrane	structural molecule activity	6.67	1.14
34	alpha-1 acid glycoprotein	AGP	extracellular region	binding	6.65	-0.52
35	angiogenin, ribonuclease, RNase A family	ANG /// RNASE4	extracellular region	nucleic acid binding	6.58	3.78
36	insulin-like growth factor binding protein 2	IGFBP2	extracellular region	insulin-like growth factor binding	6.52	0.74
37	matrix metallopeptidase 13 (collagenase 3)	MMP13	extracellular region	metalloendopeptidase activity	6.48	-1.23
38	similar to mKIAA1077 protein	LOC535166	---	---	6.45	3.46
39	G protein-coupled receptor 88	GPR88	integral to membrane	---	6.43	6.14
40	hypothetical protein LOC780785	C3orf57	---	---	6.41	3.28
41	protease, serine, 12 (neurotrypsin)	PRSS12	membrane	catalytic activity	6.4	1.89

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
42	major histocompatibility complex,	BOLA-DMB	membrane	---	6.39	4.39
43	apolipoprotein B mRNA editing enzyme,	APOBEC3B	---	catalytic activity	6.39	3.89
44	IgM	LOC444876	---	---	6.31	5.89
45	Acyl-CoA synthetase family member 3	ACSS3	mitochondrion	catalytic activity	6.28	3.38
46	ceruloplasmin (ferroxidase)	CP	extracellular space	ferroxidase activity	6.24	3.22
47	chemokine (C-C motif) ligand 2	CCL2	extracellular region	G-protein-coupled receptor binding	6.23	-0.81
48	ceruloplasmin (ferroxidase)	CP	extracellular space	ferroxidase activity	6.21	2.9
49	interleukin 12A	IL12A	extracellular region	cytokine activity	6.19	2.82
50	angiopoietin 1	ANGPT1	extracellular region	receptor binding	6.18	4.08
51	SEH1-like (S. cerevisiae)	SEH1L	---	---	6.15	1.52
52	killer cell lectin-like receptor subfamily B,	KLRB1	---	receptor activity	6.14	1.65
53	ABI gene family, member 3 (NESH) binding	ABI3BP	---	---	6.14	3.23
54	Alpha-galactosyltransferase 1	GGTA1	Golgi apparatus	transferase activity	6.13	4.09
55	fibrinogen-like 2	FGL2	extracellular region	receptor binding	6.1	2.16
56	PYD and CARD domain containing	PYCARD	extracellular region	cysteine-type endopeptidase activity	6.1	4.63

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
57	Putative hydroxypyruvate isomerase	LOC515954	---	---	6.1	4.83
58	apolipoprotein A-I	APOA1	extracellular region	lipid transporter activity	6.09	5.8
59	collagen, type XI, alpha 1	COL11A1	extracellular region	structural molecule activity	6	5.5
60	collagen, type I, alpha 1	COL1A1	extracellular region	extracellular matrix structural constituent	5.99	0.74
61	glial fibrillary acidic protein	GFAP	cytoplasm	structural molecule activity	5.98	5.2
62	GABA A receptor, alpha 1	GABRA1	plasma membrane	GABA-A receptor activity	5.97	3.79
63	interleukin 2 receptor, alpha	IL2RA	membrane	receptor activity	5.97	2.84
64	Matrix metallopeptidase 13 (collagenase 3)	MMP13	extracellular region	metalloendopeptidase activity	5.96	-1.13
65	phospholipid transfer protein	PLTP	---	lipid binding	5.93	-1.06
66	collagen, type XI, alpha 1	COL11A1	extracellular region	structural molecule activity	5.92	5.96
67	complement component 3	C3	extracellular region	endopeptidase inhibitor activity	5.86	0.86
68	S100 calcium binding protein A8	S100A8	cytoplasm	calcium ion binding	5.85	1.7
69	alpha-galactosyltransferase 1	GGTA1	Golgi apparatus	transferase activity	5.83	3.44
70	complement component 1, r subcomponent	C1R	---	catalytic activity	5.82	1.74
71	selenoprotein P, plasma, 1	SEPP1	extracellular region	selenium binding	5.81	1.17

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
72	apolipoprotein B	APOB	---	lipid transporter activity	5.8	1.26
73	aldo-keto reductase family 1, member C4	AKR1C4	cytoplasm	oxidoreductase activity	5.8	-0.01
74	chemokine (C-C motif) ligand 2	CCL2	extracellular region	G-protein-coupled receptor binding	5.8	-2.15
75	collagen, type XI, alpha 1	COL11A1	extracellular region	structural molecule activity	5.78	6.01
76	lipopolysaccharide binding protein	LBP	extracellular region	lipopolysaccharide binding	5.78	1.9
77	fibronectin type III domain containing 1	FNDC1	cytoplasm	nucleotide binding	5.74	2.12
78	TIMP metallopeptidase inhibitor 4	TIMP4	extracellular region	enzyme inhibitor activity	5.72	4.48
79	Regulated endocrine-specific protein 18	RESP18	extracellular region	---	5.71	1.51
80	SAM domain, SH3 domain	SAMSN1	---	---	5.7	3.69
81	protease, serine, 35	PRSS35	extracellular region	catalytic activity	5.7	2.6
82	T cell receptor, alpha	TRA@	---	receptor activity	5.68	0.85
83	transketolase-like 2	TKTL2	cytoplasm	catalytic activity	5.67	5.85
84	prostaglandin F receptor (FP)	PTGFR	plasma membrane	signal transducer activity	5.66	3.07
85	complement factor B	CFB	extracellular region	catalytic activity	5.63	2.1
86	homer homolog 2 (Drosophila)	HOMER2	cytoplasm	actin binding	5.54	3.67

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
87	laminin, beta 1	LAMB1	basement membrane	extracellular matrix structural constituent	5.53	2.73
88	Potassium conductance calcium-activated channel	KCNMB 1	voltage-gated potassium channel complex	ion channel activity	5.51	1.71
89	Solute carrier family 10	SLC10A 1	membrane fraction	bile acid:sodium symporter activity	5.5	4.87
90	myosin IB	MYO1B	myosin complex	nucleotide binding	5.46	4.62
91	Septin 14	SE14	septin complex	nucleotide binding	5.45	4.02
92	G-protein signaling modulator 2	GPSM2	---	---	5.45	4.75
93	pregnancy-associated glycoprotein 14	PAG14	---	aspartic-type endopeptidase activity	5.45	4.37
94	G protein-coupled receptor 173	GPR173	cytoplasm	signal transducer activity	5.43	3.89
95	tumor necrosis factor (ligand) superfamily,	TNFSF1 3	membrane	tumor necrosis factor receptor binding	5.42	4.62
96	Malate dehydrogenase 1B, NAD (soluble)	MDH1B	---	catalytic activity	5.4	3.21
97	Tripartite motif-containing 54	TRIM54	intracellular	protein binding	5.4	5.78
98	G protein-coupled receptor 92	GPR92	plasma membrane	signal transducer activity	5.38	1.95
99	Parkinson disease 7	PARK7	nucleus	protein binding	5.38	4.5
100	crystallin, gamma S	CRYGS	---	structural constituent of eye lens	5.37	3.28
101	sialic acid binding Ig-like lectin 1,	SIGLEC 1	---	---	5.37	3.94

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
102	sema domain, transmembrane domain	SEMA6D	membrane	receptor activity	5.33	3.91
103	receptor activity modifying protein 3	RAMP3	integral to membrane	protein transporter activity	5.32	1.05
104	transmembrane protein 176B	TMEM176B	---	---	5.3	0.65
105	prostaglandin D2 synthase 21kDa (brain)	PTGDS	extracellular region	prostaglandin-D synthase activity	5.28	0.59
106	colony stimulating factor 2	CSF2	extracellular region	cytokine activity	5.27	4.93
107	Similar to MAM domain containing 2	LOC539199	membrane	---	5.26	3.57
108	versican	VCAN	extracellular region	binding	5.26	2.43
109	dickkopf homolog 3 (Xenopus laevis)	DKK3	extracellular region	---	5.24	2.72
110	Protein kinase, AMP-activated, alpha 2	PRKAA2	---	protein serine	5.2	4.25
111	FYVE, RhoGEF and PH domain containing 4	FGD4	intracellular	Rho guanyl-nucleotide exchange factor activity	5.15	4.36
112	grancalcin, EF-hand calcium binding protein	GCA	---	calcium ion binding	5.14	4.3
113	Adenosine deaminase, RNA-specific	ADAR	intracellular	double-stranded RNA binding	5.14	0.25
114	Interferon-induced protein 44	IFI44	---	---	5.13	2.58
115	angiotensin II receptor, type 2	AGTR2	integral to membrane	signal transducer activity	5.13	4.62
116	similar to HEPH	LOC510736	---	copper ion binding	5.12	4.49

No	Gene Title	Gene Symbol	GO Process	GO Component	Day 4 FC	Day 14 FC
117	neurexin 1	NRXN1	membrane	calcium ion binding	5.11	4.92
118	UDP glycosyltransferase 8	UGT8	---	transferase activity	5.09	2.09
119	casein alpha s1	CSN1S1	extracellular region	transporter activity	5.09	4.35
120	S100 calcium binding protein A12	S100A12	---	calcium ion binding	5.09	1.89
121	olfactomedin-like 3	OLFML3	extracellular region	---	5.07	3.94
122	MHC class I antigen	BOLA-NC1	membrane	---	5.07	1.45
123	angiogenin, ribonuclease, RNase A family, 5	ANG	extracellular region	nucleic acid binding	5.05	2.4
124	dipeptidyl-peptidase 4	DPP4	extracellular region	aminopeptidase activity	5.05	3.32
125	BEN domain containing 5	BEND5	Golgi apparatus	---	5.02	1.6
126	cytokeratin 19	LOC514812	intermediate filament	structural molecule activity	5.02	0.69

Supplementary Table 2. **High fold change genes in the extracellular domain.** The genes shortlisted for further analysis were referenced in the National Center for Biotechnology Information (NCBI) index and based on the reported gene sequence; the primers were designed using the Primer Express Software supplied by Applied Biosystems.

No	Gene	NCBI Reference	Gene Name	Primers
1	Col1A1	NM_001034039	collagen, type I, alpha 1	Forward Primer : CATGACCGAGACGTGTGGAA Reverse Primer : TTGCCGTTGTCGCAGACA
2	Col1A2	NM_174520	collagen, type I, alpha 2	Forward Primer : CAGTCAAGAACTGGTACAGAAATTCC Reverse Primer : GGTACCACCGTTGATAGTTTCTCCTA
3	ECM1	NM_001099706	extracellular matrix protein 1	Forward Primer : CGCACAAACCGCTTGGA Reverse Primer : AGAACCGGGTCACTGAGTCTTC
4	MMP13	NM_174389	matrix metallopeptidase 13	Forward Primer : TCCGCGGAGAAACACTGATC Reverse Primer : TTCAACCTGCTGAGGATGCA
5	MXRA8	NM_001075830	matrix-remodelling associated 8	Forward Primer : CCTGCTCTGGAGACTTGTGCTT Reverse Primer : CCTGATGGCCCTGAAGACA
6	NID1	NM_001101155	nidogen 1	Forward Primer : CTTCCACCCCCACAAGCA Reverse Primer : TGAGACAGGGCGGAAGTGA
7	SFRP1	NM_174460	secreted frizzled-related protein 1	Forward Primer : CGTGCGAGCCGGTCAT Reverse Primer : TCGGGAAACTTGTCGCACTT
8	VCAN	NM_181035	versican	Forward Primer : AGCTGCATGCCGCCTATG Reverse Primer : TCCGTAGGTCCGGACTCCTT

Bibliography

1 Frank Barry, R. E. B., Beishan Liu, J. Mary Murphy. Chondrogenic Differentiation of Mesenchymal Stem Cells from Bone Marrow: Differentiation-Dependent Gene Expression of Matrix Components *Journal of Cellular Biochemistry* **79**, 89-102 (2000).

2 Sanchez, C., Deberg, Michelle,Piccardi, Nathalie,Msika, Philippe,Reginster, Jean-Yves,Henrotin, Yves. Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes *Osteoarthritis Cartilage* **13**, 988-997 (2005).

3 Grassel S, A. N. Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation. *Frontiers in Bioscience* **12**, 4946-4956 (2007).

4 Spalazzi JP, D. K., Jiang J, Lu HH. Osteoblast and chondrocyte interactions during coculture on scaffolds. *IEEE Eng Med Biol Mag* **22**, 27-34 (2003).

5 Jeanine Hendriks, J. R., Clemens A. van Blitterswijk. Co-culture in cartilage tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine* **1**, 170-178 (2007).

6 Kohei Tsuchiya, G. C., Takashi Ushida, & Takeo Matsuno, T. T. The effect of coculture of chondrocytes with mesenchymal stem cells on their cartilaginous phenotype in vitro. *material science and engineering C* **24**, 391-396 (2004).

7 Hoemann, C. D., El-Gabalawy, H. & Mckee, M. D. In vitro osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathologie Biologie* **57**, 318-323, doi:Doi 10.1016/J.Patbio.2008.06.004 (2009).

8 Mary B. Goldring, K. T., Kosei Ijiri. The Control of Chondrogenesis. *Journal of Cellular Biochemistry* **97**, 33-44 (2006).

9 Mutsumi Takagi, T. K., Satoru Koizumi, Haruka Hirose, , Shin-ichi Kondo, M. F., Kosei Ueno,Misawa Hiroaki, & Yoichiroh Hosokawa, H. M., Shigeyuki Wakitani. Correlation between cell morphology and aggrecan gene expression level during differentiation from mesenchymal stem cells to chondrocytes *Biotechnology Letters* **30**, 1189-1195 (2008).

10 Boskey, A. L., Stiner, D., Binderman, I. & Doty, S. B. Type I collagen influences cartilage calcification: An immunoblocking study in differentiating chick limb-bud mesenchymal cell cultures. *Journal of Cellular Biochemistry* **79**, 89-102 (2000).

11 Heng BC, C. T., Lee EH. Directing stem cell differentiation into the chondrogenic lineage in vitro. *Stem Cells* **22**, 1152-1167 (2004).

12 Nathalie Ortega, D. J. B., Zena Werb. Matrix remodeling during endochondral ossification. *Trends in Cell Biology* **14**, 86-93 (2004).

13 James, C. G. *et al.* Genome-Wide Analyses of Gene Expression during Mouse Endochondral Ossification. *PLoS One* **5**, -, doi:ARTN e8693
DOI 10.1371/journal.pone.0008693 (2010).

14 Colnot, C. Cellular and molecular interactions regulating skeletogenesis. *Journal of Cellular Biochemistry* **95**, 688-697 (2005).

15 M. N. Kozhevnikova , A. S. M. a. V. I. S. Molecular and genetic regulation of osteogenic differentiation of mesenchymal stromal cells. *Biology Bulletin* **35**, 223-232 (2008).

16 Sophie Lacombe-Gleize, M. G., Sylvie Demignot, Christiane Hecquet and Monique Adolphe. Implication of TGFβ1 in co-culture of chondrocytes-osteoblasts. *In Vitro Cellular & Developmental Biology - Animal* **31**, 649-652 (1995).

17 Thompson, A. D., Betz, M. W., Yoon, D. M. & Fisher, J. P. Osteogenic Differentiation of Bone Marrow Stromal Cells Induced by Coculture with Chondrocytes Encapsulated in Three-Dimensional Matrices. *Tissue Engineering Part A* **15**, 1181-1190, doi:Doi 10.1089/Ten.Tea.2007.0275 (2009).

18 Gertensfeld. Osteogenic Differentiation is Selectively Promoted by Morphogenetic Signals from Chondrocytes and Synergized by a Nutrient Rich Growth Environment. *Connective Tissue Research* (2003).

19 Gertensfeld. Chondrocytes Provide Morphogenic Signals That Selectively Induce Osteogenic Differentiation of Mesenchymal Stem Cells. *JBMR* (2002).

20 Handschel. Prospects of micromass culture technology in tissue engineering. (2007).

21 UNA CHEN, M. K. Differentiation of Mouse Embryonic Stem Cells In Vitro: 111. Morphological Evaluation of Tissues Developed After Implantation of Differentiated Mouse Embryoid Bodies *Developmental Dynamics* **197**, 217-226 (1993).

22 Hwang, N. S., Varghese, S., Puleo, C., Zhang, Z. & Elisseeff, J. Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells. *J Cell Physiol* **212**, 281-284, doi:10.1002/jcp.21052 (2007).

23 Jiang, J., Nicoll, S. B. & Lu, H. H. Co-culture of osteoblasts and chondrocytes modulates cellular differentiation in vitro. *Biochem Biophys Res Commun* **338**, 762-770, doi:S0006-291X(05)02238-2 [pii]
10.1016/j.bbrc.2005.10.025 (2005).

24 Stanton, L. A., Sabari, S., Sampaio, A. V., Underhill, T. M. & Beier, F. p38 MAP kinase signalling is required for hypertrophic chondrocyte differentiation. *Biochem J* **378**, 53-62, doi:10.1042/BJ20030874
BJ20030874 [pii] (2004).

25 Woods, A. & Beier, F. RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. *J Biol Chem* **281**, 13134-13140, doi:M509433200 [pii]
10.1074/jbc.M509433200 (2006).

26 Ghosh, P. *et al.* Pentosan polysulfate promotes proliferation and chondrogenic differentiation of adult human bone marrow-derived mesenchymal precursor cells. *Arthritis Res Ther* **12**, R28, doi:ar2935 [pii] 10.1186/ar2935 (2010).

27 Kumar, D. & Lassar, A. B. The transcriptional activity of Sox9 in chondrocytes is regulated by RhoA signaling and actin polymerization. *Mol Cell Biol* **29**, 4262-4273, doi:MCB.01779-08 [pii] 10.1128/MCB.01779-08 (2009).

28 Steinert, A. *et al.* Chondrogenic differentiation of mesenchymal progenitor cells encapsulated in ultrahigh-viscosity alginate. *J Orthop Res* **21**, 1090-1097, doi:S0736026603001001 [pii] 10.1016/S0736-0266(03)00100-1 (2003).

29 Helms, C. I. C. a. J. A. A molecular analysis of matrix remodeling and angiogenesis during long bone development. *Mechanisms of Development* **100**, 245-250 (2001).

30 Renny Franceschi, D. J. Transcriptional Regulation of Osteoblasts. *Ann N Y Acad Sci.* **1116**, 196-207 (2007).

31 Gerstenfeld, L. C. *et al.* Chondrocytes provide morphogenic signals that selectively induce osteogenic differentiation of mesenchymal stem cells. *J Bone Miner Res* **17**, 221-230, doi:10.1359/jbmr.2002.17.2.221 (2002).

32 Wei, A. *et al.* Differentiation of rodent bone marrow mesenchymal stem cells into intervertebral disc-like cells following coculture with rat disc tissue. *Tissue Eng Part A* **15**, 2581-2595, doi:10.1089/ten.TEA.2008.0458 10.1089/ten.TEA.2008.0458 [pii] (2009).

33 Sophie Lacombe-Gleize, M. G., Sylvie Demignot, Christiane Hecquet, & Adolphe, M. Implication of TGFβ1 in Co-Culture of Chondrocytes-Osteoblasts. *In Vitro Cellular & Developmental Biology. Animal* **31**, 649-652 (1995).

34 Grassel, S. *et al.* Coculture between periosteal explants and articular chondrocytes induces expression of TGF-beta1 and collagen I. *Rheumatology (Oxford)* **49**, 218-230, doi:kep326 [pii] 10.1093/rheumatology/kep326 (2010).

35 Zuacke, F. Cartilage Oligomeric Matrix Component (COMP) and Collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes. *Biochemistry* **358**, 17-24 (2001).

36 Rai, M. F. *et al.* Molecular and phenotypic modulations of primary and immortalized canine chondrocytes in different culture systems. *Res Vet Sci* **87**, 399-407, doi:S0034-5288(09)00090-3 [pii] 10.1016/j.rvsc.2009.04.008 (2009).

37 MF, R. Molecular and phenotypic modulations of primary and immortalized canine chondrocytes in different culture systems. *Research in Veterinary Science* **87**, 399 (2009).

38 Hwang, N. S. *et al.* Effects of three-dimensional culture and growth factors on the chondrogenic differentiation of murine embryonic stem cells. *Stem Cells* **24**, 284-291, doi:2005-0024 [pii] 10.1634/stemcells.2005-0024 (2006).

39 M. Grellier, L. B., and J. Amédée. Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. *Trends in Biotechnology* **27**, 562-571 (2009).
40 Cancedda, R., Castagnola, P., Cancedda, F. D., Dozin, B. & Quarto, R. Developmental control of chondrogenesis and osteogenesis. *International Journal of Developmental Biology* **44**, 707-714 (2000).
41 Nakaoka R, H. S., Mooney DJ. Regulation of chondrocyte differentiation level via co-culture with osteoblasts. *Tissue Eng* **12**, 2425-2433 (2006).
42 Ilmer M, K. M., Geissler C, Jochum M, Neth P. Human osteoblast-derived factors induce early osteogenic markers in human mesenchymal stem cells. *Tissue Eng Part A* **15**, 2397-2409 (2009).
43 Frank Barry, R. E. B., Beishan Liu, J. Mary Murphy. Chondrogenic Differentiation of Mesenchymal Stem Cells from Bone Marrow: Differentiation-Dependent Gene Expression of Matrix Components. *Experimental Cell Research* **268**, 189-200 (2002).
44 Granchi D, O. G., Leonardi E, Devescovi V, Baglìo SR, Osaba L, Baldini N, Ciapetti G. GENE EXPRESSION PATTERNS RELATED TO OSTEOGENIC DIFFERENTIATION OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS DURING EX VIVO EXPANSION. *Tissue Eng Part C Method* (2009).
45 Thompson AD, B. M., Yoon DM, Fisher JP. Osteogenic differentiation of bone marrow stromal cells induced by coculture with chondrocytes encapsulated in three-dimensional matrices. *Tissue Eng Part A* **15**, 1181-1190 (2009).
46 Artur Lichtenberg, G. D., Thorsten Walles, Michael Maringka, & Stefanie Ringes-Lichtenberg, A. R., Heike Mertsching, Axel Haverich. A multifunctional bioreactor for three-dimensional cell (co)-culture. *Biomaterials* **26**, 555-562 (2005).
47 M. Adelaide Asnaghi, P. J., Manuela T. Raimondi, Sally C. Dickinson, , Louisa E.N. Rees , T. G., Tristan A. Cogan, Amanda Dodson, Pier Paolo Parnigotto, , Anthony P. Hollander , M. A. B., Maria Teresa Conconi, Paolo Macchiarini, & Mantero, S. A double-chamber rotating bioreactor for the development of tissue-engineered hollow organs: From concept to clinical trial. *Biomaterials* **30**, 5260-5269 (2009).
48 Botchwey, E. A., Pollack, S. R., Levine, E. M. & Laurencin, C. T. Bone tissue engineering in a rotating bioreactor using a microcarrier matrix system. *J Biomed Mater Res* **55**, 242-253, doi:10.1002/1097-4636(200105)55:2<242::AID-JBM1011>3.0.CO;2-D [pii] (2001).
49 Williams, C. & Wick, T. M. Endothelial cell-smooth muscle cell co-culture in a perfusion bioreactor system. *Annals of Biomedical Engineering* **33**, 920-928, doi:Doi 10.1007/S10439-005-3238-0 (2005).
50 Inanc, B., Elcin, A. E. & Elcin, Y. M. Osteogenic induction of human periodontal ligament fibroblasts under two- and three-dimensional culture conditions. *Tissue Eng* **12**, 257-266, doi:10.1089/ten.2006.12.257 (2006).
51 Jin, Q. M. *et al.* Cementum engineering with three-dimensional polymer scaffolds. *J Biomed Mater Res A* **67**, 54-60, doi:10.1002/jbm.a.10058 (2003).

52 Duffy, G. P., Ahsan, T., O'Brien, T., Barry, F. & Nerem, R. M. Bone marrow-derived mesenchymal stem cells promote angiogenic processes in a time- and dose-dependent manner in vitro. *Tissue Eng Part A* **15**, 2459-2470, doi:10.1089/ten.TEA.2008.0341 (2009).

53 Goldstein, A. S., Juarez, T. M., Helmke, C. D., Gustin, M. C. & Mikos, A. G. Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. *Biomaterials* **22**, 1279-1288, doi:S0142961200002805 [pii] (2001).

54 Stiehler, M. *et al.* Effect of dynamic 3-D culture on proliferation, distribution, and osteogenic differentiation of human mesenchymal stem cells. *J Biomed Mater Res A* **89**, 96-107, doi:10.1002/jbm.a.31967 (2009).

55 Gomes, M. E., Sikavitsas, V. I., Behravesh, E., Reis, R. L. & Mikos, A. G. Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds. *J Biomed Mater Res A* **67**, 87-95, doi:10.1002/jbm.a.10075 (2003).

56 Holtorf, H. L., Jansen, J. A. & Mikos, A. G. Flow perfusion culture induces the osteoblastic differentiation of marrow stroma cell-scaffold constructs in the absence of dexamethasone. *J Biomed Mater Res A* **72**, 326-334, doi:10.1002/jbm.a.30251 (2005).

57 Zeng, Y., Lee, T. S., Yu, P. & Low, H. T. Numerical simulation of mass transport in a microchannel bioreactor with cell micropatterning. *J Biomech Eng* **130**, 031018, doi:10.1115/1.2913231 (2008).

58 Yu, X., Botchwey, E. A., Levine, E. M., Pollack, S. R. & Laurencin, C. T. Bioreactor-based bone tissue engineering: the influence of dynamic flow on osteoblast phenotypic expression and matrix mineralization. *Proc Natl Acad Sci U S A* **101**, 11203-11208, doi:10.1073/pnas.0402532101
0402532101 [pii] (2004).

59 Chen, X., Xu, H., Wan, C., McCaigue, M. & Li, G. Bioreactor expansion of human adult bone marrow-derived mesenchymal stem cells. *Stem Cells* **24**, 2052-2059, doi:2005-0591 [pii]
10.1634/stemcells.2005-0591 (2006).

60 Jing, D. *et al.* Hematopoietic stem cells in co-culture with mesenchymal stromal cells--modeling the niche compartments in vitro. *Haematologica* **95**, 542-550, doi:haematol.2009.010736 [pii]
10.3324/haematol.2009.010736 (2010).

61 Sikavitsas, V. I., Temenoff, J. S. & Mikos, A. G. Biomaterials and bone mechanotransduction. *Biomaterials* **22**, 2581-2593, doi:S0142961201000023 [pii] (2001).

62 Rubin, J., Rubin, C. & Jacobs, C. R. Molecular pathways mediating mechanical signaling in bone. *Gene* **367**, 1-16, doi:S0378-1119(05)00601-3 [pii]
10.1016/j.gene.2005.10.028 (2006).

63 Bidwell, J. P., Alvarez, M., Feister, H., Onyia, J. & Hock, J. Nuclear matrix proteins and osteoblast gene expression. *J Bone Miner Res* **13**, 155-167, doi:10.1359/jbmr.1998.13.2.155 (1998).

64 S. N. BHATIA, U. J. B., M. L. YARMUSH, M. TONER. Effect of cell–cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells *The FASEB Journal* **13**, 1883-1900 (1999).

65 Moon, J. J., Hahn, M. S., Kim, I., Nsiah, B. A. & West, J. L. Micropatterning of poly(ethylene glycol) diacrylate hydrogels with biomolecules to regulate and guide endothelial morphogenesis. *Tissue Eng Part A* **15**, 579-585, doi:10.1089/ten.tea.2008.0196 (2009).

66 Lee, J. Y. *et al.* Integrating sensing hydrogel microstructures into micropatterned hepatocellular cocultures. *Langmuir* **25**, 3880-3886, doi:10.1021/la803635r (2009).

67 Kim, K., Yeatts, A., Dean, D. & Fisher, J. P. Stereolithographic bone scaffold design parameters: osteogenic differentiation and signal expression. *Tissue Eng Part B Rev* **16**, 523-539, doi:10.1089/ten.TEB.2010.0171 (2010).

68 Buckwalter JA, G. M., Cooper RR, Recker R. Bone biology. I: Structure, blood supply, cells, matrix, and mineralization. *Instr Course Lect* **45** (1995).

69 Franceschi RT, G. C., Xiao G, Roca H, Jiang D. Transcriptional regulation of osteoblasts. *Ann N Y Acad Sci.* **1116**, 196-207 (2007).

70 Colnot, C. I. & Helms, J. A. A molecular analysis of matrix remodeling and angiogenesis during long bone development. *Mech Dev* **100**, 245-250, doi:S0925477300005323 [pii] (2001).

71 Grundberg, E. *et al.* Systematic assessment of the human osteoblast transcriptome in resting and induced primary cells. *Physiol Genomics* **33**, 301-311, doi:00028.2008 [pii]
10.1152/physiolgenomics.00028.2008 (2008).

72 Hattori, T. *et al.* SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. *Development* **137**, 901-911, doi:137/6/901 [pii]
10.1242/dev.045203 (2010).

73 Kronenberg, H. M. Developmental regulation of the growth plate. *Nature* **423**, 332-336, doi:10.1038/nature01657
nature01657 [pii] (2003).

74 Andrew Thompson, M. B., Diana Yoon, John Fisher. Osteogenic differentiation of bone marrow stromal cells. *Tissue Engineering Part A* (2009).

75 Gerstenfeld, L. C. & Shapiro, F. D. Expression of bone-specific genes by hypertrophic chondrocytes: implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. *J Cell Biochem* **62**, 1-9, doi:10.1002/(SICI)1097-4644(199607)62:1<1::AID-JCB1>3.0.CO;2-X [pii]
10.1002/(SICI)1097-4644(199607)62:1<1::AID-JCB1>3.0.CO;2-X (1996).

76 J. A. Buckwalter, R. R. C., M. J. Glimcher, R. Recker. Bone Biology, PART II: FORMATION, FORM, MODELING, REMODELING, AND REGULATION OF CELL FUNCT1ON. *Journal of Bone and Joint Surgery* **77** (1995).

77 Wei A, C. S., Tao H, Brisby H, Lin Z, Shen B, Ma DD, Diwan AD. Differentiation of rodent bone marrow mesenchymal stem cells into intervertebral disc-like cells following coculture with rat disc tissue. *Tissue Eng Part A* **15**, 2581-2595 (2009).
78 Matsuda, C., Takagi, M., Hattori, T., Wakitani, S. & Yoshida, T. Differentiation of Human Bone Marrow Mesenchymal Stem Cells to Chondrocytes for Construction of Three-dimensional Cartilage Tissue. *Cytotechnology* **47**, 11-17, doi:10.1007/s10616-005-3751-x (2005).
79 Hazel Tapp, R., JaneAIngram, Marshall Kuremsky, EdwardNHanleyJr and & Gruber, H. Adipose-derived mesenchymal stem cells from the sand rat: transforming growth factor beta and 3D co-culture with human disc cells stimulate proteoglycan and collagen type I rich extracellular matrix *Arthritis Res Ther* **10**, R89 (2008).
80 Lee, S. J. *et al.* Identification of proteins differentially expressed during chondrogenesis of mesenchymal cells. *FEBS Lett* **563**, 35-40, doi:10.1016/S0014-5793(04)00243-1
S0014579304002431 [pii] (2004).
81 Yamane, S., Cheng, E., You, Z. & Reddi, A. H. Gene expression profiling of mouse articular and growth plate cartilage. *Tissue Eng* **13**, 2163-2173, doi:10.1089/ten.2006.0431 (2007).
82 Birgit Kulterer, G. F., Anita Jandrositz, Fatima Sanchez-Cabo, Andreas Prokesch, Christine Paar, Marcel Scheideler, Reinhard Windhager, Karl-Heinz Preisegger, Zlatko Trajanoski. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics* **8** (2007).
83 Gregory, C. A., Gunn, W. G., Peister, A. & Prockop, D. J. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* **329**, 77-84, doi:10.1016/j.ab.2004.02.002
S0003269704001332 [pii] (2004).
84 Ho, A. D., Wagner, W. & Franke, W. Heterogeneity of mesenchymal stromal cell preparations. *Cytotherapy* **10**, 320-330, doi:794006432 [pii]
10.1080/14653240802217011 (2008).
85 Bosnakovski, D. *et al.* Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells. *Cell Tissue Res* **319**, 243-253, doi:10.1007/s00441-004-1012-5 (2005).
86 Cheng, S. L., Yang, J. W., Rifas, L., Zhang, S. F. & Avioli, L. V. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *Endocrinology* **134**, 277-286 (1994).
87 Ashburner, M. *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* **25**, 25-29, doi:10.1038/75556 (2000).
88 Sasano, Y. *et al.* Gene and protein expressions of type I collagen are regulated tissue-specifically in rat hyaline cartilages in vivo. *Eur J Morphol* **39**, 149-154 (2001).

89 Ortega, N., Wang, K., Ferrara, N., Werb, Z. & Vu, T. H. Complementary interplay between matrix metalloproteinase-9, vascular endothelial growth factor and osteoclast function drives endochondral bone formation. *Dis Model Mech* **3**, 224-235, doi:dmm.004226 [pii]
10.1242/dmm.004226 (2010).

90 Ishizeki K, K. T., Fujiwara N, Otsu K, Harada H. Expression of osteogenic proteins during the intrasplenic transplantation of Meckel's chondrocytes: A histochemical and immunohistochemical study. *Arch Histol Cytol.* **72**, 1-12 (2009).

91 Danielle J. Behonick, Z. X., Shirley Lieu, Jenni M. Buckley, Jeffrey C. Lotz, Ralph S. Marcucio, Zena Werb, Theodore Miclau, Ce´ line & Colnot, C. I. Role of Matrix Metalloproteinase 13 in Both Endochondral and Intramembranous Ossification during Skeletal Regeneration. *PLoS One* **2**, e1150 (2007).

92 Schreiweis, M. A. *et al.* A proteomic analysis of adult rat bone reveals the presence of cartilage/chondrocyte markers. *J Cell Biochem* **101**, 466-476, doi:10.1002/jcb.21196 (2007).