Wnt Signaling in Post-Natal Chondrogenesis

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INTRODUCTION

Healthy cartilage is essential for proper joint function. Pain-free movement is important for patients' well-being, since impairment affects one's ability to pursue work and leisure activities. Cartilage damage can occur through trauma, pathological states such as arthritis, or the normal wear that accompanies aging. Treatment for cartilage defects is hindered by the limited capacity of that tissue to undergo self-repair and healing. Attempts at self-repair result in local proliferation and increased matrix synthesis by chondrocytes, but the new tissue lacks the biomechanical properties necessary for long-term stability. Therefore, there is a need for innovative cartilage repair techniques.

In adults, chondrocytes maintain the extracellular matrix that gives cartilage its unique mechanical properties. Chondrocytes are long-lived and the development of new cells that are capable of producing cartilage *de novo* (i.e., chondroblasts) is not a normal part of adult cartilage physiology. One hypothesis is that synthesis of hyaline cartilage is coupled to skeletal growth. This hypothesis predicts that differentiation of new chondroblasts will be required to achieve cartilage repair. Therefore, a better understanding of the molecular mechanisms that regulate post-natal chondroblast differentiation would have a high impact on the design of strategies for cartilage repair.

PRENATAL VS. POSTNATAL CHONDROBLAST DIFFERENTIATION

Studies of embryonic skeletal development have provided a wealth of information on prenatal chondroblast differentiation. As in other embryonic tissues, chondroblasts differentiate in an environment that is rich with developmental cues. Both positive and negative signals are present. These signals include growth factors (such as TGF-β and the bone morphogenetic proteins) and matrix components (hyaluronan, perlecan, collagens) that act to induce a genetic program in target mesenchymal cells. Perturbation of these signals disrupts chondrogenesis.

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In vitro systems have been developed for induced chondrocyte differentiation of post-natal cells (bone marrow stroma, fat, etc.). Post-natal chondroinduction can be accomplished *in vivo* with implants of demineralized bone. This material induces endochondral osteogenesis and is used clinically for orthopedic and oral surgical applications. From animal studies,[1] it has been shown that an early step is attraction of cells that attach to particles of demineralized bone powder (DBP) and convert to chondroblasts. My collaborators, Drs. Julie Glowacki and Shuichi Mizuno, have developed an *in vitro* collagen sponge culture device that mimics the geometry and density of *in vivo* DBP implants. With this culture system, it is possible to begin to define cellular events that occur during chondroinduction by DBP.

GENE EXPRESSION STUDIES IN AN *IN VITRO* MODEL OF POST-NATAL CHONDROBLAST DIFFERENTIATION INDUCED BY DEMINERALIZED BONE

In our model of induced chondrocyte differentiation,[2] human dermal fibroblasts (hDFs) are cultured in a bilaminate collagen sponge with demineralized bone powder (DBP)

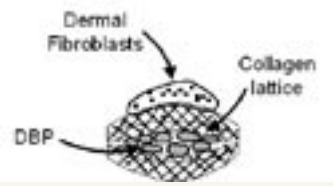

Figure 1. Schematic of DBP/collagen sponge culture device. A portion of demineralized bone powder (DBP) is held between two layers of porous collagen lattice. Control sponges consist of a single layer of collagen. Dermal fibroblasts (10^6 cells in 50 l of culture medium) are placed on the upper surface of the sponge. The cells migrate through the collagen and attach to the particles of DBP.

(**Figure 1**). After 7 days, hDFs that are associated with DBP show a chondroblast phenotype: the cells are surrounded by cartilage-like matrix that contains collagen type II, aggrecan, and typical proteoglycans.[2,3] We hypothesized that DBP activated a genetic program in target hDFs that is related to the chondroblast phenotype. To begin to define this program, we examined changes in gene expression induced by DBP that precede the chondroblast phenotype. Three days after hDFs are seeded onto sponges, the cells have established interactions with DBP but do not express cartilage-specific genes.[4] Therefore, the 3-day time point was selected for these studies.

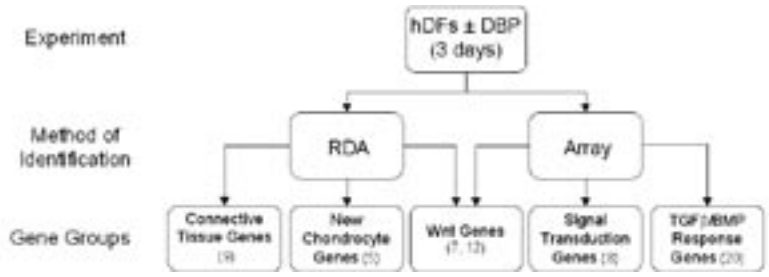

Figure 2. Overview of experiments to identify genes whose expression is altered in human dermal fibroblasts (hDFs) cultured in collagen sponges with demineralized bone powder (DBP). Two methods of gene expression analysis, representational difference analysis (RDA) and cDNA macroarrays (Array) identified several functional groups of genes.

Two different methods were used to identify genes that are affected by DBP (**Figure 2**). First, representational difference analysis (RDA), a PCR-based method of subtractive hybridization, was used to gain an overview of the cellular processes altered by DBP.[4-6] Second, cDNA macroarrays were used to test specific hypotheses about DBP's effects on specific functional classes of genes.[7,8]

These studies on DBP-altered gene expression revealed several insights into early events in chondroblast differentiation in this experimental system (Figure 2). Expression of many functional classes of genes (cytoskeletal and matrix components, growth factors, protein synthesis and secretion machinery, transcriptional regulators) is altered by DBP. The diversity of these functional classes supports our hypothesis that DBP activates a programmatic shift in cell physiology.[4] Altered expression of genes that encode matrix components (collagens, collagen-modifying enzymes) and cell-matrix interacting proteins (collagen receptors) suggests that an early step in post-natal chondrogenesis is the creation of a supportive extracellular environment. This step may be necessary because, unlike the programmed milieu of embryonic tissues, postnatal tissues lack the regulatory signals to promote chondrogenesis.[9]

In the course of these gene expression studies, we identified two members of the Wnt gene family whose expression was altered by DBP.[7] The fact that Wnt genes were altered by DBP was striking because of the importance of that gene family in embryonic chondroblast differentiation and chondrogenesis. Therefore, the role of Wnt components in chondroinduction by DBP was explored further.

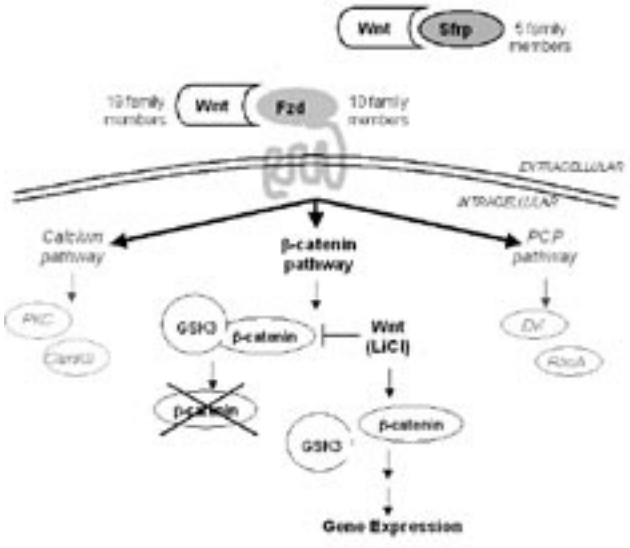

Figure 3. Simplified schematic of Wnt components and signaling pathways. Members of the Wnt protein family interact with Frizzled (Fzd) receptors. Secreted, Frizzled-related proteins (Sfrp) can also bind to Wnt and antagonize interactions with Fzd. At least three Wnt signaling pathways have been identified: calcium, -catenin, and planar cell polarity (PCP). In the -catenin pathway, Wnt binding of Fzd prevents the glycogen synthase kinase 3 (GSK3)-mediated destruction of -catenin. This step can be mimicked by lithium chloride (LiCl). The resulting increase in intracellular -catenin levels triggers a signaling cascade that ultimately affects gene expression in the cell.

THE Wnt FAMILY, SIGNALING AND EMBRYONIC CHONDROGENESIS

The *WNT* gene family encodes secreted proteins that interact with cellular receptors (encoded by *FZD* genes). Wnt activity can be modulated by secreted, frizzled-related proteins (encoded by *SFRP* genes) that compete with Fzd to bind Wnts. These genes' families (hereafter referred to as Wnt pathway components) form a regulatory network (**Figure 3**) that regulates cell fate and function in many tissues.

Intracellular signaling pathways that are activated by Wnt proteins are complex. At least three pathways are known: the so-called β-catenin, calcium, and planar cell polarity pathways (Figure 3). Of these, the β-catenin pathway has been studied in greatest detail. In the absence of Wnt, β-catenin is targeted for destruction via an interaction with glycogen synthase kinase 3β (GSK-3β). When some of the Wnt proteins bind Fzd receptors, GSK-3β activity is inhibited and cytosolic β-catenin levels increase. This allows the downstream signaling events to proceed that culminate in altered gene expression. Treatment of cells with lithium chloride (LiCl) results in inhibition of GSK-3β,[10] thereby mimicking Wnt signaling via the β-catenin pathway (Figure 3).

During embryonic skeletogenesis, Wnt components act as both positive and negative regulators of key events, including chondroblast differentiation, chondrocyte maturation, and joint formation.[11] The importance of Wnt components in prenatal chondroblast differentiation, and our finding that some Wnt family members were affected by DBP, suggested that the Wnt network might regulate post-natal chondroblast differentiation. Therefore, the DBP/collagen sponge model was used to test this hypothesis.[12]

Wnt GENES CONTRIBUTE TO CHONDROINDUCTION OF POST-NATAL FIBROBLASTS BY DBP

The potential role of Wnt genes in post-natal chondroblast differentiation of hDFs was explored by characterizing Wnt component gene expression during culture with DBP, and by activating the Wnt/β-catenin signaling pathway in the absence of DBP. Primers were designed for PCR amplification of all known human *WNT*, *FZD*, and *SFRP* genes. Gene expression levels of Wnt components were measured in hDFs cultured in DBP/collagen and control collagen sponges by semi-quantita-

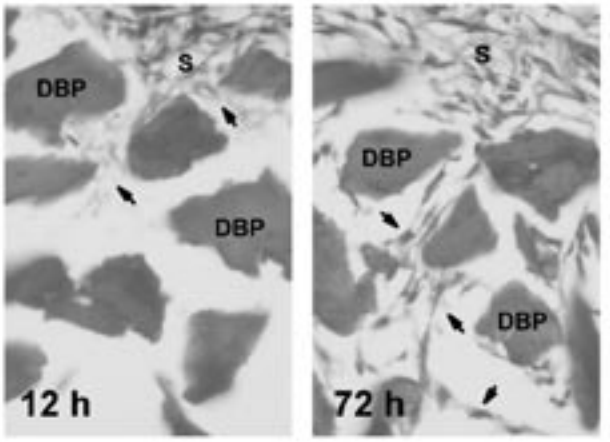

Figure 4. Migration of hDFs into DBP/collagen sponges. Cells were seeded onto sponges and cultured for 12 or 72 hours (4 m-thick paraffin sections, Safranin O stain). Arrows indicate hDFs that have migrated into the packet of DBP contained within the sponge. A portion of the upper layer of collagen in the sponge (S) is visible at the top of each image.

tive RT-PCR. Prior to hDFs' interactions with DBP -- 12 hours after cells were seeded onto the sponges (**Figure 4**) -- Wnt component gene expression levels were similar in DBP/collagen and control collagen sponges. After hDFs' interactions with DBP were well established (72h after seeding), altered expression of several *WNT* and *FZD* genes was observed: two genes were upregulated (average 175% of control) and three genes were downregulated (average 40% of control). Expression of *SFRP* genes was not affected by DBP on day 3. Many of the genes whose expression was changed by DBP on day 3 also showed altered expression on days 7 and 14, when the hDFs produced cartilage-like matrix. In contrast, Wnt component genes whose expression was not changed by DBP on day 3 did not show altered expression on either day 7 or 14. These results showed that a subset of Wnt pathway components is altered during chondroinduction (day 3) and chondrogenesis (days 7-14) by DBP.

Several of the Wnt components that were altered by DBP are known to activate the β-catenin pathway. Therefore, the contribution of Wnt/β-catenin signaling to chondroblast differentiation was assessed by exposing hDFs in collagen sponges (without DBP) to LiCl. On day 10, histology showed significantly more metachromatic matrix in LiCl-treated sponges than in sodium chloride- (NaCl) treated controls. The LiCl-treated sponges also tended to contain more sulfated proteoglycan than NaCl-treated controls. Therefore, activation of Wnt signaling via the β-catenin pathway was sufficient to increase proteoglycan synthesis by hDFs cultured in collagen sponges. Taken together, these results on Wnt gene expression and signaling in hDFs implicate the Wnt regulatory network as mediators of post-natal chondroblast differentiation by demineralized bone.

FUTURE DIRECTIONS

In this study, activation of Wnt signaling via the β-catenin pathway appeared to promote chondrogenesis. Another study, from a group led by Dr. Glowacki, also found that activating Wnt/β-catenin signaling increased chondrogenesis in human cells.[13] Results from other experimental systems that use mouse, chick, or rabbit, cells have shown both stimulatory[14,15] and inhibitory effects[16,17] of Wnt/β-catenin signaling on chondrogenesis. The effect of Wnt/β-catenin signaling is likely affected by cell type, maturational stage, and species. Recent data showing that the Wnt/calcium signaling pathway can antagonize the β-catenin pathway[18,19] may also explain the observed duality of Wnt/β-catenin signaling. The net effect of DBP-induced changes in Wnt component expression on signaling pathways in this experimental system has yet to be determined. The potential roles for accessory proteins such as Lrp/arrow co-receptors must also be defined. Nevertheless, these results suggest that Wnt signaling contributes to chondroblast differentiation induced by demineralized bone.

ACKNOWLEDGEMENTS

I thank J. D. Clark II and R. MacLean for technical assistance, and J. Glowacki, S. Mizuno, and S. Zhou for their contributions to this work. The Wnt studies were supported by a grant from the Aircast Foundation (#SA-501).

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