Regulation of cartilage cell fate determination and homeostasis

A thesis

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ABSTRACT

We first studied the role of Shh in patterning the somite during early embryonic development. We found that differing levels of Shh signaling elicit differing responses from somitic cells: the lowest level of Shh signaling allows dermomyotomal gene expression, intermediate levels induce loss of dermomyotomal markers and activation of myogenic differentiation, and higher levels induce loss of myotomal markers and activation of sclerotomal gene expression. We also found that under high levels of Wnt signaling, Shh signals act to maintain the expression of dermomyotomal and myotomal markers. One sclerotomal gene induced by high Shh signaling is Nkx3.2. Forced expression of Nkx3.2 blocks expression of dermomyotomal marker Pax3 both in vitro and in vivo. Conversely, expression of Pax3 in somites can block Shh-mediated induction of sclerotomal gene expression. In summary, Pax3 and Nkx3.2 set up mutually repressing somitic cell fates..

Muscle is a tissue located in close proximity to cartilage tissue. We found that chondrocytes cultured with C2C12 muscle cells exhibited increased production of cartilage matrix proteins. Furthermore, muscle cell-conditioned medium led to increased cartilage matrix production, suggesting that muscle cells secrete pro-chondrogenic factors. Furthermore, we showed that muscle cells diminish the response of chondrocytes to arthritis-associated pro-inflammatory cytokines IL-1β and TNFα. We found that chondrocytes cocultured with muscle cells or cultured in muscle cell-conditioned medium significantly enhanced the expression of cartilage matrix proteins (collagen II and collagen IX) by interfering with IL-1β and TNFα downstream signaling mediators (including NFkappaB, ESE-1, Cox-2, and

GADD45beta), and causing the attenuated expression of cartilage-degrading enzymes (MMPs and ADAMTS4). This result may ultimately lead to the discovery of novel factors that regulate cartilage formation and homeostasis, and provide insights into improving the strategies for regenerating cartilage.

Lastly we describe a mechanism by which we investigate the mechanism by which these muscle progenitor cells adopt a cartilage fate. We show that chick muscle satellite cells can be converted to express cartilage matrix proteins in vitro when cultured in chondrogenic medium containing TGFβ or BMP2. Ectopic expression of the myogenic factor Pax3 prevents chondrogenesis in these cells, while chondrogenic factors Nkx3.2 and Sox9 promote this cell fate transition. We found that Nkx3.2 and Sox9 repress the activity of the Pax3 promoter. A reverse function mutant of Nkx3.2 blocks the ability of Sox9 to both inhibit myogenesis and induce chondrogenesis, suggesting that Nkx3.2 is required for Sox9 to promote chondrogenesis. Finally, in an in vivo mouse model of fracture healing using lineage-traced muscle progenitor cells, Nkx3.2 and Sox9 are significantly upregulated while Pax3 is significantly downregulated in the muscle progenitor cells that give rise to chondrocytes during fracture repair.

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1.1.1 Somite formation

During embryonic development, all tissues originate from very naïve precursor cells that go on to differentiate into their respective pre-determined cell fates. Elucidating the molecular mechanisms by which this differentiation into specialized tissue types occurs is crucial not only to the understanding of development, but also to the understanding of the regeneration of adult tissues as well as the deviations in those differentiation pathways that lead to disease states.

In the vertebrate embryo, there are three primary tissue types known as germ layers: endoderm, ectoderm, and mesoderm [1, 2]. These germ layers ultimately give rise to all differentiated tissues in the body. Fundamentally, the endoderm forms most of the internal organs such as the stomach and the lungs, the ectoderm forms the nervous tissue and epidermis, and the mesoderm ultimately becomes muscle, the skeleton, the dermis of skin, and connective tissue including blood cells [1, 2].

The mesoderm that forms on either side of the neural tube is called paraxial mesoderm [3]. Paraxial mesoderm separates into spherical divisions known as somites. Somites bud periodically, and differentiate in a caudal to rostral fashion [4, 5]. Somites start out as immature balls of cells that have the capacity to form multiple tissue types. As they continue to develop they differentiate into dorsal and ventral domains based on the various signals that

are secreted from neighboring embryonic structures. Interestingly, early experiments in which immature somites were rotated 180° in order to expose the dorsal somite to those signals secreted from ventrally located tissues and vice versa, demonstrated that flipped cells differentiated into the tissue type specified by their new location. These results indicate that specification of appropriate somitic cell fate is dependent on local environmental cues [4, 6].

1.1.2 Morphogens pattern the somite into the sclerotome, myotome and dermomyotome

Morphogens are one type of signaling molecule that serve to intricately pattern the nascent somite in a concentration dependent manner thereby yielding different cell fates [7]. They are usually secreted from a localized source, which gets diffused throughout a tissue thereby forming a concentration gradient with those cells closest in position to the source receiving a high concentration of the morphogen and those cells farther away receiving a relatively low concentration. Cells then begin to differentiate according to the amount of morphogen they are exposed to by producing specific cellular responses and ultimately turning on target genes. Distinct cell fates appear as the outcome of subsequent gene expression [8].

The expression and function of many patterning molecules in somite differentiation have been defined. The major morphogens that act on the somites are Sonic Hedgehog (Shh), Wingless (Wnt) [9], and Bone Morphogenetic Protein (BMP). These factors act in concert to pattern the dorsal and ventral domains of the somite [3]. These dorsal and ventral domains then compartmentalize further to delineate specified tissue types. The discrete regions of the dorsal domain are termed the dermomyotome,

which will eventually become dermis of the back, and the myotome, which yields the skeletal muscle of the trunk. In the ventral domain lies the sclerotome, which develops into the vertebrae and ribs.

Shh is secreted by the notochord and the ventral neural tube, and plays a vital role in the specification of the sclerotomal compartment as well as the chondrogenesis of the cells within it [10-13]. Mice in which Shh is specifically knocked out have diminished sclerotomal compartments which result in the complete absence of the vertebral column [11]. Furthermore, exogenous expression of Shh during somite cell fate specification is sufficient to expand the domain of the sclerotome at the expense of the other two tissue types [14]. Shh knockout embryos also have much reduced expression of muscle marker Myf5, suggesting that Shh is also implicated in the specification of the myotome[11, 15, 16]. Later studies further characterized the role of Shh in this process, and it was determined that during the differentiation of the myotomal compartment, Shh is a non-essential factor in the induction of myogenesis, but serves a more prominent function in promoting both the survival and proliferation of those cells already committed to the myogenic fate. Decreased Myf5 expression in the myotome was determined to be the result of decreased proliferation and increased apoptosis of myogenic progenitors and not because of a failure to properly differentiate [17].

Wnt signaling, which is secreted by the ectoderm and dorsal neural tube, is also required for myotome formation [18]. Mouse double knockouts of Wnt-1 and Wnt-3a also exhibit a decrease in Myf5 expression in the somite [19]. This then suggests that the myotome, which is in the medial

compartment of the somite, essentially receives competing signals from external dorsal and ventral tissues, which serve to specify muscle cell fate [20].

In *in vivo* experiments in which cells expressing low levels of exogenous BMP4 were placed in the area of presomitic mesoderm, the expression of Sim1 was induced, which is a marker specific to the lateral domain of the somite. Moreover, relatively high levels of BMP4 were sufficient to transform the entire somite into lateral plate mesoderm, which demonstrates not only the importance of BMP concentration and potency on the differentiation of embryonic tissues [21-23].

1.1.3 Somitic cell fate specification: the dermomyotome

The aforementioned somitic compartments; dermomyotome, myotome and sclerotome; are each visible by their induced gene expression. The dermomyotomal compartment has been the least well-studied relative to the myotome and sclerotome. (Scaal and Christ, 2004). Early markers of the dorsally located dermomyotome include Pax3 and Pax7. The dermomyotome is believed to develop as a result of Wnt signaling secreted by the nearby ectoderm [24, 25], as blocking Wnt signaling results in a significantly thinner dermis. Furthermore, it was shown that expression of En1, a dermal marker, requires the presence of surface ectoderm in a chick embryo model [26, 27]. Additionally, transcription factor cDermo-1 is expressed in the dermis and has been described as feather primordia. Forced expression of Dermo-1 in embryonic chicken dermis resulted in a thickened dermis normally observed in skin that contains feathers [28].

1.1.4 Somitic cell fate specification: the myotome

The myotome, or presumptive muscle also expresses Pax3 and Pax7 during the initial specification of muscle cell fate, but later begins to upregulate Myf5, MyoD and Myosin Heavy Chain (MHC)[29-32]. Induction of these cell type specific factors is often vital to the survival of the adult tissue. For example, Mouse knockouts of Pax3 completely lack all limb muscles, as Pax3 is involved in patterning the migration of muscle precursors to the limb bud [33]. Conversely, loss of Pax7 does not affect the development of embryonic muscle, but does however impair the induction and maintenance of muscle satellite cells in the adult [34, 35]. Downstream of Pax3 and -7 is Myf5. Mice deficient in Myf5 expression exhibit severe defects in myotome formation before embryonic day 10.5, after which their phenotype is rescued upon the endogenous upregulation of MyoD expression, producing mice with relatively normal muscle phenotypes [36, 37]. Mouse knockouts of MyoD, conversely have completely normal muscle development during embryogenesis, but lose the capacity to regenerate muscle during adulthood, as MyoD is required for the proper differentiation of muscle satellite cells [38, 39]. Removing both of these factors produces Myf5/MyoD double mutant mice that cannot generate any skeletal muscle progenitors [40]. Humans carrying mutations of the gene encoding for myosin heavy chain exhibit early onset myopathy, which indicates that this gene is essential for the normal function of muscle [41]. Taken together, these results suggest that the transcriptional activation of these morphogen-induced genes is a tightly controlled process; one in which each induced factor plays an important role in the specification and maintenance of its respective tissue type.

1.1.5 Somitic cell fate specification: the sclerotome

Markers of the sclerotome include Pax1, Pax9, Nkx3.2 and Sox9. While their expression domains differ slightly, they all become activated in the ventral portion of the somite that delineates the presumptive cartilage. Pax1 and Pax9 are crucial factors in the development of the axial skeleton [42]. Pax1 and Pax9 have similar homology and expression domains in the initial sclerotome of the somite. Despite this, they are not completely redundant. Pax9 knockout mice have no obvious skeletal malformations, while Pax1 deficient mice lack the ventral components of the vertebral column [43-45]. However, double knockouts of both Pax1 and -9 have a much more severe phenotype than that of Pax1 knockouts alone [46]. Double Pax1/9 mutant cells in the sclerotome fail to initiate chondrogenesis and exhibit a decreased rate of proliferation. Later work demonstrated that Pax1 and Pax9 are both necessary and sufficient for the expression of another important sclerotomal transcription factor, Nkx3.2 [47].

Sox9 is a potent transcriptional activator that has a well-characterized role in chondrogenesis. In humans, haploinsufficiency of Sox9 results in campomelic dysplasia, a disease that is often fatal, in which the skeleton fails to form properly resulting in bowed and shortened long bones [48, 49]. In a chimeric mouse model, Sox9 -/- cells present in cartilaginous elements failed to express cartilage markers Collagen II and aggrecan [50]. Furthermore, conditionally knocking out Sox9 in mouse limb buds resulted in a complete absence of cartilage and bone elements in the limb [51]. Sox9 has been shown to directly activate the promoters of cartilage matrix genes Collagen II and aggrecan [52-54]. Sox9 has also been shown to induce the expression of

another sclerotome marker, Nkx3.2 [55-58]. Mouse knockouts of Nkx3.2 have major defects in formation of the axial skeleton, which is likely caused by a decrease in Sox9 expression in the somite [55, 56, 59, 60]. Induction of either Nkx3.2 or Sox9 in the somite of chicken embryos is sufficient to cause ectopic cartilage formation [60].

Interestingly, despite the continuous diffusion of morphogens throughout the entire somite, these markers become expressed in very distinct patterns with well-defined borders between the cartilage and muscle precursor zones [8]. While morphogen gradients are well-characterized sources of patterning of somitic cell fates, the way in which those cells interpret those signals and subsequently turn on their respective expression profiles is not fully understood. Furthermore, the potential interrelationship between the primordia of these distinct tissues has also not been explored.

1.2 Interrelationship between muscle and cartilage

1.2.1 Muscle and cartilage precursors are juxtaposed in the somite

Muscle and cartilage progenitors, the myotome and sclerotome respectively, are juxtaposed during development, and have been proposed to influence the mutual development of each other. A number of mouse mutants in which muscle-specific markers are knocked out have been studied that exhibit very dramatic skeletal phenotypes.

1.2.2 Muscle marker knockouts have decreased skeletal size

For example, knocking out Pax7, a transcription factor first expressed in the dermomyotome of the somite with an additional and perhaps more

important role in the specification of postnatal muscle stem cells or muscle satellite cells, produces mice with a decreased skeletal size as well as a curvature of the spine [35]. Mouse models of Duchenne muscular dystrophy in which muscle markers utrophin and dystrophin have been specifically knocked out also exhibit a reduction in length of skeletal elements [61]. Interestingly, in accordance with mouse models of the disease, patients with Duchenne muscular dystrophy are also significantly shorter in stature [62-64]. Myogenin, another muscle-specific transcription factor expressed in the myotome, is a later stage marker of muscle differentiation that is involved in both development and repair. Knocking out myogenin causes perinatal lethality due to incomplete muscle formation [65], however, postnatal deletion of this marker yields mice with phenotypically normal muscle tissue [66]. Interestingly, this removal of myogenin postnatally causes a dramatically reduced skeleton size relative to controls, suggestive of a non-cell autonomous effect with respect to the effect of myogenin-deficient muscle on neighboring cartilage and bone elements.

1.2.3 Muscle tissue likely exerts an influence on cartilage tissue

These phenomena potentially suggest that there is some interplay between the presumptive muscle and cartilage in both the embryo and the developing young vertebrate. It seems plausible also that this interaction may involve paracrine signaling between muscle and cartilage that can have multiple effects on various aspects of the development of each tissue including but not limited to proliferation, migration of precursor cells and differentiation. Despite these multiple mouse knockout studies, the direct role

of muscle cells on cartilage differentiation and maintenance has not been thoroughly explored.

1.3 Cartilage growth in the embryo as compared to the adult

1.3.1 Cartilage growth is efficient during development: endochondral ossification

After the initial specification of cell fates during somite development, the dermomyotome, myotome and sclerotome each go on to fully develop into their respective mature tissue types. As previously mentioned, cells within the sclerotomal compartment ultimately form the axial skeleton. Briefly, cells within the sclerotome first migrate to surround the developing notochord, which ultimately becomes the vertebral body within the axial skeleton. These precursor cells first become cartilage before becoming mature bone by a process known as endochondral ossification. This process is also responsible for the formation of all of the long bones within the appendicular skeleton as well [67]. Endochondral ossification occurs by the condensation of mesenchymal tissue, which is highly influenced by signaling molecules such as BMPs. During this phase, cells begin to first express matrix molecules such as collagen II and aggrecan as well as the potent chondrogenic activator Sox9. As chondrogenesis continues, cells begin to proliferative rapidly and to produce more matrix proteins. Once the cartilage anlagen is fully formed, the cells in the center begin to mature or hypertrophy, expressing Collagen X. These cells eventually mineralize and die, at which point osteoclasts infiltrate to chew up the existing matrix while osteoblasts replenish it with new bony matrix high in Collagen I. Angiogenesis occurs in the center of the new bone thereby creating the bone marrow cavity. This part of the process is known as primary ossification. Secondary ossification

utilizes similar mechanisms, however it occurs at the ends of the long bones. Secondary ossification centers ultimately yield growth plate cartilage, which serves to allow for expansion and growth until adulthood, at which point the vertebrate is fully developed. Growth plate cartilage retains identifiable zones with well-characterized markers of expression (see Figure 2). Growth plate cartilage completely erodes following adolescence in humans, however articular cartilage is maintained throughout life via largely unknown mechanisms. This static tissue is relatively stable until challenged with injury, disease and/or old age.

1.3.2 Cartilage repair is insufficient in the adult

In the adult, cartilage lacks the ability to efficiently repair itself. This could be due to its avascularity, as well as a shortage of stem cells resident in the articular cartilage tissue itself. Only when the damage becomes severe enough that it penetrates the subchondral bone do blood cells from the underlying marrow become activated to repair cartilage damage, however at that point the damage is so extensive that the repaired tissue becomes scar-like fibrocartilage that lacks the mechanical strength and integrity of the native hyaline articular cartilage. For these reasons, it is vital to understand the developmental processes involved in early cartilage formation as this knowledge could also potentially be harnessed to facilitate cartilage repair in the adult.

1.4 Role of inflammation in cartilage and bone healing and disease

1.4.1 Arthritis - cartilage destruction by pro-inflammatory cytokines

The most prevalent cartilage-related disease in adults is arthritis.

Arthritis is the most common cause of disability in the United States. Arthritis is primarily a disease of the elderly, as it is speculated that over 70% of arthritis patients in North America are over the age of 65. With an ever-aging population the incidence of this disease expected to increase dramatically[68, 69].

Arthritis is characterized by swelling and inflammation of the joint, severe pain, and ultimately immobility. The most common form of arthritis, osteoarthritis, is most often the result or joint trauma and/or old age. Other arthritis forms include rheumatoid arthritis, inflammatory arthritis, and other related autoimmune diseases. There is no cure for rheumatoid or osteoarthritis [70]. In extreme cases in which joint cartilage is almost completely eroded, joint replacement is the only suitable treatment option as the hyaline cartilage is completely beyond repair. However, in more moderate cases there are treatment strategies that aim to alleviate pain as well as to repair any articular cartilage damage caused by disease.

Arthritis is generally caused and/or exacerbated by the infiltration of inflammatory cytokines within the joint cavity. In normal cartilage, there is a well-maintained balance between anabolic and catabolic factors. In arthritic cartilage, this balance becomes disrupted. Initially this results in an overall increase in both matrix synthesis and destruction, as the chondrocytes try to rapidly compensate for the onslaught of matrix damage brought on by inflammation [71]. However, over time the balance tips again in favor of the matrix degrading enzymes, resulting in severe cartilage damage that is incapable of proper repair. Osteoarthritic chondrocytes have been shown to generate a variety of MMPs in response to pro-inflammatory cytokine

stimulation, the most prominent being MMP-3 (stromelysin), MMP-8 (collagenase-2), and MMP-13 (collagenase-3) [72, 73].

Furthermore, the expression of molecules that antagonize the activity of MMPs, TIMPs, is markedly lower in the diseased tissue. It is not only the collagen fibril component of the matrix that becomes degraded. A family of enzymes containing A Disintegrin-like And Metalloproteinase-like domain (ADAM) specifically cleave glycosaminoglycans. The most well-characterized aggrecanase1 and 2, which are ADAMs enzymes that bind specifically to chondroitin sulfate [74-76].

Other phenotypic changes relating to an altered extracellular matrix involves chondrocyte dedifferentiation. This is essentially the conversion of a healthy chondrocyte, to a cell that more closely resembles a fibroblast. Dedifferentiated chondrocytes halt the expression of cartilage matrix genes Collagen II and aggrecan, and begin to secret a matrix more consistent with that of fibroblasts, specifically collagens I, III and V [77]. Unlike in the case of the altered anabolic/catabolic balance previously described, these cells are very actively secreting matrix, however that matrix no longer maintains the integrity of healthy cartilage matrix.

Another change in matrix composition occurs when chondrocytes switch from resident stable articular chondrocytes into hypertrophic chondrocytes. Articular cartilage lesions can often penetrate the subchondral bone, thereby triggering not only an influx of inflammatory cytokines, but can also initiate an angiogenic response. These cells go from secreting Collagen

II to Collagen X, at which point the matrix often calcifies, becoming more similar to bone matrix [78]. This type of matrix damage is also detrimental in that calcified articular cartilage lacks the ability to endure sustained mechanical forces.

Lastly, not only the matrix profile is affected by arthritis, the chondrocytes within the matrix often experience various phenotypic changes. Studies have shown that arthritic chondrocytes have a low proliferative capacity relative to healthy ones [79]. Furthermore, nitric oxide (NO) radicals have been shown to be upregulated in OA chondrocytes. While its role in the pathogenesis have not been fully characterized, it has been hypothesized that NO may induce the apoptosis of both articular cartilage and synovial cells [80-84]. While the cartilage repair process is inherently flawed, cartilage growth during development is robust and well organized. Studying the mechanisms that are utilized during the period when cartilage growth and differentiation is most efficient, during development, will allow us to develop strategies to treat cartilage-related diseases in the adult.

1.4.2 Current treatment options for arthritis

Current treatments include medications such as non-steroidal anti-inflammatory drugs like ibuoprofen, which are used to help control the pain and inflammation associated with arthritis. While this type of treatment may provide temporary protection against these pro-inflammatory cytokines, it is generally not effective for long term use [85-88]. Further research has identified growth various factors that have anti-inflammatory effects on cartilage tissue. For example, TGFβ1 has been shown to prevent TNFα-induced collagen degradation in bovine cartilage [89, 90]. Similarly, insulin-

like growth factor 1 (IGF-1) has been shown to prevent the induction of MMP-13 expression in IL-1β treated human chondrocytes [91]. Furthermore, glucosamine, an amino sugar involved in the synthesis of glycosaminoglycans such as aggrecan, has been proposed as an inhibitor of cartilage destruction and as a potential supplement in the treatment of arthritis. Interestingly, Gouze et. al. demonstrated that treatment with glucosamine attenuated the degradative effects of IL-1β in articular chondrocytes isolated from rats [92].

Despite management of symptoms using anti-inflammatory therapies, oftentimes the disease can still progress further. Especially in the case of a localized injury to the articular surface of joint cartilage, in which the influx of inflammation occurs at an accelerated rate [75, 76]. In addition to treating arthritis by attempting to counteract the high levels of pro-inflammatory cytokines present in the diseased joint, there are also surgical interventions that can be performed before complete joint replacement becomes necessary[84, 93].

Techniques to stimulate the underlying bone marrow are utilized when there are obvious articular cartilage defects. The purpose of these types of surgeries are to activate stem cells lying beneath the subchondral bone in order for those cells to migrate to the damaged site and facilitate articular repair. One example is microfracture surgery, which is the creation of tiny fractures in the bone which ultimately leads to the development of blood clots at the fracture sites. These blood clots contain hematopoietic stem cells that then differentiate into chondrocytes. While this surgery is an ideal treatment option as it is minimally invasive, and has relatively high success rates,

longer term studies indicate that the repaired cartilage tissue is largely made up of scar-like fibroblasts, which lacks the biomechanical integrity of normal healthy cartilage[94-97].

Another promising strategy to treat arthritis and cartilage defects is cartilage tissue engineering. Basically, this involves the culturing of cells in vitro for the purpose of transplanting improved cartilage tissue into a patient. To limit any potential concerns of host rejection the most ideal source of cells is from the patient in need of repair. These autologous cells can be harvested from the bone marrow as mesenchymal stem cells, then induced to differentiate in vitro using a TGFβ-based media in three dimensional culture. Once differentiated these cartilage-like constructs can subsequently be implanted to fix the articular defect. While this method allows for the harvesting of a large number of cells, the resulting cartilage is mechanically weak in comparison to native cartilage, and often does not integrate well into the host tissue. The other method of autologous cell transplantation involves the use of the host's own chondrocytes. Essentially, cartilage cells are isolated from the patient's healthy articular cartilage from a non-weight bearing portion of the joint. These cells are expanded in vitro until the population is of a sufficient number for transplantation. Compared to other surgical interventions, autologous chondrocyte implantation has proved to be the most reliable, however it is not without setbacks. For example, the expansion of patient chondrocytes in vitro can lead to de-differentiation of cells and a loss of the cartilage cell phenotype. While generally more mechanically robust than cartilage constructs derived from mesenchymal stem cells, autologous chondrocyte-based constructs still lack the strength of

native cartilage. Furthermore, none of the current treatment strategies address the lingering issues paramount to cartilage repair being integration of the transplanted tissue, and perhaps more importantly the effect of the constantly high levels of pro-inflammatory cytokines such as IL-1β and TNFα present in the joint that act to degrade the transplanted cartilage tissue [98]. Developing methods to circumvent inflammation will be essential in treating arthritis as well as other cartilage-related pathologies in which inflammation plays a prominent role[94, 95].

1.4.3 Inflammation in fracture healing

Bone fracture healing is another process that is highly dependent upon inflammatory cytokine regulation. There are three main phases of fracture healing. The first phase is known as the reactive or inflammatory phase. Within a few hours post-fracture, a blood clot forms at the fracture site. This blood clot serves as a reserve pool of hematopoeitic cells that secrete growth factors and various cytokines that recruit neighboring stem cells to participate in healing and fracture repair. Without this influx of inflammatory cytokines, fracture healing is often impaired, alluding to the importance of cytokine control in this process. This second phase, or reparative phase, involves the proliferation of stem-like cells that will ultimately contribute to the fracture callus. The primary source of these cells is the periosteum, the thin outer covering of the long bone. Other potential cell sources include the bone marrow as well as stem cells migrating from neighboring soft tissues such as muscle[99, 100]. All aforementioned stem cell populations have been shown to have the capacity to form cartilage. These stem-like cells form chondrocytes that secrete cartilage matrix proteins

Collagen II and Aggrecan in the fracture callus, which is subsequently replaced by calcified bone tissue. During the third and final phase known as the remodeling phase, the fracture healing site is reorganized by osteoclasts that chew up existing bone and osteoblasts that subsequently replace it with newly formed bone to restore the shape and function of the original bone. This phase continues even after the site is completely healed[101, 102].

1.4.4 Fracture healing recapitulates development

The process of fracture healing utilizes endochondral ossification, the same process that occurs during development when long bones are first formed[102, 103]. In this way, fracture healing is said to recapitulate the developmental process. A variety of signaling molecules is involved in bone healing. These include factors such as bone morphogenic proteins (BMP), transforming growth factor-ß (TGF-ß), and insulin-like growth factor II (IGF-II)[103, 104]. Understanding the interplay of these growth factors and cytokines will also help in generating approaches to address impaired fracture healing.

1.4.5 Current treatment options for impaired fracture healing

Impaired fracture healing can be a major problem – especially in the instance of non-union fractures; fractures in which the injured bone is not properly stabilized and/or gets infected; as well as fractures in individuals with pre-existing skeletal diseases such as osteoporosis or arthritis. For simple fractures, treatment generally includes stabilization of the fractured bone through the use of external casts and/or internal devices such as pins or screws to hold bone fragments firmly in place. When the breakage fails to

heal properly, other measures must be taken to ensure that the bone can fully repair itself.

Various molecules have been shown to have efficacy in bone repair, and are even used clinically. Studies in rat models have shown that treatment with parathyroid hormone (PTH (1-34)) results in an accelerated formation of the fracture callus, coincident with enhanced remodeling activity as well as increased biomechanical strength of the healed bones [105]. Furthermore, taken as a preventative measure, parathyroid hormone (1-34) has been shown to decrease the risk of vertebral and nonvertebral fractures and increases total bone mineral density in postmenopausal women with osteoporosis [106]. Taken together, these results suggest that PTH provides a promising therapeutic agent in the treatment of poorly healing bone fractures. However, the molecular downstream effects have yet to be fully characterized.

Treatment with BMP2 increased the rate of fracture callus formation in a rat femoral fracture model [107], suggestive that BMP2 may potentially accelerate the healing process. In a rabbit model of ulnar fracture healing, the fracture callus size was 20-60% bigger and the resulting repaired bone exhibited significantly increased biophysical strength upon recombinant human BMP2 treatment, as compared to controls [108]. Similarly, closed tibial fractures treated with recombinant BMP7 in a goat model of fracture healing, showed a larger callus size and enhanced biomechanical properties [109].

Substantial clinical research has been conducted on the potential usage of BMPs for fracture repair. Clinical trials in which recombinant BMP7 was administered to non-union fractures across various location have indicated that there was a 92% rate of proper healing with no complications [110]. Other studies have utilized recombinant BMP2 delivered via intramedullary placement of a collagen sponge. Those patients in treatment groups reported a significantly faster healing time as well as a lower incidence of reparative interventions [111]. These clinical studies indicate that BMP is both safe and efficacious for use in humans. Taken together, while treatment of non-healing fractures using BMP has tremendous therapeutic potential, the molecular mechanisms involved downstream of BMP signaling and the subsequent effects on various localized cell types must first be thoroughly examined.

While these therapeutic options such as PTH and BMPs offer a means to potentially enhance the actual process of fracture healing, like in the case of arthritis, there is often a lot of pain and inflammation associated with this condition. As with arthritic patients, it is commonly advised to take NSAIDs or COX-2 inhibitors to alleviate symptoms. However, as the infiltration of cytokines is the initial step in fracture healing, interference with this process could have negative consequences. Simon et. al. found that indeed treatment with a COX-2 inhibitor resulted in the impairment of fracture healing, suggesting that the pro-inflammatory cytokines induced during the inflammation phase of bone healing as required for proper healing [112]. Understanding the interplay of inflammatory cytokines and growth factors is

essential in developing effective strategies to treat both bone healing as well as arthritis.

1.5 Muscle and the inflammatory response

1.5.1 Muscles secrete anti-inflammatory factors

Muscle tissue has also been shown to be a secretory organ that potentially has important roles in the inflammatory response. Mass spectrometry proteomic analysis of media conditioned by C2C12 muscle cells identified secretory proteins that have been implicated in various cellular processes including matrix remodeling, cellular proliferation, migration and TGFβ signaling [113]. Muscle is a reported source of cytokines such as IL-6 [114]; matrix metalloproteinases (MMPs), which are enzymes that degrade matrix proteins such as in the degradation of Collagen II that happens during arthritis and fracture healing; as well as MMP inhibitors, which inhibit the activity of MMPs [115, 116]. A study of the inflammatory response during skeletal muscle repair indicated that injured muscle recruited pro-inflammatory monocytes upon initial muscle injury, but that these cells soon switched their phenotype to become anti-inflammatory monocytes that then secreted TGFβ1. Furthermore, they demonstrated that co-culture of muscle cells with pro-inflammatory monocytes caused muscle cell proliferation, while co-culture with anti-inflammatory monocytes induced differentiation of muscle cells, suggesting that inflammatory cytokines can have multiple effects on cellular processes, and that muscle cells are sensitive to these tightly regulated mechanisms of cytokine signaling [117].

This regulation of the inflammatory response by muscle tissue has clinical significance as well – particularly and perhaps most strikingly with

regard to its effect on cartilage metabolism. Centronuclear myopathy, a disease characterized by severely weakened muscle, has been shown to be correlated with articular fibrosis of the cartilage of the temporomandibular joint (TMJ), suggesting perhaps that the altered secretory profile of the defective muscle in these patients yields phenotypic changes in the immediately juxtaposed cartilage tissue [118]. Furthermore, Slemenda et. al. concluded that muscle weakness was a causal risk factor for osteoarthritis in a cohort of women [119]. While these clinical studies do not definitively provide a direct role of muscle in the regulation of adjacent cartilage tissue, they do provide sufficient evidence to warrant further study to characterize the interaction of these tissues under inflammatory conditions.

1.5.2 Muscle cells as a potential therapy: arthritis and fracture healing

Muscle cells may ultimately be harnessed as a therapeutic tool. Further study of the anti-inflammatory properties of muscle cells may make them an attractive candidate for the treatment of arthritis. Muscle secreted factors could not only attenuate the response of chondrocytes in an inflamed arthritic joint, they could also potentially promote host integration of engineered cartilage constructs. Furthermore, given their capacity to form cartilage as well as respond to inflammatory stimuli, stem-like progenitor cells from muscle could also be used to enhance fracture healing. Once fully elucidated, it may be possible to utilize muscle cell-mediated cartilage regulation for the purpose of treating cartilage-based injury and disease.

In summary, the relationship between muscle and cartilage is an intricate one – from development, into adulthood and also during disease

states. Analysis of the signaling pathways that guide the differentiation and target gene expression of both tissue types have widespread implications – not only for the basic understanding of various processes but also for usage in potential translational applications.

1.6 Chapter 1 figures and legends

Figure 1.1

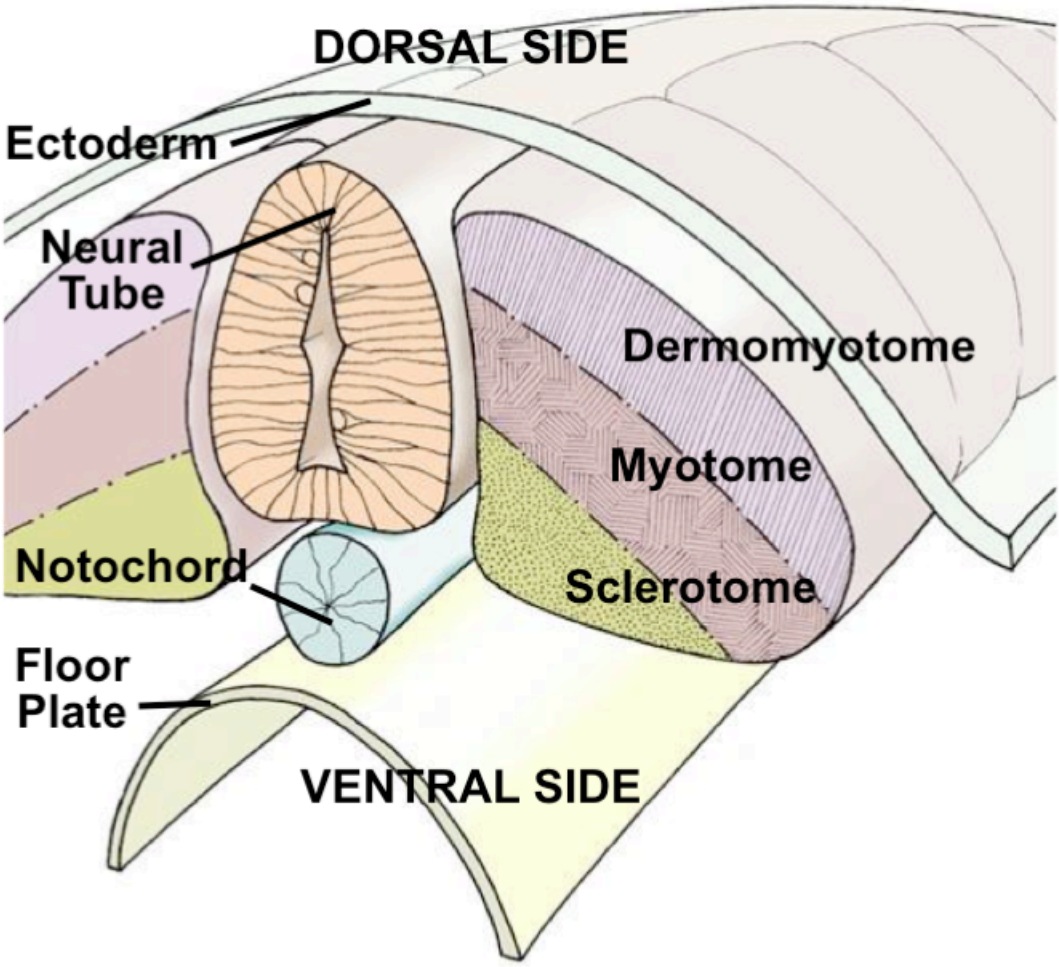


Fig. 1. Schematic illustration of the cross-section through a somite-stage embryo. Various signaling molecules secreted from neighboring structures such as the ectoderm, neural tube, notochord, and floor plate serve to pattern the somite into dermomyotome, myotome and sclerotome cell fates. Modified from http://www.bionalogy.com/skeletal_system.htm.

Figure 1.2

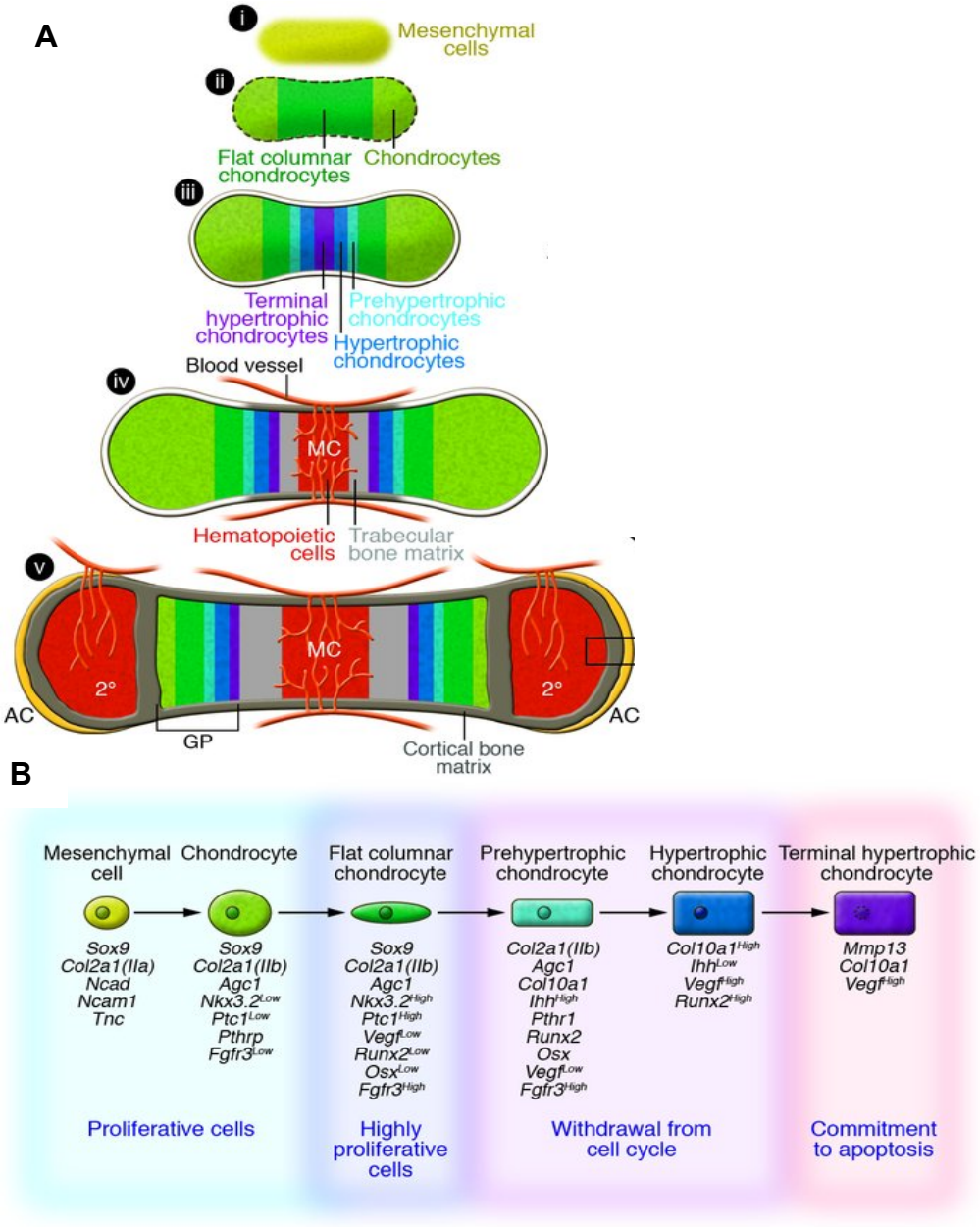

A
i
Mesenchymal cells
ii
Flat columnar chondrocytes
Chondrocytes
iii
Terminal hypertrophic chondrocytes
Prehypertrophic chondrocytes
Hypertrophic chondrocytes
Blood vessel
iv
MC
Hematopoietic cells
Trabecular bone matrix
v
2°
MC
2°
AC
AC
GP
Cortical bone matrix
B
Mesenchymal cell
Chondrocyte
Flat columnar chondrocyte
Prehypertrophic chondrocyte
Hypertrophic chondrocyte
Terminal hypertrophic chondrocyte
Sox9 Col2a1(IIa) Ncad Ncam1 Tnc
Sox9 Col2a1(IIb) Agc1 Nkx3.2Low Ptc1Low Pthrp Fgfr3Low
Sox9 Col2a1(IIb) Agc1 Nkx3.2High Ptc1High Vegfl.Low Runx2Low OsxLow Fgfr3High
Col2a1(IIb) Agc1 Col10a1 IhhHigh Pthr1 Runx2 Osx Vegfl.Low Fgfr3High
Col10a1High IhhLow Vegfl.High Runx2High
Mmp13 Col10a1 Vegfl.High
Proliferative cells
Highly proliferative cells
Withdrawal from cell cycle
Commitment to apoptosis

Fig. 2. Stages and respective markers of cartilage and bone development during endochondral ossification. (A) The process begins with mesenchymal cell condensation (i); followed by chondrocyte differentiation and the formation of the cartilage template of the long bone (ii). These chondrocytes then mature and undergo hypertrophy (iii). The primary ossification center becomes vascularized and bone formation begins (iv). The secondary ossification centers then form, and the marrow cavity develops (v). (B) Schematic of the intracellular changes that occur during this process. Markers are listed for each stage of chondrocyte differentiation. Modified from Zuscik et. al. [67]

CHAPTER 2: A gradient of Shh establishes mutually repressing somitic cell fates induced by Nkx3.2 and Pax3

2.1 Introduction

In the vertebrate embryo, somites give rise to multiple tissues including cartilage, bone and muscle of the vertebral column. Somites are pairs of spherical structures located adjacent to the neural tube of the developing embryo. They are formed by periodic budding from the presomitic mesoderm, the timing of which is a highly controlled event[120-122]. A newly formed somite consists of epithelial cells at the periphery, with a small number of mesenchymal cells in the center (somitocoele)[123]. As a somite matures, the cells within the somite are separated into ventral and dorsal domains. The sclerotome is formed from cells located in the ventral domain of the somite, where the once epithelial cells become mesenchymal. Cartilage and bone of the vertebrae and ribs are derived from the sclerotome. The dermomyotome is formed in the dorsal domain of the somite, where the cells remain epithelial. As somite maturation proceeds, the dermomyotome will give rise to both the dermis and an interstitial layer of differentiated skeletal muscle, the myotome [122].

The initial specification of the dorsal and ventral somitic cell fates is vital because it determines the number of precursors that will give rise to either cartilage, muscle or dermal cells. When a somite first forms, all the epithelial cells in the immature somite have the potential of adopting a sclerotomal or a dermomyotomal cell fate. In somite rotation experiments, when a newly formed somite was rotated 180°, the cells were reprogrammed to become either sclerotome or dermomyotome based upon their new

location[4, 6], indicating that cell fate had not yet been determined in these newly formed somites and that local environmental signals induce the appropriate somitic cell fate. Multiple extracellular signals expressed in the surrounding tissues determine somite cell fates [122]. Wnt proteins secreted from the dorsal neural tube (Wnt1 and Wnt3a) and from the surface ectoderm (Wnt4, Wnt6 and Wnt7a) promote the formation of the dermomyotome and myotome [12, 24, 124-128]. Ectopic expression of Wnt proteins has been noted to induce the expression of both Pax3 and Pax7, which are expressed throughout the dermomyotome and in proliferative myogenic precursors that migrate into the myotome [12, 24, 125, 126, 129, 130]. Furthermore, Wnt signals promote the expression of myotome-specific markers, including MyoD, Myf5 and Myosin Heavy Chain, in a PKA or PKC-dependent manner [127, 131, 132]. In addition to inducing the dorsal somite fate, Wnt proteins have also been documented to inhibit the expression of the sclerotomal marker Pax1 [12, 24, 133].

Sonic Hedgehog (Shh), a signaling molecule secreted by both the notochord and floor plate of the neural tube, is essential to induce formation of the sclerotome and cartilage differentiation of ventral somitic cells[11-14]. In the Shh knockout mouse, sclerotome formation is compromised, and no vertebral column is formed[11]. Similarly, mice lacking all hedgehog signaling due to a loss of the hedgehog signal transducer smoothened, fail to express the sclerotomal markers Pax1 and Nkx3.2 [134] . Moreover, gain-of-function studies have demonstrated that ectopic expression of Shh induced the expression of Pax1 while inhibiting the expression of the dermomyotomal marker Pax3[10, 14].

Shh also promotes the myotome cell fate[16, 127, 135-137]. Mouse embryos lacking Shh or its receptor smoothened display markedly decreased Myf5 expression, suggesting that Shh is essential for muscle formation[11, 134]. Several lines of evidence support the idea that myogenesis in the epaxial (i.e., medial) domain of the myotome requires the combination of both Shh and Wnt signaling (reviewed by Bailey et. al. [138]). A molecular understanding of how such signals can synergistically activate Myf5 expression can be explained by the dual requirement for both TCF and GLI binding sites in the enhancer element that drives Myf5 expression in the epaxial myotome[18].

Shh signals may promote myotome formation by promoting the maturation of dermomyotomal cells to become MyoD/Myf5 expressing myotomal cells[139, 140]. In zebrafish, Shh signaling is necessary for Pax3/7-expressing cells in the dermomyotome to transition into MyoD-expressing cells; in the absence of Shh signaling, Pax3 and Pax7-expressing cells in the somite increase in number but fail to activate the myogenic[139, 140]. Therefore, Shh is required for the differentiation of Pax3/7-expressing dermomyotomal cells into myotomal cells and for the subsequent loss of Pax3/7 gene expression in these differentiated myocytes. These findings in zebrafish are consistent with earlier findings in chicken embryos which indicate that ectopic expression of Shh in the somite induced loss of Pax3 expression while promoting premature myogenic differentiation[141]. Thus Shh acts in the somite to drive sclerotome formation, repress the expression of dermomyotomal gene expression, and induce the maturation of the dermomyotome into differentiated myotomal cells.

Prior studies have indicated that the dorsal–ventral identity of somitic cells is established by both antagonistic and synergistic activities of Wnt and Shh signals[12, 13, 20]. Fan and Tessier-Levigne demonstrated that signaling by cells expressing either Wnt1 or Shh, when positioned adjacent to somites, is able to travel through the entire distance of a young somite and that these signaling pathways can induce mutually exclusive patterns of dermomyotomal or sclerotomal gene expression, respectively[12, 13]. Thus cells within the somite must interpret Wnt signals, secreted by both the neural tube and surface ectoderm, and Shh, secreted by the notochord and floor plate, to establish dorsal and ventral somitic fields with clear borders. However, it is not clear from these prior studies whether Shh and Wnt act as morphogens to set up these different cell types, with distinct fates being designated by differing levels of signaling by these factors. To examine this issue more thoroughly, we have examined the response of somitic cells to varying levels of Shh in the presence of constant Wnt signaling. We find that differing levels of Shh elicit differing responses from somitic cells, with very low levels of Shh working in combination with Wnt signals to maintain dermomyotomal gene expression, slightly higher levels inducing both loss of dermomyotomal markers and activation of myogenic differentiation, and yet higher levels inducing the loss of myotomal markers and the activation of sclerotomal gene expression. In addition, we have found that in the presence of high levels of Wnt signaling, instead of inducing sclerotomal markers, Shh acts to maintain the expression of dermomyotomal and myotomal markers.

Two of the sclerotomal genes induced by high levels of Shh signaling are Nkx3.2 and Sox9, which are both known to promote cartilage

formation[58, 60, 142]. Ectopic expression of either Nkx3.2 or Sox9 can induce ectopic cartilage formation in the chick embryo[58, 60]. Mice lacking Nkx3.2 (a.k.a., Bapx1) display a severe reduction of somitic Sox9 expression and defects in axial cartilage formation, suggesting that Nkx3.2 promotes chondrogenesis by inducing Sox9 expression.[56, 59, 143] Sox9 in turn activates the chondrogenic program by directly activating expression of cartilage-specific genes such as collagen II and aggrecan, and Sox9-deficient cells are incapable of chondrogenic differentiation[52, 54, 59, 143, 144]. However because both Nkx3.2 and Sox9 are expressed during early phases of somite patterning prior to cartilage differentiation, it is not clear whether Nkx3.2 and Sox9 act only on a pre-existing ventral somite population to promote chondrogenesis or possibly also play an earlier role in demarcating the ventral somite field. Consistent with this latter notion, we have found that ectopic expression of Nkx3.2 blocks the expression of both dermomyotomal and myotomal markers in vitro and in vivo. Pax3 is a transcription factor expressed in the dermomyotome and subsequently in proliferating cells within the myotome [129, 145-148] and plays an important role in promoting muscle differentiation[30, 129, 147]. We have found that forced expression of Pax3 in somites can block Shh-mediated induction of sclerotomal gene expression and chondrocyte differentiation in vitro. Thus we propose that varying levels of Shh signals act as a morphogen to elicit differing responses from somitic cells, and that Pax3 and Nkx3.2 set up mutually repressing cell states that promote either dermomyotome/myotome or sclerotome differentiation, respectively.

2.2 Materials and methods

2.2.1 Materials

White leghorn chicken eggs were obtained from Charles River Laboratory (Willmington, MA) and Hy-Line International (Elizabethtown, PA). Mouse anti-Pax3, anti-Pax7 and anti-Myosin antibodies were purchased from Developmental Studies Hybridoma Bank. Rabbit anti-HA and rabbit anti-V5 antibodies were purchased from Sigma. Rabbit anti-GFP antibody was purchased from Abcam. N-Shh was produced as described in Zeng et al. (2002). Human BMP4 protein was purchased from R&D. Wnt3a conditioned medium was produced from Wnt3a-secreting cells (ATCC) as instructed. RatB1a control and RatB1a-Wnt1 cells were gifts from Dr. Jan Kitajewski (Columbia University). Alkaline phosphatase virus (RCAS (A)-AP) and Pax3 virus (RCAS (A)-AP) were generated as described in Maroto et al. (1997). Plasmids for making in situ hybridization probes have been described before[23, 58]. For the construction of RCAS (B)-Sox9V5 plasmid, chicken Sox9 sequence (from PCR) was cloned into pBluescript-V5 vector (kindly provided by Dr. Tony Ip, UMass) so that V5 was tagged at the C-terminus of Sox9. Then pBluescript-Sox9V5 was cut with EcoRI and XbaI, and blunt ended, and subsequently cloned into the RCAS-B viral vector. Electroporation vector pMES-GFP was kindly provided by Dr. Tom Schultheiss (Beth Israel Deaconess Medical Center). pMES-Nkx3.2 was generated by PCR cloning of chicken Nkx3.2 into BamHI cut pMES vector. pMES-Sox9V5 was generated by cloning EcoRI and XbaI cut fragment from pBluescript-Sox9V5 into SmaI cut pMES-GFP vector. Nkx3.2 virus was generated using RCAS (B)-Nkx3.2HA plasmid as described[60]. Viruses were produced according to the standard protocol[14], and were titered by direct

visualizing GFP expression (in the case of RCAS-GFP production) or indirect immunocytochemistry using anti-HA or anti-V5 antibodies. All viruses used have reached a titer of 10^8 particles/ml.

2.2.2 Embryo explant culture and viral infection

Somite explants from chicken embryos of HH stage 10 were excised as described[58]. Briefly, chicken embryos were pinned onto an agar dish with the ventral side up. The embryos were treated with dispase (Roche, 2 mg/ml) to dissociate the germ layers. Tungsten needles were used to dissociate the endoderm layer and to slice out the mesodermal somite tissues. When culturing the somites with the surface ectoderm, no dispase was used. The explants were incubated in 5 µl concentrated virus stock (titer of 10^8/ml) for 30 min–1 h on ice before being transferred onto the collagen gel[58]. The collagen gel was constructed as a lower collagen layer and an upper collagen layer, with the somite explants in the middle. The composition of the collagen layers is: 30% rat tail collagen I (BD biosciences) and 1× DMEM. A total of 25 µl of collagen gel mixture was used for each explant. After the collagen solidified at room temperature, explants in collagen gel were cultured in 400 µl of α-MEM somite culture medium (with 10% FBS, 1% penstrep, 5% chick embryo extract, 1 ng/ml bFGF) in 4-well dishes (Nunc) at 37 °C for 1–6 days before being harvested for either RT-PCR analysis or immunocytochemistry[149]. Results from Fig. 1 were obtained using only embryos of 11 somites. Co-cultures with control Rat1B cells or Rat1B-Wnt1 cells were performed according to Münsterberg et al. (1995). Briefly, the Rat1B cells were scraped off tissue culture grade plastic and subsequently plated onto an agar dish containing medium for 4 h to overnight. Aggregates

of Rat1B cells (which form on agar dishes) were cut into small pieces and co-cultured together with the presomitic mesoderm explants in the collagen gel.

2.2.3 RT-PCR analysis

Explants were processed by the RNeasy mini kit from Qiagen[58]. Primer sequences have been described before[23, 58]. All PCR analyses were normalized based on GAPDH expression quantified by a phospho-imager (BioRad).

2.2.4 Immunocytochemistry

Embryo explants were fixed with 4% paraformaldehyde and incubated with primary antibodies overnight[149]. After washing with PBST, they were incubated with secondary antibodies (conjugated with Alexa 488 (green) or 594 (red) from Invitrogen) overnight, followed by repeated washing the day after. Electroporated embryos were serial-sectioned first on the cryostat (10 μm thick, Microm HM560) before immunostaining. The sections on slides were incubated overnight with primary antibodies, and for 4 h with the secondary antibodies.

2.2.5 In situ hybridization

Whole mount in situ hybridization was performed as described[60]. After whole mount in situ, the embryos were sectioned on the cryostat (Microm HM560) at 10 μm.

2.2.6 Electroporation

For in ovo electroporation, plasmid DNA of 1 μg/ml concentration was injected into the lumen of the newly formed somites using a micromanipulator (Parker Picospritzer II), and immediately electroporated using the electroporator (BTX ECM830) on chicken HH Stage 14–16 embryos. The electrodes are Genetrodes by Genetronics, model 514. Electroporation conditions are: 30 V, 20 ms pulse length, 5 pulses with 600 ms intervals. After the operation, the window of the shell was sealed with scotch tape, and allowed to incubate at 37 °C for 2 days before the embryos were beheaded and fixed for immunocytochemistry.

2.2.7 Imaging

In situ hybridization and immunocytochemistry were visualized and photographed on the following microscopes: Olympus IX71 inverted microscope, Nikon Eclipse 800 compound microscope, Leica MZ16F stereo microscope and Leica TCS SP2 AOBS confocal microscope.

2.3 Results

2.3.1 Shh gradient determines dorsal–ventral somitic cell fates

To determine the effect of varying levels of Shh signaling on the specification of somitic cell fates, we cultured chicken presomitic mesoderm explants (isolated from stage 10 chicken embryos) with a constant amount of Wnt3a-conditioned medium and increasing levels of the N-terminal fragment of Sonic Hedgehog (N-Shh), which is soluble and a potent Shh agonist[150]. We employed Wnt3a-conditioned medium[151] in these cultures, to ensure that each of the explants would receive the same amount of Wnt3a signal.

Following incubation of the explants in increasing concentrations of N-Shh, we assayed the expression of both dorsal and ventral somite markers by RT-PCR analysis. We varied the N-Shh concentration in the culture medium from 34 ng/ml–2 μg/ml. We believe this level of Shh signaling reflects a physiological level of Shh signal transduction, as downstream markers induced by N-Shh were expressed at similar levels as in somite explants cultured in the presence of notochord (a source of endogenous Shh; data not shown). Our RT-PCR analysis indicated that Patched 2 (Ptc2), a previously described Hedgehog responsive gene[152], displayed a gradual increase in expression in response to increasing levels of N-Shh (Fig. 1A, lanes 1–8). In contrast, the expression of dermomyotomal and myotomal markers responded to increasing levels of N-Shh in a complicated and biphasic pattern. While a low level of N-Shh (34 ng/ml) induced the expression of Pax3 in somitic explants (Fig. 1A, lane 2), further increasing levels of N-Shh (67 ng/ml–250 ng/ml) led to a steady decrease of Pax3 expression (Fig. 1A, lanes 3–6). Consistent with these results, an earlier analysis of mouse somite explants showed that a low level of Shh at 2.5 nM (which is equivalent to 35 ng/ml) did not inhibit Pax3 expression, while higher levels of Shh at 25 nM (equivalent to 350 ng/ml) inhibited expression of murine Pax3 (Fan et al., 1997). Interestingly, the loss of Pax3 gene expression induced by increasing levels of N-Shh correlated with the activation of MyoD and Myf5 at these intermediate concentrations of N-Shh (67–250 ng/ml) (Fig. 1A, lanes 3–6). While intermediate levels of N-Shh-induced MyoD and Myf5 expression (Fig. 1A, lanes 4–6), concentrations of N-Shh at or above 500 ng/ml led to a precipitous loss of both MyoD and Myf5 expression (Fig. 1A, lanes 7–8).

Expression of the sclerotomal markers, Nkx3.2, Sox9 and Pax1, were induced to maximal levels by a threshold intermediate concentration of N-Shh (125–500 ng/ml) (Fig. 1A, lanes 3–5). Induction of Pax1 required greater levels of N-Shh than did either Nkx3.2 or Sox9 (Fig. 1A, lanes 3–5). These findings suggest that sclerotome gene expression requires a threshold level of Shh signaling, and that the induction of Pax1 requires a higher concentration of Shh, as compared to that of Nkx3.2 and Sox9. These in vitro findings are consistent with the fact that compared to other sclerotomal markers, Pax1 is only highly expressed in the ventral-medial domain of the sclerotome, next to the notochord that secretes Shh, and therefore in the region of the somite exposed to the highest level of Shh.

We were puzzled by the fact that low levels of N-Shh (when combined with Wnt3A) induced the expression of Pax3 in somite explants, as the dermomyotome expression domain of Pax3 is expanded in mutant mice embryos lacking Shh[11, 153]. We considered the possibility that N-Shh might induce the expansion of a small population of Pax3-positive cells within the somite, because Shh is known to both induce somitic cell proliferation and block apoptosis[17, 135, 154]. To explore this possibility, we cultured the presomitic mesoderm explants for either one day or five days in Wnt3a conditioned medium either in the presence or in the absence of Shh (125 ng/ml). After only one day of culture, explants treated with either control conditioned medium or with medium containing only Shh failed to express Pax3 (Fig. 1B, panels 1 and 2). In striking contrast, after one day of culture Pax3 protein was easily detected in explants treated with Wnt3a alone (Fig. 1B, panel 3). Addition of N-Shh (125 ng/ml) did not significantly alter the

expression level of Pax3 induced by Wnt3a conditioned medium, possibly because this level of Shh was not very high (Fig. 1B, panel 4). This finding is consistent with prior reports indicating Wnt signaling alone is sufficient to induce Pax3 expression in explants of murine presomitic mesoderm cultured for 24 h[24]. Furthermore, work in zebrafish employing mutants in the hedgehog pathway or the hedgehog antagonist, cyclopamine, indicates that the induction of Pax3 or Pax7 expression in the dermomyotome of this species does not require hedgehog signaling[139, 140]. In marked contrast to explants cultured for only one day, presomitic mesoderm explants cultured for five days did not express detectable Pax3 protein when cultured in Wnt3a conditioned medium alone (Fig. 1B, panel 7). However, like the one-day cultures, the explants cultured for five days in the combination of Wnt3a-conditioned medium and N-Shh (125 ng/ml) expressed significant levels of Pax3 (Fig. 1B, panel 8). This finding is consistent with our RT-PCR data shown in Fig. 1A, where the explants were analyzed after five days of culture. Thus our analysis suggests that Shh may be required to maintain Pax3 expression in explants of presomitic mesoderm cultured for five days in Wnt3a conditioned medium, although it may not be required to initiate Pax3 expression in explants cultured for only one day in Wnt3a conditioned medium. To investigate whether more mature somites also require exogenous Shh to express Pax3, we cultured somites IV–VI for five days in either the absence or presence of Wnt3a conditioned medium. In contrast to explants of presomitic mesoderm, high levels of Pax3 were detectable in explants of somite IV to VI that had been cultured for five days in Wnt3a conditioned medium (Fig. 1C). Interestingly, these more mature somites behave in vitro as if they have already been exposed to Shh signals in

vivo[127]. Therefore, our analyses suggest that lower levels of Shh signaling act in combination with Wnt signals to promote the maintenance of Pax3 expression in somites cultured in vitro.

We propose that a gradient of Shh patterns somitic cells to adopt different cell fates (Fig. 1D). Working in combination with Wnt signaling, the lowest level of Shh signal helps to maintain the expression of the dermomyotomal marker, Pax3, in vitro, while higher levels of Shh signaling both repress the expression of Pax3 and induce the expression of MyoD and Myf5. Greater levels of Shh signaling repress the expression of myotomal markers while allowing the continued expression of sclerotomal makers, which are apparently activated by a threshold level of Shh signaling. These in vitro findings are consistent with the in vivo expression patterns for Pax3, Myf5, MyoD, Nkx3.2, Sox9 and Pax1, which are expressed in progressively more ventralized somitic domains, respectively (Fig. 1D).

2.3.2 Comparison of somitic expression of Pax3, Nkx3.2 and Sox9

Our RT-PCR results led us to hypothesize that a relatively low concentration of N-Shh (as low as 67 ng/ml) can induce factors that antagonize Pax3 expression. As Sox9 and Nkx3.2 were induced by intermediate concentrations of N-Shh that repressed the expression of Pax3 (Fig. 1A, lanes 3 and 4), we considered the possibility that either Sox9 or Nkx3.2 may act to repress the expression of Pax3. To determine if Sox9 and Nkx3.2 are inhibitors of Pax3 expression, we first directly compared their expression with that of Pax3 during the period when the dorsal–ventral somite domains are determined. Whole mount in situ hybridization analysis of

stage 10 chicken embryos revealed that Pax3 is expressed in the dorsal neural tube, throughout the rostral presomitic (-I somite) mesoderm and in dorsal regions of more mature somites (Figs. 2A and B). In the first somite, Pax3 is expressed throughout the somite, with higher levels of expression in the dorsal domain; as the somite matures, Pax3 expression is gradually restricted to the dorsal domain[29, 155, 156]. In contrast to Pax3, Nkx3.2 expression is absent from the presomitic mesoderm, but is strongly expressed in the ventral region of somite III as well as in the somitocoele (Figs. 2A and B, panels 5–8). Interestingly, the loss of Pax3 expression from the ventral region of somite III correlates with the induction of Nkx3.2 expression in this somitic domain (Fig. 2B, compare panels 2 and 6). Unlike Pax3 and Nkx3.2, Sox9 exhibits a more dynamic expression pattern during somite maturation. Sox9 is initially expressed throughout the presomitic mesoderm before the budding of the first somite; its expression largely diminishes in the next two somites and only begins to reappear starting from the third somite in the anterior ventral domain (Figs. 2A and B, panels 9–12). Sox9 is strongly expressed in the ventral region of more mature somites (somite V–VII) (Figs. 2A, and B, panel 9). Our expression analyses indicate that dermomyotomal and sclerotomal gene expression is segregated into dorsal and ventral domains well before the morphological changes indicative of a differentiated dermomyotome and myotome. The restriction of Pax3 expression to the dorsal somite correlates with the induction of Nkx3.2 in the ventral region of the newly formed somite. In contrast, Sox9 expression is not yet expressed at high levels in the ventral domain of the somite until well after Pax3 expression is already restricted to the dorsal somite and Nkx3.2 is strongly expressed in the ventral somitic domain. These dynamic expression

patterns raise the possibility that the induction of Nkx3.2 in the ventral somite may act to both inhibit Pax3 expression and augment that of Sox9 in this region of the somite.

2.3.3 Nkx3.2 and Sox9 inhibit Wnt3a-induced Pax3 expression

To explore whether Nkx3.2 or Sox9 has the potential to directly or indirectly repress the expression of dermomyotomal genes in the ventral somite, we examined the effect of retrovirally-expressed Nkx3.2 and Sox9 on the expression of a dorsal marker, Pax3, in isolated somites. In the embryo, Pax3 expression is induced by Wnt proteins secreted by either the neural tube (Wnt1, Wnt3a) or the surface ectoderm (Wnt4, Wnt6 and Wnt7a)[9, 23, 24, 155]. Consistent with these prior observations, culture of somites IV–VI from stage 10 chick embryos, which behave as if they have already seen a Shh signal in vivo[20], expressed robust levels of Pax3 in response to Wnt3a conditioned medium alone (Fig. 1C). Thus, we investigated whether the expression of retroviral encoded Nkx3.2 or Sox9 would blunt the ability of Wnt3a conditioned medium to induce the expression of Pax3 in such cultured somites. Robust expression of Pax3 (red) was induced by Wnt3a conditioned medium in somite explants infected with a control virus encoding GFP (RCAS-GFP) (Fig. 3A, panels 1–4). Extensive overlap was found between GFP and Pax3 expression, indicating that ectopic GFP expression does not inhibit Pax3 expression. In contrast, similarly cultured somites infected with a retrovirus encoding HA-tagged Nkx3.2 (RCAS-Nkx3.2HA) displayed markedly reduced Pax3 expression (Fig. 3A, panels 5–8). Indeed, almost no Nkx3.2HA-expressing cells (green) expressed Pax3 (red), suggesting that forced Nkx3.2 expression strongly inhibits Pax3 expression in somitic cells

(Fig. 3A, panels 5–8). While somites infected with a retrovirus encoding V5-tagged Sox9 (RCAS-Sox9V5) still expressed Pax3, the majority of cells that expressed viral Sox9V5 did not express Pax3 (Fig. 3A, panels 9–12). Some of the Sox9V5-expressing cells appear to form clusters that are devoid of Pax3 expression (Fig. 3A, panels 9–12). It is possible that ectopic Sox9 expression has converted these cells into proliferating cartilage cells (see below) [58].

We quantified this immunostaining result by calculating the percentage of virus-expressing cells that were Pax3-positive (Fig. 3B). In GFP-infected explants, 89% of the GFP-expressing cells were Pax3-positive. In contrast, only 1% of the Nkx3.2-expressing cells were Pax3-positive, indicating that ectopic Nkx3.2 strongly inhibits the expression of Pax3 induced by Wnt3a. In RCAS-Sox9V5 infected explants, only 38% of the Sox9-expressing cells were Pax3-positive, suggesting that ectopic Sox9 represses, but does not completely inhibit, Wnt3a-induced Pax3 expression (Fig. 3B). Because virally encoded Sox9 is known to activate expression of endogenous Nkx3.2 in infected somites[58], it is possible that the reduced level of Pax3 expression observed in RCAS-Sox9V5 infected cells reflects the induction of Nkx3.2 in these cultures.

As prior work has established that forced expression of either Nkx3.2 or Sox9 can activate chondrogenesis in somite explants cultured in medium containing BMPs [60], we investigated whether somites cultured in Wnt3a conditioned medium and infected with either RCAS-Nkx3.2HA or RCAS-Sox9V5 have similarly adopted a cartilage cell fate. To assay for chondrocyte differentiation, we evaluated collagen II expression in these explants via

immunocytochemistry. While neither RCAS-GFP-infected cells nor RCAS-Nkx3.2HA-infected cells expressed any collagen II (Fig. 3C, panels 1–8), RCAS-Sox9V5-infected cells were all collagen II-positive (Fig. 3C, panels 9–12). This finding suggests that ectopic expression of Nkx3.2 is not sufficient to activate the chondrocyte differentiation program in somites cultured in Wnt conditioned medium, while expression of ectopic Sox9 is able to activate this differentiation program in similarly cultured cells.

It is possible that RCAS-Nkx3.2-infected cells did not adopt a cartilage fate due to an insufficient amount of BMP in the medium, as prior work has indicated that Nkx3.2 requires a higher amount of BMP4 than does Sox9 to induce chondrogenesis [58]. To examine if Nkx3.2 failed to induce collagen II expression due to an insufficient amount of BMP in the culture medium, we administered exogenous BMP4 to RCAS-Nkx3.2HA-infected somites exposed to Wnt3a conditioned medium. In the presence of both exogenous BMP4 and Wnt3a conditioned medium, RCAS-Nkx3.2HA infected cells still failed to express any detectable collagen II (Fig. 3D, panels 5–8). In contrast, RCAS-Nkx3.2 infected somites cultured in the presence of exogenous BMP4 and in the absence of Wnt3a conditioned medium expressed detectable levels of collagen II (Fig. 3D, panels 9–12). These findings indicate that Wnt3a conditioned medium blocks the ability of Nkx3.2 to induce cartilage formation even in the presence of exogenous BMP4.

2.3.4 Nkx3.2, but not Sox9, inhibits surface ectoderm-induced Pax3 expression

As Pax3 expression in the somite is strongly induced by signals from the surface ectoderm, we examined the effects of exogenous Nkx3.2 and

Sox9 on the induction of Pax3 by signals from this tissue[23, 131]. Culture of somites IV–VI (but not somites I–III) with the adjacent surface ectoderm induces the expression of Pax3 in such explants [23]. We thus infected explants of somites IV–VI cultured with the adjacent surface ectoderm with either RCAS-GFP, RCAS-Nkx3.2HA or RCAS-Sox9V5. While we observed extensive overlapping expression of GFP (green) and Pax3 (red) (Fig. 4A, panels 1–4) in the RCAS-GFP-infected explants, very few of the RCAS-Nkx3.2HA-infected explants expressed Pax3 (Fig. 4A, panels 5–8). Unlike RCAS-Nkx3.2HA-infected explants, explants infected with RCAS-Sox9V5 exhibited robust Pax3 expression, and many of the Sox9V5 positive cells were also Pax3 positive (Fig. 4A, panels 9–12), indicating that ectopic Sox9 does not inhibit the expression of Pax3 induced by the surface ectoderm. Quantification of the immunostaining result described above indicated that 96% of the GFP-infected cells expressed Pax3, while only 4% of the Nkx3.2HA-expressing cells were Pax3-positive, and 70% of cells that expressed Sox9V5 were Pax3-positive (Fig. 4B). Therefore, our results indicate that ectopic Nkx3.2 expression efficiently inhibits Pax3 expression induced by signals from the surface ectoderm as compared to ectopic Sox9 expression, which only slightly dampens Pax3 expression in these same cultures.

We determined whether ectopic expression of either Nkx3.2 or Sox9 was able to drive cartilage differentiation of somites cultured with adjacent surface ectoderm. While we were unable to detect expression of the cartilage-specific marker collagen II in somite/ectoderm explants infected with RCAS-GFP (Fig. 4C, panels 1–4), explants infected with RCAS-Nkx3.2HA

occasionally exhibited low levels of collagen II expression (in less than 1% of the infected cells) (arrows, Fig. 4C, panels 5–8). In contrast, explants infected with RCAS-Sox9V5 robustly expressed collagen II (Fig. 4C, panels 9–12). In the presence of exogenous BMP4, however, RCAS-Nkx3.2HA induced a significant amount of collagen II expression in somite/ectoderm explants (Fig. 4D, panels 5–8), confirming again that Nkx3.2 requires BMP signaling to induce chondrogenesis[58]. Taken together, our findings indicate that ectopic Nkx3.2 blocks the induction of Pax3 by either soluble Wnt3A or signals from the surface ectoderm without inducing chondrocyte differentiation. Nkx3.2-mediated repression of Pax3 expression is therefore similar to the loss of Pax3 expression in the ventral domain of the newly formed somite, which occurs well before chondrogenesis takes place in these same cells. In contrast, Sox9, which efficiently induces collagen II expression does not block Pax3 expression.

2.3.5 Ectopic Nkx3.2 inhibits the expression of dermomyotomal markers in vivo

Our in vitro findings with somite explants indicate that Nkx3.2 can efficiently inhibit the expression of Pax3 in response to either Wnt3a or signals from the dorsal ectoderm; in contrast, Sox9 can decrease but not block the expression of this dorsal somite marker. To evaluate whether ectopic expression of Nkx3.2 can similarly block expression of dorsal somitic markers in vivo, we electroporated Nkx3.2 into the dorsal somite cells of chick embryos via in ovo electroporation. Nkx3.2 was cloned into the electroporation vector pMES, which includes an IRES-GFP module that enables GFP to be co-expressed in the same cells as Nkx3.2[22, 157].

pMES-Nkx3.2 was subsequently electroporated into the dorsal cells of newly formed somites in stage 13 to 15 chicken embryos. To confirm ectopic Nkx3.2 expression in dorsal somite cells following electroporation of pMES-Nkx3.2, we performed whole mount in situ hybridization (ISH) analysis using an Nkx3.2 RNA probe. Transverse sections of these electroporated embryos revealed that endogenous Nkx3.2 was expressed in ventral somite cells and that exogenous Nkx3.2 was expressed in dorsal somite cells (Fig. 5A). For comparison, we also electroporated a Sox9-encoding plasmid (pMES-Sox9V5) into the newly formed somites. To confirm the ectopic expression of Sox9 in the dorsal somite cells, we performed immunocytochemistry employing an anti-V5 antibody. Indeed, the epithelial cells in the dorsal lateral domain of the somite expressed ectopic Sox9V5 (red), and were also GFP-positive (green) (Fig. 5B). Therefore, both Nkx3.2 and Sox9 were targeted to the dorsal somite cells, and our immunocytochemistry result reassured us that we could use GFP expression to identify cells co-expressing either ectopic Nkx3.2 or Sox9 plus GFP.

To determine the effects of ectopic Nkx3.2 on dorsal somitic gene expression, we evaluated the expression of dermomyotomal markers Pax3 and Pax7[129]. We observed disrupted expression of Pax3 and Pax7 in the dermomyotome following ectopic expression of Nkx3.2 (Fig. 5C, panels 5–6). We are confident that ectopic Nkx3.2 was targeted into the dorsal Pax3 and Pax7-positive cell population, because Nkx3.2-expressing cells lie dorsal to the myosin-positive myotome cells (data not shown). Examination at higher magnification indicated that dorsal cells expressing Nkx3.2 (green) no longer expressed Pax3 and Pax7 (red) (Fig. 5C, panels 7–8). In contrast, Pax3 and

Pax7 expression were not perturbed in the dermomyotome of embryos electroporated with either GFP or Sox9 expression vehicles (Fig. 5C, panels 1–4 and 9–12). Thus ectopic expression of Nkx3.2 was able to inhibit the expression of Pax3 and Pax7 in vivo, suggesting that Nkx3.2 prevents the adoption of dorsal somitic cell fates in vivo. Quantification of the immunostaining result following in ovo electroporation indicated that in cells electroporated with GFP alone, 76% expressed Pax3 and 80% expressed Pax7 (Fig. 5D). In contrast, in cells electroporated with Nkx3.2, only 3% expressed Pax3 and only 7% expressed Pax7. In cells electroporated with Sox9V5, 51% expressed Pax3 and 66% expressed Pax7 (Fig. 5D). Therefore, these in vivo results are consistent with our in vitro studies and together indicate that ectopic expression of Nkx3.2, but not Sox9, efficiently inhibits expression of both Pax3 and Pax7.

In addition, we evaluated whether ectopic expression of Nkx3.2 in the dorsal somite promotes these cells to adopt a ventral cell fate by examining the expression of the sclerotomal marker, Sox9. Following electroporation of Nkx3.2 into the dorsal somite, we observed expression of endogenous Sox9 around the notochord and in the neural tube and in addition, noted ectopic expression of Sox9 in dorsal somitic cells, indicating that Nkx3.2 had reprogrammed these dorsal cells to adopt a ventral cell fate (Fig. 5E, arrow). In summary, we have found that in vivo expression of Nkx3.2 in dorsal somitic cells strongly inhibited expression of Pax3 and Pax7, and induced ectopic Sox9 expression.

2.3.6 High level of Wnt signaling inhibits Hedgehog-induced Nkx3.2 expression

Our findings indicate that forced expression of Nkx3.2 can repress the expression of the dermomyotomal markers Pax3 and Pax7 in vitro and in vivo. Therefore, in order for the somite to differentiate normally, Nkx3.2 expression has to be excluded from the dorsal domain. Since prior reports have indicated that Wnt proteins could inhibit the expression of the sclerotome marker Pax1[12, 24, 133], we investigated whether Wnt signals would similarly inhibit Nkx3.2 expression. Because in the presence of Wnt3a conditioned medium, Shh is still able to induce Nkx3.2 expression in cultured presomitic mesoderm (Fig. 1A), we suspected that the Wnt signals in such conditioned medium may not be long-lived enough or of sufficient intensity to block sclerotomal gene expression. Therefore, to examine whether high levels of Wnt signaling could inhibit Nkx3.2 expression induced by Shh, we surrounded explants of presomitic mesoderm with aggregates of Wnt1-expressing cells and added soluble N-Shh (125 ng/ml) to the cultures simultaneously. The explants were harvested after five days of culturing. As observed previously, Wnt signals alone failed to induce the expression of either dermomyotomal (i.e., Pax3) or myotomal (i.e., MyoD and Myf5) markers in presomitic mesoderm explants cultured for five days (Fig. 6A, lane 2). Intermediate level of Shh signal alone (125 ng/ml) also failed to induce Pax3, Myf5 and MyoD, but strongly induced the expression of Nkx3.2 (Fig. 6A, lane 3). In striking contrast, the combination of Shh and high levels of Wnt signals led to robust expression of Pax3, Myf5 and MyoD, and simultaneously inhibited the expression of Nkx3.2 (Fig. 6A, lane 4), demonstrating that Wnt signals inhibit the expression of Nkx3.2. Interestingly, we observed that co-culture of presomitic mesoderm with Wnt1-expressing cells did not blunt the induction of Patched2, indicating that Wnt signals did

not attenuate the expression of all Shh responsive genes in these cultures (Fig. 6A, lane 4).

We speculate that the co-expression of Pax3, MyoD, and Myf5 in these cultures (Fig. 6A, lane 4, as well as Fig. 1A, lane 4) likely reflects a differential exposure of the somitic cells on the surface of the somite versus those in the interior of the somite to either Wnt, Shh, or both signals which manifests itself as a combination of cell fate responses to different levels of these signals. While this experimental set up does not allow for the equal exposure of all somitic cells to the same levels of Wnt/Shh signaling, it does mimic the in vivo situation, in which different somitic domains are differentially exposed to different levels of Wnt and Shh signals, and therefore adopt different fates. In summary, these results suggest that a high level of Wnt signals (from both dorsal neural tube and surface ectoderm) promote Shh-mediated expression of both dermomyotomal and myotomal markers at the expense of the sclerotomal marker Nkx3.2, and would thus act to restrict Nkx3.2 expression to the ventral domain of the somite. These findings are consistent with and extend prior work which has indicated that Wnt and Shh signals can act antagonistically to induce dermomyotomal (Pax3) versus sclerotomal (Pax1) gene expression, respectively[10, 12-14].

2.3.7 Forced expression of Pax3 blocks Shh-mediated Nkx3.2 expression and chondrogenesis

As discussed above, surrounding presomitic mesoderm with aggregates of Wnt1-expressing cells biases the response of the presomitic cells to Shh signals to promote Pax3 expression at the expense of the sclerotomal marker Nkx3.2. Because Pax3 is sufficient to induce myogenesis

in somites[30], we wondered whether the continuous expression of Pax3 was acting to simultaneously repress Shh-mediated induction of Nkx3.2 and other sclerotomal markers. To examine this issue, we infected presomitic mesoderm with either Pax3 virus (RCAS-Pax3) or control virus alkaline phosphatase (RCAS-AP). We subsequently challenged these infected explants to activate sclerotomal/chondrocyte differentiation markers by the addition of N-Shh (250 ng/ml). As noted previously[30], infection of presomitic mesoderm with RCAS-Pax3 efficiently activated the expression of MyoD (Fig. 6B, lanes 2 and 4). While N-Shh induced the expression of both sclerotomal markers (i.e., Nkx3.2 and Sox9) and cartilage differentiation markers (i.e., aggrecan, epiphycan and collagen IX) in RCAS-AP-infected cultures (Fig. 6B, lanes 1 and 3), the expression of both sclerotomal and cartilage markers were markedly repressed in the RCAS-Pax3-infected explants (Fig. 6B, lanes 2 and 4). In contrast, the expressions of Patched2 and Pax1 in these cultures were either not affected or only slightly dampened by the expression of exogenous Pax3 (Fig. 6B, compare lanes 1 and 2, and lanes 3 and 4), suggesting that the effects of Pax3 were not due to attenuated Hedgehog signal transduction per se. These findings indicate that forced expression of Pax3 can both repress N-Shh-mediated induction of a subset of sclerotomal markers (Nkx3.2 and Sox9) and block cartilage differentiation of presomitic mesoderm. The ability of forced Pax3 expression to block both sclerotome induction and cartilage differentiation is consistent with our findings that high levels of Wnt signaling (plus N-Shh) can both promote stable expression of Pax3 and similarly repress the induction of the sclerotomal marker Nkx3.2 (Fig. 6A).

2.4 Discussion

2.4.1 Shh acts in a morphogen-like manner to pattern dorsal–ventral cell fates in the somite

Our work suggests that a Shh gradient determines the dorsal–ventral pattern of cell fates within the somite (Fig. 7). In the presence of a constant amount of Wnt signals, increasing levels of Shh induce a graded response in somitic cells; low levels of Shh promote the expression of the dermomyotomal marker Pax3, intermediate levels of Shh induce Myf5 and MyoD expression with a concomitant loss of Pax3 expression, and higher levels of Shh inhibit the expression of dermomyotomal/myotomal markers while promoting the expression of sclerotomal markers. Superimposed on this graded response to Shh signals, our findings also suggest that the relative level of Wnt signaling can modify the response of somitic cells to Shh signals; with high levels of Wnt signaling acting to repress the induction of sclerotomal genes while promoting the induction of both dermomyotomal and myotomal gene expression. Our work suggests that a Shh concentration gradient works in a morphogen-like fashion to differentially pattern dorsal–ventral somitic fates. A morphogen is defined as a signaling molecule that acts directly on a cohort of equivalent cells to elicit specific responses depending on its concentration[8]. Our in vitro analysis of somites cultured for several days in constant levels of soluble Wnt3a plus increasing levels of Shh has demonstrated that increasing thresholds of Shh sequentially promote dermomyotomal, myotomal and sclerotomal gene expression. Because our analysis of gene expression was performed after 5 days in culture it is possible that differing levels of Shh can modulate either the induction/maintenance of differential gene expression in these cultures or the

differential survival/proliferation of distinct cell types. Indeed we noted that low levels of Shh are apparently not necessary for Wnt-mediated induction of Pax3, but only for the maintenance of Pax3 expression in these cultures. Prior work has indicated that a gradient of Shh protein is deposited within the developing somite[7], and that mouse embryos that lack Smoothened, the receptor for hedgehog signaling, failed to express the sclerotomal markers Pax1 and Nkx3.2 and exhibited much reduced expression of the myotomal marker Myf5, suggesting that Shh acts directly on somitic cells to specify their cell fates [134]. Since Shh diffuses throughout the somite [12, 13]and acts directly on these cells, we propose that Shh acts in a morphogen-like fashion to specify different somitic cell fates by either inducing or maintaining the expression of dermomyotomal, myotomal and sclerotomal genes at relatively greater levels of Shh signaling, respectively. Indeed this is similar to the established role for Shh in patterning cell fates in the ventral neural tube[158-160].

We were surprised to find that low levels (as low as 34 ng/ml) of Shh promoted Pax3 expression in the presomitic mesoderm in our long-term cultures (five days), even though Wnt signals alone were sufficient to induce the expression of Pax3 in somites cultured for only one day. In contrast to this apparent in vitro requirement for Shh signals to maintain somitic Pax3 expression, both Shh null mice embryos or chicken embryos with early notochord ablation display an expansion of Pax3 expression[3, 11, 161], suggesting that Shh negatively regulates Pax3 expression in the dermomyotome. In addition, loss of Hedgehog signaling in zebrafish embryos resulted in an overproduction of Pax3/Pax7-positive cells in the

dermomyotome of the somite[139, 140], again suggesting that hedgehog signals are required to restrict the expression of these dermomyotomal Pax genes. In light of these in vivo findings, we suggest that the signals required to initiate versus maintain the expression of Pax3 may be distinct and that Shh signals may only be necessary to maintain either the expression of Pax3 or the survival and expansion of Pax3 expressing cells in explants of presomitic mesoderm that lack other signals that perform such functions in vivo. Indeed it is possible that Shh signals act to maintain the viability of Pax3 expressing presomitic mesodermal cells cultured in vitro, as is apparently the case for both myotomal cells [161] and hypaxial limb muscle[17] in vivo.

In the presence of Wnt3a conditioned medium, intermediate levels of Shh (67–250 ng/ml), induced the loss of Pax3 expression concomitant with the induction of MyoD and Myf5. This finding is very reminiscent of the requirement for hedgehog signals to induce the differentiation of Pax3/Pax7-positive dermomyotomal cells in zebrafish [139, 140]. Indeed, morpholino-based knockdown of MyoD and Myf5 in zebrafish is sufficient to enhance the expression of Pax3/7 [140], indicating that these myogenic regulatory factors (MRFs) are necessary to repress the expression of these dermomyotomal Pax genes. Our finding that intermediate levels of Shh simultaneously promote the induction of MRFs and the loss of Pax3 expression is completely consistent with both these recent reports in zebrafish and with earlier findings in the chick indicating that ectopic Shh signals promote premature skeletal muscle differentiation of dermomyotomal cells [141]. A clear focus for the future will be to clarify the details of how hedgehog signaling leads to MRF induction in Pax3/7 expressing dermomyotomal cells and how the induction

of MRFs leads to a reciprocal down-regulation of Pax3/7 expression. Intermediate levels of N-Shh, in addition to inducing the activation of MRF expression in somites cultured in Wnt3a conditioned medium, also induced the expression of sclerotomal markers in these cultures. While we were unable to address whether the same or different cells express sclerotomal versus myotomal markers in these cultures (due to the absence of good antibodies against chick sclerotomal markers), we think it is most likely that somitic cells in these cultures express only one program of gene expression and not both simultaneously (discussed below).

Finally, high levels of Shh (greater than 500 ng/ml of N-Shh) led to the loss of all dermomyotomal and myotomal markers in presomitic mesoderm cultured in the presence of Wnt3a conditioned medium. In concert with the loss of all dermomyotomal and myotomal marker expression at these relatively high concentrations of Shh, expression of the sclerotomal markers Pax1, Nkx3.2 and Sox9 were maintained at these levels of Shh signaling. Prior to this work, Münsterberg et al demonstrated that increasing N-Shh concentrations up to 200 ng/ml promoted the expression of both MyoD and Pax1 in cultured chick somites[127]. Our work is consistent with that report, and we have observed that even higher concentrations of Shh can act to repress MyoD gene expression while continuing to induce that of sclerotomal markers. Our finding is also consistent with in ovo notochord implantation in chick embryos[3]. In such a study, heterotopic grafting of a notochord between the neural tube and the dorsal somite only induced MyoD expression in cells that were located a short distance away from the implant [3]. In addition to noting a graded response of dermomyotomal, myotomal

and sclerotomal markers to differing levels of Shh, we also observed that expression of various sclerotomal markers displayed a requirement for differing threshold levels of Shh. Expression of Pax1 was induced by a higher concentration of Shh as compared to that of Nkx3.2 and Sox9. Indeed, prior work has indicated that induction of the sclerotomal markers Pax1 and Twist1 in cultured mouse somites required different levels of Shh [13]. A requirement for higher levels of Shh to induce Pax1 expression (compared to Nkx3.2 and Sox9) is consistent with the localized expression of Pax1 to the medial-ventral domain within the sclerotome, as compared to other sclerotome markers which are expressed more laterally[14, 162].

2.4.2 Nkx3.2 and Pax3 induce mutually repressive somitic cell fates

Somites are divided into dorsal and ventral domains with clear borders marked by differential gene expression, even before the morphological distinction of dermomyotome and sclerotome (see Fig. 2). Importantly, the expression of the dermomyotomal marker Pax3 is initially expressed throughout the dorsal–ventral extent of the presomitic mesoderm and its expression is extinguished in the ventral somite in response to Shh [11, 161] concomitant with the activation of Nkx3.2 in this somitic domain (this work). In contrast, Sox9 and Pax3 are transiently co-expressed in the presomitic mesoderm throughout the dorsal–ventral axis. We have found that forced expression of the Shh-induced transcriptional repressor, Nkx3.2, can block the expression of Pax3 induced by either Wnt3a or signals from the surface ectoderm in explanted chicken somites without inducing cartilage differentiation in such cells. Furthermore, we have found that ectopic expression of Nkx3.2 in the dorsal somite was capable of inhibiting Pax3 and

Pax7 expression in vivo. In contrast, forced expression of Sox9, which induced robust chondrocyte differentiation of somites, attenuated but did not efficiently inhibit Pax3 gene expression induced by either Wnt3a conditioned medium or signals from the surface ectoderm. These findings are consistent with the expression profile of Sox9 and Nkx3.2 in the embryo, and suggest that Shh restricts the expression of dermomyotomal genes such as Pax3 and Pax7 in part by inducing the expression of Nkx3.2 in the ventral somite [60].

Forced Nkx3.2 expression can block the induction of dermomyotomal markers by Wnt signals without inducing cartilage differentiation in somites. The absence of cartilage differentiation in such explants may be a consequence of a Wnt-mediated repression of Sox9 gene expression[163-166]. In prior work, we demonstrated that ectopic Nkx3.2 induces the expression of Sox9 in somites cultured in the absence of exogenous Wnt signals [58]. Apparently, Wnt3a prevents Nkx3.2 from inducing collagen II expression (this work) perhaps by promoting β-catenin-mediated Sox9 degradation [167]. In contrast, Wnt3a did not block chondrogenesis in RCAS-Sox9-infected explants, as exogenous Sox9 may be present at high enough levels to overcome this degradation. Furthermore, we showed that Nkx3.2 requires a higher level of BMP4 to promote chondrogenesis (this work and Zeng, 2002). Thus, our findings suggest that induction of a ventral somitic cell fate by Nkx3.2 consists of two steps: (1) in the presence of high levels of Wnt signals, and a low level of BMP4, Nkx3.2 represses dorsal somite gene expression (i.e. Pax3) in the absence of inducing cartilage differentiation, and (2) in the absence of Wnt signaling and the presence of a higher level of BMP4, Nkx3.2 induces the chondrocyte differentiation program.

We and others have reported that Sox9 expression in the somite is induced by Sonic hedgehog (Shh)[58, 136]. Although it is known that Shh acts through Gli proteins to induce Sox9 expression, it was not clear how this takes place. Our past work indicated that Nkx3.2, which is downstream of Pax1 and likely Gli proteins[47, 136], induced Sox9 expression in whole somite explants. However, our RT-PCR analysis did not exclude the possibility that Nkx3.2 selectively amplifies a population of ventral somitic sclerotomal precursor cells [58]. In our current work, we found that ectopic Nkx3.2 expression in the dorsal somite cells was able to induce Sox9 expression while repressing Pax3 and Pax7 expression. Therefore, our findings strongly suggest a role for Nkx3.2 in the initiation of Sox9 gene expression in cartilage progenitors in the embryo. This notion is consistent with a study in 10T1/2 cells, which suggested that Nkx3.2 promotes the expression of Sox9 during the chondrogenic differentiation of these cells [168], and is also consistent with observations in Nkx3.2 null mouse embryos, where reduced Sox9 expression was observed [59].

While Nkx3.2 knockout mice embryos displayed reduced expression of Sox9 in the sclerotome, it is not known whether expression of dorsal somite markers such as Pax3 or Pax7 is found in more ventral positions in such animals[59, 143, 169]. Gene expression analysis in mice embryos lacking both Nkx3.2 and the closely related gene, Nkx3.1 (which is also transiently expressed in the sclerotome[57, 170], revealed that the expression of Myf5 is still restricted to the myotomal region in the developing somites of these mutant embryos and is not expressed in the ventral-most somitic domain in the combined absence of Nkx3.2 and Nkx3.1 (data not shown,

L.Z., A. B. L., and Hans Arnold). Thus, it seems likely that other Shh-induced factors in addition to Nkx3.2 may also restrict expression of dermomyotomal and myotomal markers to the dorsal somite. Consistent with this idea, it has been reported that the Wnt-antagonist, Sfrp2, is also induced by Shh in the sclerotome and can prevent Wnt1 or Wnt4 from inducing Pax3 expression in mouse somites [171]. In the chick, Sfrp2 has been reported to be expressed in the dermomyotome [172], in the sclerotome [173], or in different somite compartments in different developmental stages [174], while Sfrp1 is expressed in the ventral somite [174]. Thus, it is possible that Shh-mediated induction of Nkx3.2 and Sfrps in the sclerotome may act in parallel to block Wnt-mediated induction of dermomyotomal and myotomal markers in ventral regions of the somite.

Co-culture of somites with Wnt1-producing cells alters the response of somitic cells to Shh signals, promoting expression of the dermomyotomal/myotomal genes Pax3 and Myf5, and repressing the induction of Nkx3.2 and other sclerotomal markers (see also [12, 24, 133]). Pax3 plays an essential role in somitic myogenesis, where it activates the expression of myogenic markers MyoD and Myf5 as well as FGF signaling[30, 32, 131, 175]. Pax3 mutant embryos exhibit loss of muscle progenitors in the epaxial and hypaxial dermomyotome and fail to form limb muscle [129, 147]. We found that forced expression of the dermomyotomal marker Pax3 both promoted myogenesis and blocked Shh-induced expression of both Nkx3.2 and other markers of cartilage differentiation. While RT-PCR analysis of such cultures did not exclude the possibility that ectopic Pax3 induced a clonal expansion of muscle precursors in such somite

explants, genetic evidence has indicated that the combined loss of both Pax3 and its homolog Pax7 resulted in either the apoptosis of dermomyotomal cells or the re-specification of these cells into collagen II-expressing cartilage cells [129]. Thus, we propose that the stable expression of Pax3 or Pax7 results in the commitment of somitic cells to the dermomyotomal/myotomal cell fate and precludes the induction of both sclerotomal markers (Sox9 and Nkx3.2) and cartilage differentiation of these cells in response to Shh signals. Conversely, our results indicate that forced expression of Nkx3.2 induces a cell state that is incompatible with dermomyotomal/myotomal gene expression. Thus the stable induction of either Pax3/Pax7 or Nkx3.2 induce mutually repressing cell states that exclude the co-expression of both dermomyotomal/myotomal and sclerotomal markers in the same cell, and thereby ensures that somitic cells eventually become committed to give rise to either dermomyotomal/myotomal or sclerotomal derivatives but not both (Fig. 7). A similar logic has apparently been employed to pattern the neural tube, where dorsally restricted Pax genes and ventrally restricted Nkx family members both mutually inhibit one another's expression and promote either distinct dorsal or ventral neural tube cell fates, respectively[176].

2.5 Chapter 2 figures and legends

Figure 2.1

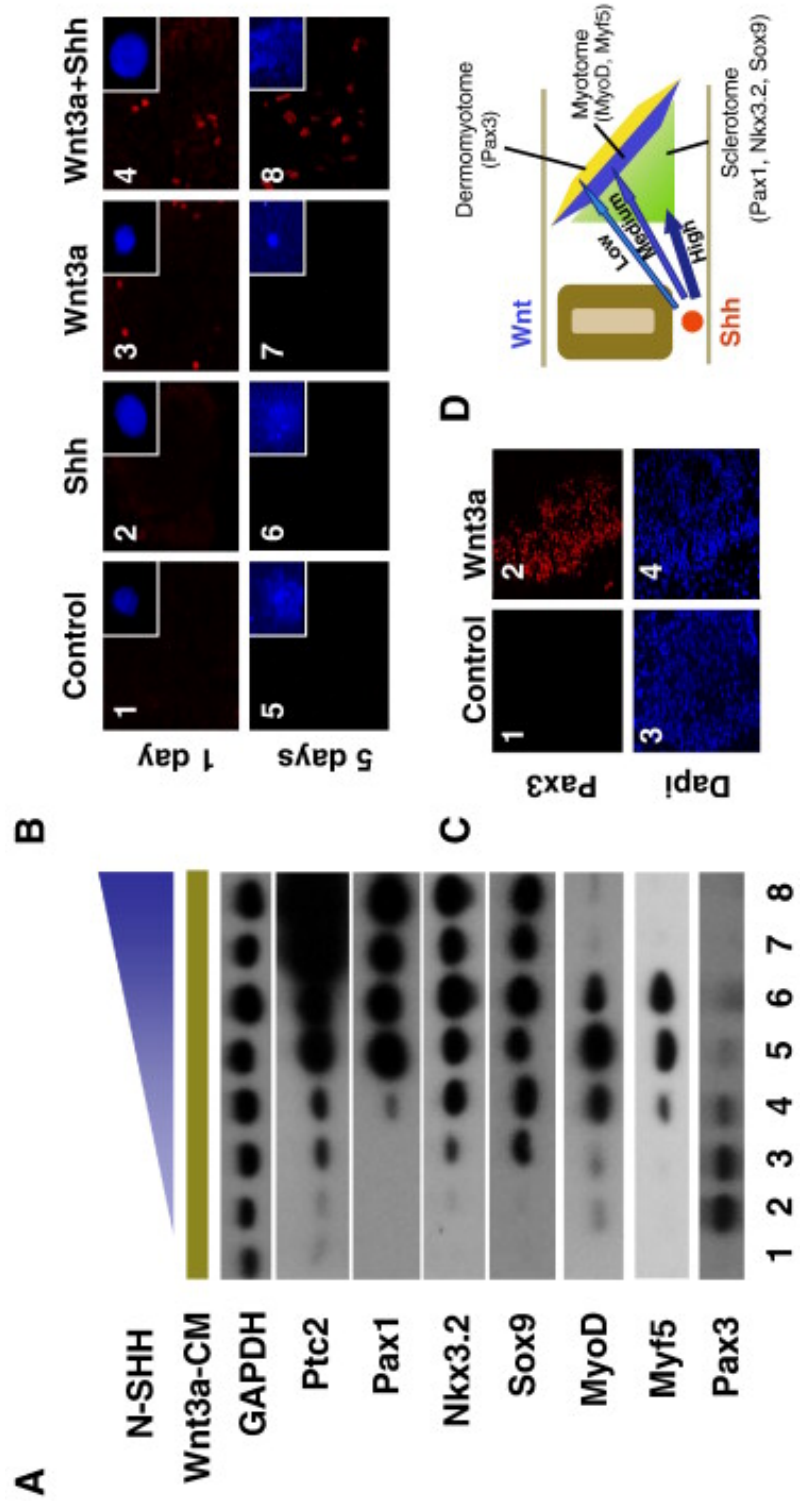
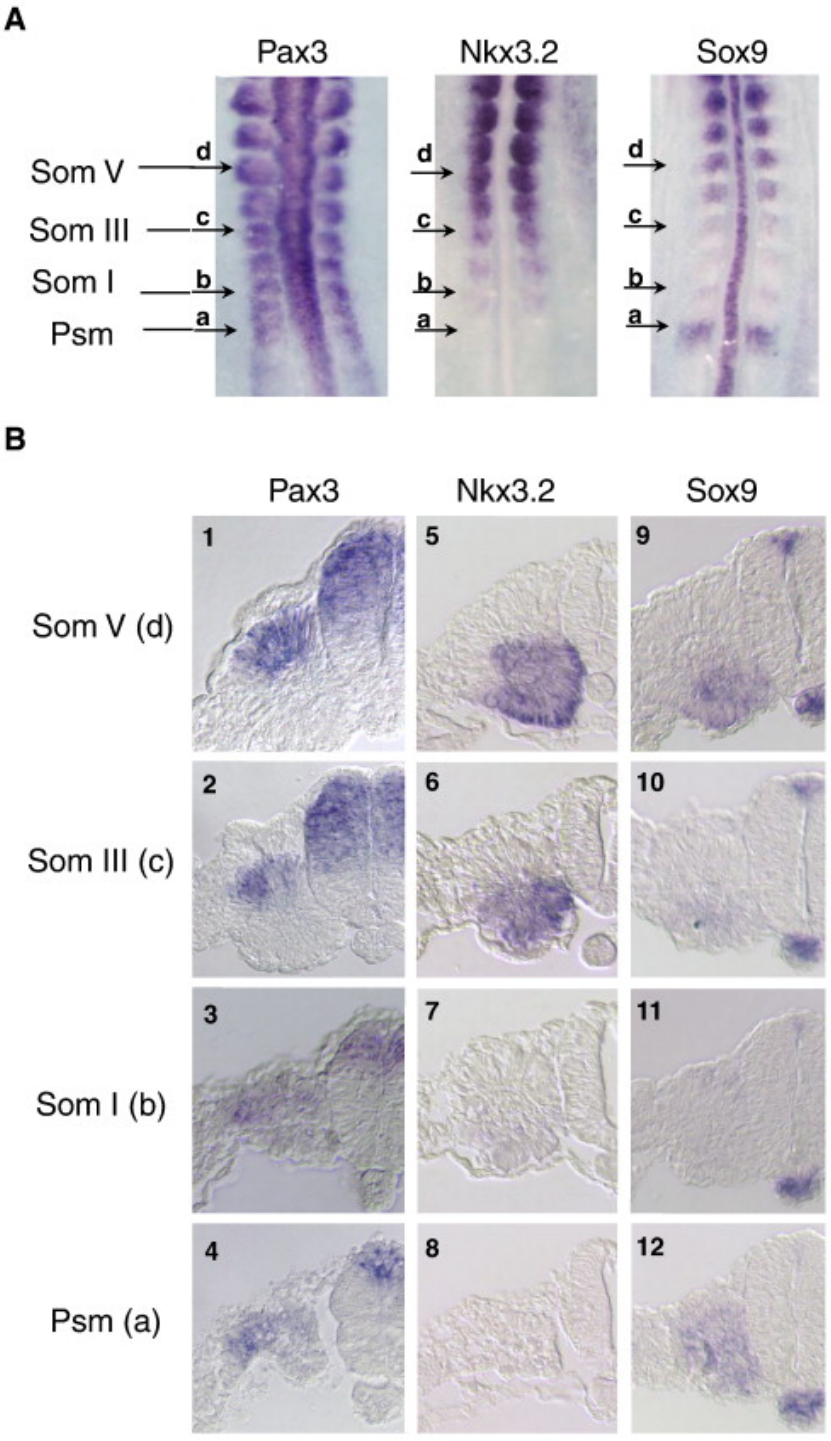


Fig. 1. Shh concentration gradient patterns dorsal–ventral fates of the somite. (A) In the presence of constant levels of Wnt3a signals, differing levels of Shh are necessary to induce either dorsal or ventral somite markers. Presomitic mesoderm explants were cultured for five days in Wnt3a-conditioned medium and increasing amounts of N-SHH. Lane 1: no N-SHH. Lane 2: 34 ng/ml N-SHH. Lane 3: 67 ng/ml N-SHH. Lane 4: 125 ng/ml N-SHH. Lane 5: 250 ng/ml N-SHH. Lane 6: 500 ng/ml N-SHH. Lane 7: 1 μg/ml N-SHH. Lane 8: 2 μg/ml N-SHH. Gene expression was assayed by RT-PCR. (B) Shh is required to maintain but not initiate the expression of Pax3 in presomitic mesoderm exposed to Wnt signals. Presomitic mesoderm explants from stage 10 chicken embryos were cultured for either one day (panels 1–4) or five days (panels 5–8) in either control conditioned medium (panels 1 and 5), control conditioned medium containing 125 ng/ml N-SHH (panels 2 and 6), Wnt3a-conditioned medium (panels 3 and 7), or the combination of Wnt3a-conditioned medium plus 125 ng/ml N-SHH (panels 4 and 8). Pax3 expression was analyzed by immunocytochemistry and confocal microscopy. The inset in each panel represents a whole mount Dapi staining of the explant. The Dapi images were taken at the same magnification. Few somitic cells migrated out of the explants at day 1. By day 5, the explants were much larger due to cell proliferation and migration. It is evident that more cells were present in Shh-treated explants. (C) Wnt3a alone is sufficient to induce Pax3 expression in somite IV–VI. Somite IV–VI explants from stage 10 chicken embryos were cultured for five days in either control conditioned medium (panels 1 and 3) or Wnt3a-conditioned medium (panels 2 and 4). Confocal images of Pax3 expression (panels 1 and 2) and DAPI staining (panels 3 and 4) are displayed. (D) Model showing differing levels of Shh signal lead to differing cell fates in the somite.

Figure 2.2


A
Pax3
Nkx3.2
Sox9
Som V
Som III
Som I
Psm
d
c
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a
B
Pax3
Nkx3.2
Sox9
Som V (d)
Som III (c)
Som I (b)
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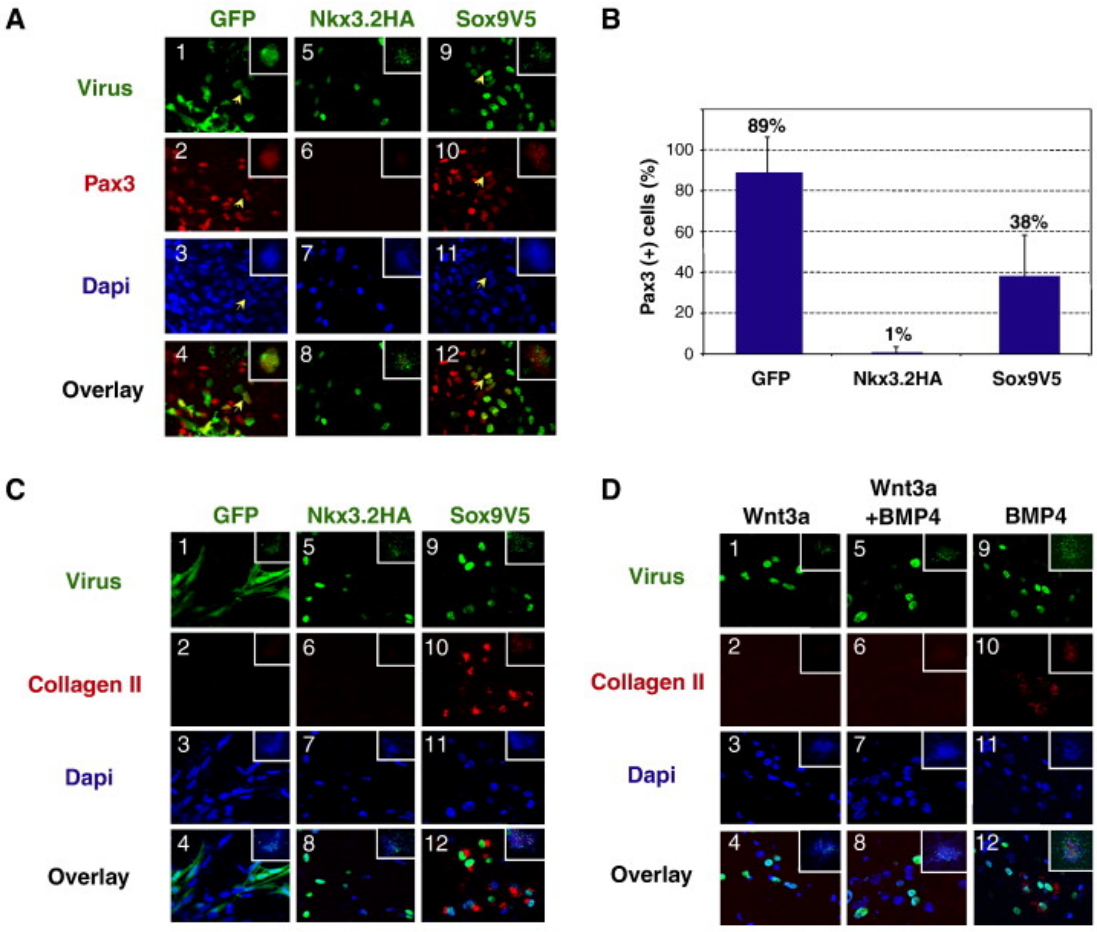
Fig. 2. In situ hybridization analysis of Pax3, Nkx3.2 and Sox9. (A) Whole mount in situ hybridization analysis of Pax3, Nkx3.2 and Sox9 on 12 somite (stage 10) chick embryos. Arrows mark the caudal to rostral levels of the somitic mesoderm. a, presomitic mesoderm (Psm). b, somite I. c, somite III. d, somite V. (B) Sections of Pax3, Nkx3.2 and Sox9 ISH embryos at various axial levels. Panels 1–4, Pax3 expression. Panels 5–8, Nkx3.2 expression. Panels 9–12, Sox9 expression. Sections at the presomitic mesoderm level are shown in panels 4, 8 and 12. Sections at somite I level are shown in panels 3, 7 and 11. Sections at somite III level are shown in panels 2, 6 and 7. Sections at somite V level are shown in panels 1, 5 and 9.

Figure 2.3


A
GFP
Nkx3.2HA
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B
Pax3 (+) cells (%)
100
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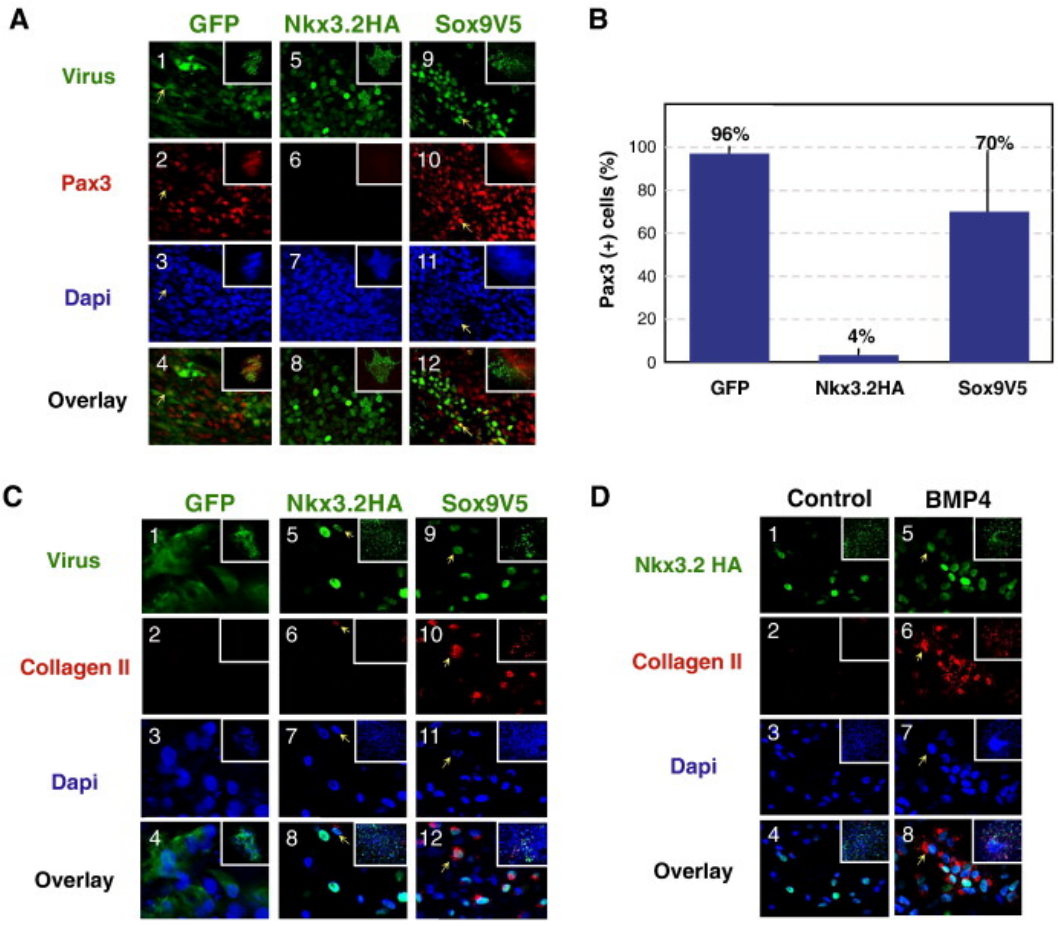
40
20
0
89%
1%
38%
GFP
Nkx3.2HA
Sox9V5
C
GFP
Nkx3.2HA
Sox9V5
Virus
Collagen II
Dapi
Overlay
D
Wnt3a
Wnt3a +BMP4
BMP4
Virus
Collagen II
Dapi
Overlay

Fig. 3. Nkx3.2 and Sox9 inhibit Wnt3a-induced Pax3 expression in somite explants. (A) Somite explants immunostained for Pax3 expression following infection with retroviral encoded Nkx3.2HA, Sox9V5 or GFP. Somite IV–VI explants of stage 10 chicken embryos were cultured in Wnt3a conditioned medium for 5 days. Panels 1–4, RCAS-B-GFP-infected explant, arrows indicating a cell that expressed GFP as well as Pax3. Panels 5–8, RCAS-B-Nkx3.2HA-infected explant. Panels 9–12, RCAS-B-Sox9V5-infected explant, arrows indicating a cell that expressed Sox9V5 as well as Pax3. The inset within each panel shows a low powered view of each explant. Green: GFP, Nkx3.2HA and Sox9V5. Red, Pax3. Overlay, merged view of virus-expression (GFP, Nkx3.2 and Sox9) and Pax3. Dapi images are not overlaid with virus and Pax3 images so that yellow overlapping expression is more evident. No significant changes in cell numbers in these explants were observed. (B) Quantification of the results of panel A. Percentage of virus-infected cells that express Pax3 was quantified. For each virus-infected sample, a total number of 500–1000 virus-infected cells from at least 5 different views were analyzed under the microscope. Standard deviations are shown. (C) Somite explants immunostained for collagen II expression following infection with retrovirally encoded Nkx3.2HA, Sox9V5 or GFP. Somite explants were cultured under the same condition as described in panel A. Panels 1–4, RCAS-B-GFP-infected explant. Panels 5–8, RCAS-B-Nkx3.2HA-infected explant. Panels 9–12, RCAS-B-Sox9V5-infected explant. Green: GFP, Nkx3.2HA and Sox9V5. Red, collagen II. Overlay, merged view of virus-expression (GFP, Nkx3.2 and Sox9), collagen II and Dapi. The inset within each panel shows a low powered view of each explant. (D) Nkx3.2 virus-infected cells do not adopt a cartilage fate in the presence of Wnt3a, but do express collagen II in the presence of exogenous BMP4. Somites IV–VI of stage 10 chicken embryos were cultured in either Wnt3a-conditioned medium (panels 1–4), Wnt3a-conditioned medium plus 100 ng/ml BMP4 protein (panels 5–8), or control L-cell conditioned medium plus 100 ng/ml BMP4 protein (panels 9–12). The explants were cultured altogether for 6 days before immunostaining. Green: GFP, Nkx3.2HA and Sox9V5. Red, collagen II. Overlay, merged view of virus-expression (GFP, Nkx3.2 and Sox9), collagen II and Dapi. The inset within each panel shows a low powered view of each explant.

Figure 2.4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B
Pax3 (+) cells (%)
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96%
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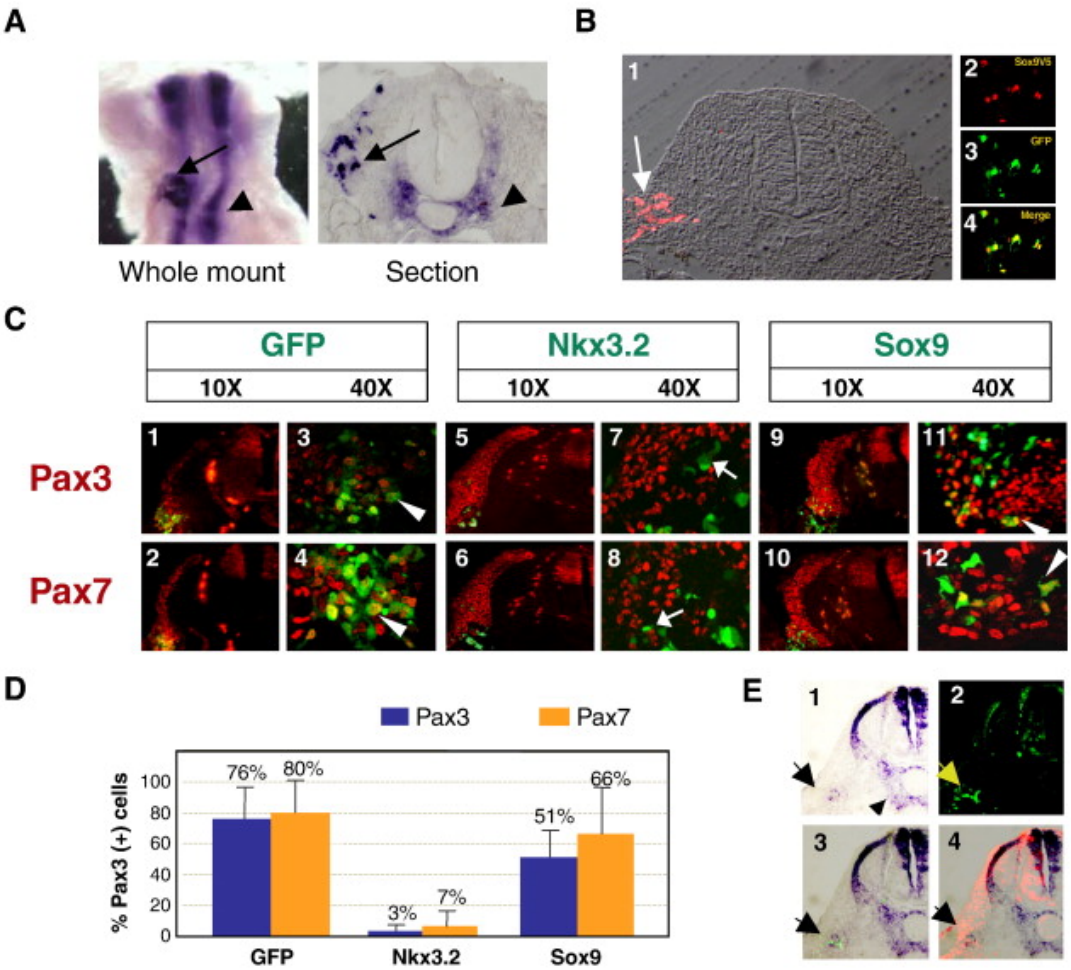
70%
GFP
Nkx3.2HA
Sox9V5
C
GFP
Nkx3.2HA
Sox9V5
Virus
Collagen II
Dapi
Overlay
D
Control
BMP4
Nkx3.2 HA
Collagen II
Dapi
Overlay

Fig. 4. Effects of Nkx3.2 and Sox9 on surface-ectoderm-induced Pax3 expression. (A) Pax3 expression in somite/ectoderm explants infected with GFP, Nkx3.2HA and Sox9V5. Stage 10 chicken embryo explants of somite IV–VI with surface ectoderm attached were cultured for 5 days. Panels 1–4, RCAS-B-GFP-infected explant. Panels 5–8, RCAS-B-Nkx3.2HA-infected explant. Panels 9–12, RCAS-B-Sox9V5-infected explant. The inset within each panel shows a low powered view of each explant. Arrows indicating cells that expressed GFP or Sox9V5 as well as Pax3. The inset within each panel shows a low powered view of each explant. Green: GFP, Nkx3.2HA and Sox9V5. Red, Pax3. Overlay, merged view of virus-expression (GFP, Nkx3.2 and Sox9) and Pax3. Dapi images are not overlaid with virus and Pax3 images so that yellow overlapping expression is more evident. No significant changes in cell numbers in these explants were observed. (B) Quantification of the results of panel A. Percentage of virus-infected cells that express Pax3 was quantified. For each virus-infected sample, a total number of 500–1000 virus-infected cells from 5 different views were analyzed under the microscope. (C) Somite/ectoderm explants immunostained for collagen II expression following infection with retrovirally encoded Nkx3.2HA, Sox9V5 or GFP. Somite explants were cultured under the same condition as described in Panel A. Panels 1–4, RCAS-B-GFP-infected explant. Panels 5–8, RCAS-B-Nkx3.2HA-infected explant. Arrow, an Nkx3.2HA-expressing cell that also expresses a low level of collagen II. Panels 9–12, RCAS-B-Sox9V5-infected explant. Green: GFP, Nkx3.2HA and Sox9V5. Red, collagen II. Overlay, merged view of virus-expression (GFP, Nkx3.2 and Sox9), collagen II and Dapi. The inset within each panel shows a low powered view of each explant. (D) Nkx3.2 virus-infected cells adopt a cartilage fate in the presence of exogenous BMP4. Stage 10 chicken embryo explants of somite IV–VI with surface ectoderm attached were cultured for 6 days in either control medium (panels 1–4) or in medium supplemented with 100 ng/ml BMP4 protein (panels 5–8). The inset within each panel shows a low powered view of each explant. Nkx3.2HA, green. Collagen II, red. Dapi, blue. Overlay, merged image of Nkx3.2HA, collagen II and Dapi.

Figure 2.5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Whole mount
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B
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2
Sox9/V5
3
GFP
4
Merge
C
GFP
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40X
Nkx3.2
10X
40X
Sox9
10X
40X
Pax3
Pax7
1
2
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7
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9
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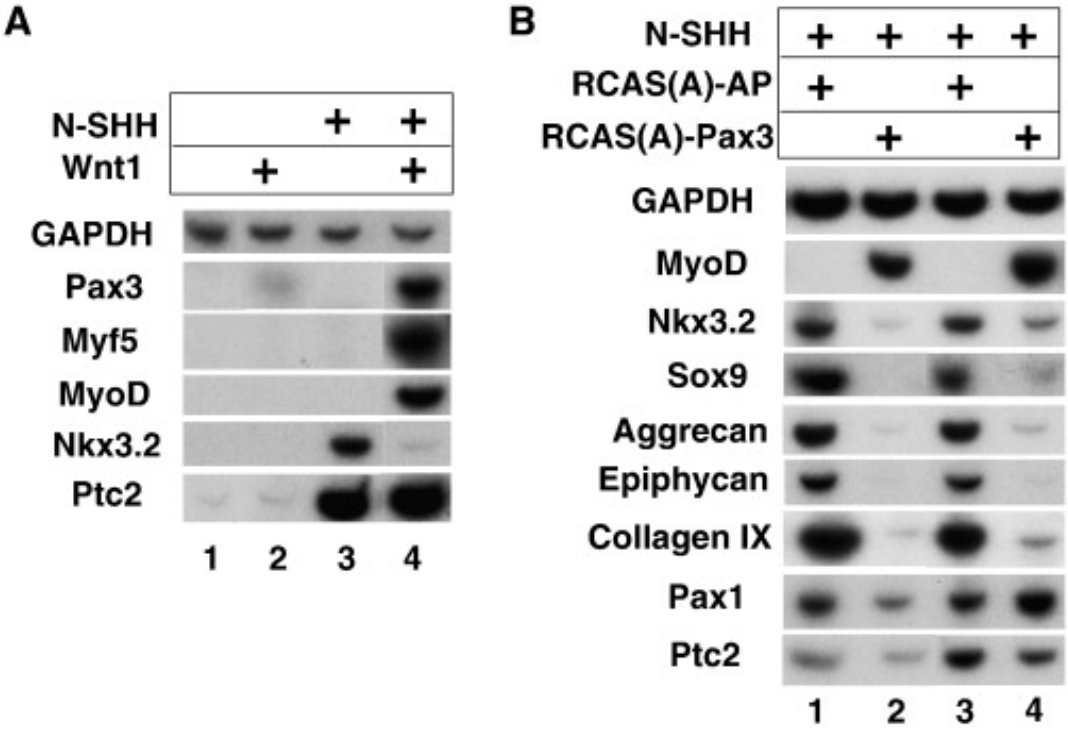
11
12
D
Pax3
Pax7
% Pax3 (+) cells
100
80
60
40
20
0
76%
80%
3%
7%
51%
66%
GFP
Nkx3.2
Sox9
E
1
2
3
4

Fig. 5. Nkx3.2, but not Sox9, inhibits the expression of Pax3 and Pax7 in ovo. (A) Ectopic expression of Nkx3.2 in the dorsal somite cells. In situ hybridization (ISH) analysis of chicken embryos electroporated with pMES-Nkx3.2-GFP into the lumen of the newly formed somites two days after electroporation, using an Nkx3.2 RNA probe. 5 embryos were analyzed and all exhibited ectopic Nkx3.2 in the dorsal somitic cells. Left panel: whole mount ISH; right panel: a cross section of the whole mount ISH embryo. Arrows: exogenous Nkx3.2 expression. Arrowheads: endogenous Nkx3.2 expression. (B) Ectopic expression of Sox9 in the dorsal somite cells. Immunocytochemistry analysis on sectioned embryos electroporated with pMES-Sox9-GFP two days after electroporation. 5 sections were analyzed and all cells that expressed GFP also expressed Sox9V5. Panel 1, V5 staining showing Sox9V5 expression in dorsal somite cells. A bright field view of the section was overlaid on the fluorescent image. Panel 2, magnified view of Sox9V5-positive cells. Panel 3, magnified view of GFP-positive cells. Panel 4, merged image (yellow) of Sox9V5 and GFP. (C) Nkx3.2, but not Sox9, inhibited Pax3 and Pax7 expression. Embryos electroporated with either pMES-GFP (vector), pMES-Nkx3.2-GFP or pMES-Sox9V5-GFP into the lumen of the newly formed somites were whole mount fixed and serial-sectioned. The sections were immunostained with antibodies against Pax3 and Pax7. Cells harboring the introduced DNA are GFP-positive, and thus are green. Only overlaid images are shown. Most of the cells that expressed the introduced plasmids were laterally located. However, the few medially-targeted cells exhibit the same phenotype as these laterally located cells. Panels 1–4, GFP electroporated embryos (panels 1–2 are 10× views, and panels 3–4 are 40× views). Panels 5–8, Nkx3.2 electroporated embryos (panels 5–6 are 10× views, and panels 7–8 are 40× views). Panels 9–12, Sox9V5 electroporated embryos (panels 9–10 are 10× views, panels 11–12 are 40× views). Arrows: cells that express introduced DNA (GFP-positive), but are not Pax3 or Pax7-positive (red). Arrowheads: cells that express introduced DNA (GFP-positive), and are also Pax3 or Pax7-positive (red), and are thus yellow. (D) Quantification of the results of panel C. Percentage of electroporated cells that express Pax3 and Pax7 was quantified. For each electroporated sample, a total number of 100–200 targeted cells from at least 3 embryos were analyzed under the confocal microscope. Only the target cells (GFP-positive) that were located in the normal Pax3 and Pax7 expression domains were used for counting. Pax3, blue. Pax7, orange. (E) Nkx3.2 induced the expression of Sox9 in the dorsal somite cells. Embryos electroporated with pMES-Nkx3.2-GFP into the lumen of the newly formed somites were subjected to whole mount in situ hybridization and sectioning, using a Sox9 RNA probe. 5 embryos were analyzed. Panel 1, in situ hybridization of Sox9. Arrowhead: exogenous Sox9 expression. Arrow: endogenous Sox9 expression. A bright field view of the section was overlaid on the fluorescent image. Panel 2, Anti-GFP staining indicating the ectopic expression of the introduced DNA pMES-Nkx3.2-GFP (arrow). Panel 3, Overlay of the bright field ISH image with the GFP immunostaining image (arrow). Panel 4, merged bright field image of ISH and immunofluorescent staining of endogenous Pax3, showing ectopic Sox9 expression within the Pax3-expressing domain.

Figure 2.6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N-SHH
Wnt1
+ + +
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Pax3
Myf5
MyoD
Nkx3.2
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1 2 3 4
B
N-SHH + + + +
RCAS(A)-AP + + +
RCAS(A)-Pax3 + +
GAPDH
MyoD
Nkx3.2
Sox9
Aggrecan
Epiphycan
Collagen IX
Pax1
Ptc2
1 2 3 4

Fig. 6. High level of Wnt signaling and Pax3 expression inhibit Nkx3.2 expression. (A) High level of Wnt signaling inhibits Nkx3.2 expression and promotes dorsal somite cell fates. RatB1a cells secreting Wnt1 were co-cultured with presomitic mesoderm explants of stage 10 chicken embryos in the presence or absence of N-SHH. Explants were harvested after 5 days culture for RT-PCR analysis of indicated genes. Lane 1, control RatB1a cells co-cultured with presomitic mesoderm explants. Lane 2, Wnt1-secreting RatB1a cells co-cultured with presomitic mesoderm explants. Lane 3, control RatB1a cells co-cultured with presomitic mesoderm explants in the presence of 250 ng/ml N-SHH. Lane 4, Wnt1-secreting RatB1a cells co-cultured with presomitic mesoderm explants in the presence of 250 ng/ml N-SHH. (B) Forced expression of Pax3 inhibits the expression of Nkx3.2 and chondrogenic markers induced by N-SHH. Presomitic mesoderm explants were infected with avian retroviruses encoding either Pax3 or Alkaline phosphatase (AP, control). 500 ng/ml N-SHH was present in all cultures. Explants were harvested after 5 days culture for RT-PCR analysis of indicated genes. Lanes 1 and 3, RCAS-A-AP-infected explants. Lanes 2 and 4, RCAS-A-Pax3-infected explants.

Figure 2.7

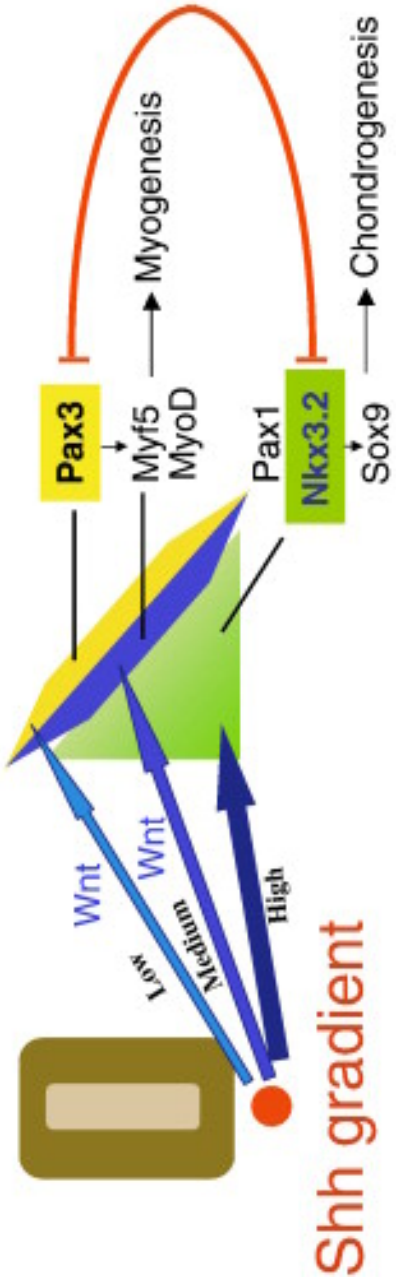
Myogenesis
Chondrogenesis
Pax3
Myf5
MyoD
Pax1
Nkx3.2
Sox9
Wnt
Wnt
Low
Medium
High
Shh gradient

Fig. 7. A gradient of Shh patterns the somite into mutually repressing muscle and cartilage cell fates. Low levels of Shh promote the maintenance of Pax3 expression induced by Wnt signals. Medium levels of Shh, along with Wnt signals, promote the expression of the myotomal factors Myf5 and MyoD. High levels of Shh promote the expression of the sclerotomal factors Nkx3.2, Sox9 and Pax1, and simultaneously repress expression of both dermomyotomal and myotomal markers. Mutual repression by Pax3 and Nkx3.2 ensures that cells adopt either a dermomyotomal or sclerotomal cell fate. While Pax3 induces the expression of MyoD/Myf5 and promotes somitic myogenesis, Nkx3.2 and Sox9 promote somitic chondrogenesis.

CHAPTER 3: The role of muscle cells in regulating cartilage matrix production

3.1 Introduction

Most of the bones in the human body are formed through the process of endochondral ossification, where the initially formed cartilage serves as a template for bone formation[177, 178]. Cartilage tissue consists of extracellular matrix (ECM) and the chondrocytes that secrete the matrix[179]. Cartilage ECM is composed mostly of proteoglycans and collagen fibers. In cartilage ECM, proteoglycans such as aggrecan and versican are bound to large quantities of glycosaminoglycans (GAG), which are highly negatively charged and thus allow cartilage to be resistant to compression[179]. The other indispensable component of cartilage ECM is collagen, which provides cartilage with the property of resisting tension. The major collagen in hyaline cartilage is collagen II, whose helical structure is stabilized by other important collagens such as collagen IX and collagen XI[180]. Deficiency of these collagens could lead to congenital skeletal disorders such as hypochondrogenesis, as well as early onset of arthritis[180-182].

Cartilage matrix production is controlled by many factors including growth hormone, parathyroid hormone related peptide (PTHrP), fibroblast growth factors (FGFs), and TGFβ family members (including BMP)[177]. These signals are either provided to the cartilage systemically (such as growth hormone), or supplied locally by both the tissues surrounding the developing cartilage and the chondrocytes themselves.

Muscle is a tissue that lies immediately next to the developing cartilage tissue in the embryo and remains in close proximity to the cartilage

template after birth[31, 122]. Multiple pieces of evidence indicate that muscle regulates skeletal development. For example, when muscle was paralyzed by botulinum toxin, which abolished muscle contraction, the chicken embryo showed abnormal joint formation and shortened bones[183]. Mouse mutants that lack muscle-specific proteins such as dystrophin/utrophin or myogenin also exhibited skeletal abnormalities such as a curved spine or a reduced size of the skeleton[61, 66, 184]. Consistent with the phenotype of these mouse mutants, short stature and scoliosis are common features of children with Duchenne Muscular Dystrophy[62, 63]. Despite these studies, it is still not clear if muscle cells directly influence cartilage matrix production, which may be the underlying mechanism of muscle-mediated skeletal regulation. Our hypothesis is that muscle cells play an important role in regulating cartilage matrix production, thereby influencing skeletal structures. We tested this hypothesis by coculturing chondrocytes with muscle cells, and showed that muscle cells provide biochemical signals to enhance cartilage matrix production.

3.2 Materials and methods

3.2.1 Cell culture

Murine myoblast (C2C12) and murine mesenchymal (NIH-3T3) cell lines were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). Chicken embryonic fibroblasts (CEF) and rat chondrosarcoma (RCS) cells were gifts courtesy of Andrew Lassar (Harvard Medical School). Bovine fetlock joint were obtained from Research 87, Inc. (Boylston, MA, USA; http://www.research87.com/home.nxg), which supplies cadaver tissues to research institutions. Bovine articular chondrocytes were

then isolated from the articular surface of the joints as previously described[185]. Briefly, cartilage pieces were digested with 1 mg/mL bovine hyaluronidase (Sigma, St. Louis, MO, USA) for 15 min followed by 30 min of 0.25% trypsin (Sigma) digestion, and finally 15 h of 2 mg/mL collagenase (Sigma) digestion. Single cell suspension was obtained by passing the cells through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA). For monolayer cultures, cells were seeded at a density of 104/well of a 24-well plate. For 3D collagen gel cultures, cells were seeded at a density of 104/collagen gel. Collagen gels were composed of 30% rat-tail collagen I (BD biosciences) and 1 × DMEM (Invitrogen).17 A total of 50 μL of collagen gel mixture was used for each 3D construct. All cocultures were seeded at a ratio of 2:1 (RCS:C2C12). Cells were cultured in DMEM with 10% FBS (Hyclone, Logan, UT, USA) and 1% pen/strep. DiI-labeling was performed according to the manufacture's protocol. Briefly, C2C12 cells were incubated with 1 μM DiI (Invitrogen, Carlsbad, CA, USA) for 5 min at 37°C, followed by 15 min at 4°C. Afterward, cells were washed repeatedly with PBS and cultured in fresh medium.

3.2.2 Conditioned media preparation

C2C12 muscle cells were cultured at a confluency of 60–90%. The conditioned medium was collected and filtered using a 0.22 μM filter (Millipore, Bedford, MA, USA) and applied immediately to chondrocyte cultures. For collecting conditioned medium from CEFs, the cells were infected with avian-retrovirus RCAS–GFP and RCAS–MyoD (constructs from Andrew Lassar, Harvard Medical School). These viruses were generated according to the standard protocol[14], and titered by directly visualizing GFP

expression (in the case of RCAS-GFP) or indirect immunocytochemistry using anti-MyoD antibody (in the case of RCAS-MyoD). Viruses with titers of at least 10^8 particles/ml were applied to CEFs at a concentration of 3 μL/500 μL culture. After 3 days of virus infection, the conditioned media were collected and filtered for subsequent use.

3.2.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

RNA was isolated from cell cultures using the RNeasy mini-kit from Qiagen (Chatsworth, CA, USA)[58]. All PCR analyses were normalized based on GAPDH expression using the iQ5 Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Primer sequences are (all are listed from 5′ to 3′): rat GAPDH (NCBI accession number NM_017008), 1131-GTTGCTGAG-GAGTCCCCA-1147 (Forw) and 1258-CCTATTCGAGAGA-AGGGA-1241 (Rev); rat Col IIa1 (NCBI accession number NM_012929.2), 1972-AAGCAAGGTGACCAGGGTATTCCT-1995 (Forw) and 2255-TTCTCGCCAACATCACCTCTGTCT-2232 (Rev); rat Col IX (NCBI accession number NM 001108675), 1961-TCGTGGATGTGGTGCTGAAGATGA-1984 (Forw) and 2100 -ATTGGGTCCCTGTTTGCCTGGATA-2083 (Rev); rat aggrecan (NCBI accession number NM_0221190.1), 1363-AAGGACTGTCTATCTGCACGCCAA-1386 (Forw) and 1487-TCACCACCCACTCCGAAGAAGTTT-1465 (Rev).

3.2.4 Histological analysis

Cultures were fixed with 4% paraformaldehyde, and then stained with hematoxylin and eosin (H&E) according to standard protocol. For alcian blue staining, cells were incubated with 1% (w/v) alcian blue overnight, followed by

repeated washes with 0.1 N HCl. Quantification of alcian blue staining was carried out by applying 4 M guanidinium chloride to the stained cells then measuring the absorbance of the resultant solution at 590 nm on a spectrophotometer[186].

3.2.5 Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde and incubated with primary antibodies overnight. The primary antibodies used in this study are mouse anti-Collagen II (generous gift from Dr. Tom Linsenmayer, Tufts University), MyoD (clone 5.8, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), Collagen IX (Developmental Studies Hybridoma Bank), Myosin Heavy Chain (MHC) (MF20 from Dev.Stud.Hyb.Bank)21 and rabbit anti-Desmin (Abcam Cat #12500). After washing with phosphate-buffered saline (PBS) with 0.1% Tween (PBST), cultures were incubated with secondary antibodies (conjugated with Alexa 488 (green) or 594 (red) from Invitrogen), followed by repeated washing. For detecting actin structures, Alexa Fluor 594-conjugated phalloidin (Invitrogen) was applied to the fixed cells at 5 units/mL for 30 min at room temperature. All cultures were counterstained with DAPI (Invitrogen).

3.2.6 Microscopy

Bright-field and fluorescent images from histological and immunocytochemistry analysis of 2D and 3D cultures were taken under the Olympus IX71 inverted microscope using Olympus DP70 digital camera and associated software. For quantification of immunofluorescent signals in 3D cultures, images were taken under the Zeiss LSM510 confocal microscope. For scanning electron microscopy analysis, 3D collagen gel culture

specimens were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 4 h and stored in buffer overnight. The samples were then postfixed in 1% osmium tetroxide for 1 h, washed, dehydrated in ethanol, and critically point dried using an Edwards Auto 306 Vacuum Evaporator equipped for general coating. The samples were sputter coated with palladium-gold and observed using an ISI DS130 scanning electron microscope at Tufts Imaging Facility.

3.2.7 Quantification of fluorescent images and statistical analysis

Relative protein levels were quantified by analyzing pixel intensity of the fluorescent images using the computer program Image J[187, 188]. Values of pixel intensity were normalized to the total chondrocyte numbers, which were determined by their round cell morphology and Collagen II protein expression. Three repeats were carried out for each experiment, and for each experiment 3–10 views of fields were photographed for quantification. For statistical analysis, the mean and standard deviation were calculated. Statistically significant differences (i.e., $p < 0.05$) were determined by one-factor analysis of variance (ANOVA) with post hoc Tukey test using the statistics software SYSTAT12 (Systat, Chicago, IL, USA).

3.2.8 Western blot analysis

For Western blot analysis, total protein lysates were obtained following a standard protocol from confluent 6cm tissue culture plates that contained roughly 3×10^6 cells[189]. The proteins were separated by SDS-PAGE using BioRad mini-gel apparatus and blotted onto nitrocellulose membranes using BioRad transfer apparatus. The membranes were blotted

with the following antibodies overnight: rabbit anti-Collagen II (Abcam, Ab34712), rabbit anti-Desmin (Abcam, Ab12500) and mouse anti-GAPDH (Abcam, Ab8245). After repeated washing, the membranes were hybridized with secondary antibodies of goat antimouse or goat antirabbit HRP conjugated antibodies (Calbiochem, LaJolla, CA, USA). The signals were developed using Pierce ECL substrate (cat# 32106), and Kodak films exposed to chemiluminescent signals were developed in Kodak M35A X-OMAT processor.

3.3 Results

3.3.1 Muscle cells promote cartilage gene expression in RCS chondrocyte cell line in 2D cocultures

We evaluated the effect of muscle cells on cartilage cells by coculturing these two cell types as monolayer cultures. For muscle cells, we selected C2C12 (mouse muscle cells), the most widely used muscle cell line in studying muscle differentiation and in muscle tissue engineering[190-192]. For cartilage cells, we selected RCS (rat chondrosarcoma cells), which has the same culture condition as C2C12 cells (DMEM with 10% FBS); and is a commonly used cell line for studying cartilage homeostasis, cell cycle control and cartilage matrix gene expression[142, 193-200]. We chose muscle and cartilage cells of mouse and rat origins respectively, as the mouse and rat species are closely related but also different which would allow us to perform RT-PCR analysis on only chondrocyte gene expression using rat-specific primers. To confirm that chondrocytes and muscle cells maintain their phenotypes when cocultured, we labeled C2C12 cells, but not RCS chondrocytes, with lineage tracer DiI. When cultured separately, these two

cell types exhibited a distinct difference in cell shape. C2C12 muscle cells have an elongated, fibroblast-like morphology, while RCS chondrocytes are rounded in shape (Fig. 1A). When cocultured, DiI-positive cells still had the fibroblast-like morphology, while all unlabeled cells maintained a round morphology. This indicates that muscle cells and chondrocytes do not change their morphology upon coculturing (Fig. 1A). Furthermore, our immunocytochemistry analysis showed that in cocultures, all RCS cells continued to express cartilage marker Collagen II[142]. Similarly, all C2C12 cells continued to express muscle marker Desmin[201] (Fig. 1B). Although some muscle cells are at the mononuclear myoblast stages, other muscle cells have fused into myotubes (Fig. 1B). The presence of both cartilage and muscle markers suggests that these two different cell lines maintained their cartilage and muscle phenotype respectively when cocultured (Fig. 1A and 1B).

We further evaluated cartilage matrix production through histological and immunocytochemistry analysis. We found that when compared with RCS chondrocytes cultured alone, chondrocytes cocultured with muscle cells exhibited stronger basophilic H&E staining and more intense alcian blue staining (Fig. 2A and B), suggestive of the presence of more glycosaminoglycans (which are negatively charged) in cocultures[179]. As we established that muscle cells maintained their muscle identity when cocultured (Fig. 1B), we believe that the increase in alican blue intensity in the coculture was due to an increased amount of cartilage matrix produced by the chondrocytes. It seems that upon coculturing, muscle cells herd the chondrocytes into clusters and the chondrocytes assume a rounder

phenotype (Fig. 2A and B). Furthermore, chondrocytes (RCS) cocultured with mouse C2C12 muscle cells expressed higher levels of collagen II and collagen IX proteins (Fig. 2C–E). To confirm our quantification result from immunofluorescent signals, we performed Western blot analysis to evaluate Collagen II protein expression in RCS chondrocytes cultured alone or cocultured with mouse muscle cells. We again found that RCS chondrocytes cocultured with C2C12 cells expressed a higher level of Collagen II (Fig. 2F). Interestingly, qRT-PCR analysis indicated that the mRNA levels of cartilage-specific genes (Collagen II, Collagen IX, and Aggrecan) were not significantly altered by the presence of muscle cells (Fig. 2F), suggesting that in our 2D cultures, muscle cells promote cartilage matrix production primarily at the posttranscriptional level.

To evaluate whether muscle cells also have the same effect on primary chondrocytes, we cultured primary bovine articular chondrocytes (BAC) with C2C12 muscle cells. We found that cocultured primary chondrocytes also exhibited stronger alcian blue staining and collagen II staining than those of chondrocytes cultured alone (Fig. 3A–D). Taken together, our results demonstrate that C2C12 muscle cells increase the expression of cartilage matrix proteins in a chondrocyte cell line as well as in primary chondrocytes.

3.3.2 Nonmuscle cells do not promote cartilage matrix production in RCS chondrocytes

We then asked whether the effect on cartilage gene expression is specific to muscle cells or if other cell types could also promote matrix production when cocultured with chondrocytes. Thus, we cocultured RCS

chondrocytes with the nonmuscle mesenchymal cell line NIH3T3. We found that NIH3T3 also herded the chondrocytes into clusters, similar to the way C2C12 muscle cells herded the chondrocytes in cocultures (Fig. 4A). Compared with RCS chondrocytes cultured alone, chondrocytes cocultured with NIH3T3 did not exhibit increased Collagen II expression after 4 days of culturing (Fig. 4A and B). Furthermore, chondrocyte-NIH 3T3 cocultures exhibited a much lower level of alcian blue staining compared with chondrocytes cultured alone (Fig. 4C). This result indicates that nonmuscle cells NIH3T3 do not promote cartilage matrix production in RCS chondrocytes. This result also suggests that confinement of chondrocytes into a smaller growth space, a process that benefits chondrocyte differentiation from progenitor cells[202], is not sufficient to promote cartilage matrix production in already formed chondrocytes.

3.3.3 Muscle cell-secreted factors promote Collagen II and Collagen IX expression

To test if muscle cells promote cartilage matrix production by releasing secreted factors into the medium, we cultured RCS chondrocytes in C2C12 muscle cell-conditioned medium. We noticed that chondrocytes cultured in the conditioned medium of C2C12 muscle cells looked rounder and less flattened than chondrocytes cultured in regular medium (Fig. 5A), similar to what we observed in muscle-cartilage cell cocultures (see Fig. 2A). This difference in morphology correlates with a change in the actin cytoskeleton, which is reflected by Phalloidin staining (Fig. 5B). It seems that chondrocytes cultured in muscle cell-conditioned medium have a more compact pattern of actin structure. This actin structure is similar to that observed in differentiated primary bovine chondrocytes, but not in

dedifferentiated chondrocytes (Supplemental Fig. 1). Furthermore, we found that chondrocytes cultured in muscle cell-conditioned medium exhibited a higher level of Collagen II and Collagen IX protein expression in our quantification analysis of the immunofluorescent signals (Fig. 5B and C). This result is further confirmed by Western blot analysis on RCS cells cultured in C2C12 conditioned medium (Fig. 5D). RCS cells cultured in both 50 and 100% C2C12 conditioned medium exhibited a significant increase in Collagen II protein expression compared with those cultured in the regular medium (Fig. 5D). These results suggest that muscle cells may secrete pro-chondrogenic factors.

We further confirmed this notion by culturing RCS chondrocytes in the conditioned medium from either a nonmuscle cell type or a converted muscle cell type. Primary CEFs are nonmuscle cells, and they can be converted into muscle cells when infected with MyoD-expressing viruses. MyoD is a master regulator of myogenesis and can transform many nonmuscle cells into muscle cells[203]. Indeed, infection of CEF by avian-specific retrovirus RCAS-MyoD induced the expression of muscle-specific markers Desmin and Myosin, while GFP-infected CEFs did not express any of the muscle markers (Fig. 6A). This shows that MyoD infection has converted the CEFs into muscle cells. When we applied CEF–MyoD-conditioned medium to the RCS chondrocytes, we observed a twofold increase in Collagen II protein expression and a fivefold increase in Collagen IX protein expression compared with CEFs treated with CEF–GFP control medium (Fig. 6B–D). Because the avian retroviruses (GFP and MyoD) present in the conditioned

medium did not infect rat cells, this result suggests that factors released from converted muscle cells promoted cartilage gene expression.

3.3.4 Muscle cells promote cartilage matrix production in RCS chondrocytes in 3D cocultures

To evaluate the effect of muscle cells on cartilage matrix production in 3D cultures, which recapitulates the in vivo situation more accurately, we seeded RCS chondrocytes and C2C12 muscle cells into 3D collagen gels. In collagen gels, RCS chondrocytes exhibited a round morphology when cultured either alone or with muscle cells (Fig. 7A). Strikingly, our bright-field and scanning electron microscopic images showed that upon coculturing, muscle cells formed a 3D lattice-like structure, separating the round chondrocytes into individual compartments (Fig. 7A). Consistent with our 2D analysis, our alcian blue staining on 3D cultures showed enhanced alcian blue staining in cocultured chondrocytes (Fig. 7B). Our immunocytochemistry analysis confirmed that the cells surrounding the chondrocytes were indeed muscle cells, as they were Desmin-positive (Fig. 7C). In addition, we found that cocultured chondrocytes had stronger Collagen II and Collagen IX protein expression (Fig. 7D). We further analyzed the mRNA expression of cartilage matrix genes by qRT-PCR. We found that muscle cells enhanced the mRNA expression of Collagen II and Aggrecan (Fig. 7E), whereas we did not observe such as an effect in our 2D cultures (Fig. 2F). However, in both 2D and 3D cultures, coculturing with muscle cells did not lead to a significant change in Collagen IX mRNA expression (Fig. 7E). It is possible that 3D culturing may favor the interaction between muscle and cartilage cells, as both cartilage and muscle tissues function in a 3D environment.

Nevertheless, both 2D and 3D culture results show that, at the protein level, muscle cells promoted cartilage gene expression in chondrocytes.

3.4 Discussion

In this investigation, we tested the hypothesis that muscle cells regulate cartilage gene expression by analyzing chondrocytes cocultured with muscle cells in 2D and 3D conditions. We showed that chondrocytes cultured with muscle cells exhibited enhanced alcian blue staining, which is indicative of increased GAG content[179]. In addition, chondrocytes cocultured with muscle cells showed elevated expression of collagen II and collagen IX proteins. We propose that muscle cells achieve this effect through secreted factors as muscle cell-conditioned medium also led to increased cartilage matrix production.

3.4.1 Muscle regulation of cartilage development

It has long been suggested that muscle could influence skeletal development through mechanical forces generated by muscle contraction[183, 204]. Support for this notion stems from earlier experiments in which muscle movement was abolished either by neurotoxin or extraction of amniotic fluid, leading to reduced skeleton size or abnormal joint structures[183, 205]. Thus, these authors suggest that the altered mechanical stimuli can be sensed by the developing cartilage. However, it is not clear how these treatments had altered cartilage matrix gene expression. Consistent with this theory, in vitro stress testings indeed show that mechanical forces can directly affect cartilage development[206, 207].

Our results suggest that muscle may also influence skeletal development by releasing biochemical signals outside the muscle cells. Indeed, muscle secretes a variety of growth factors or cytokines that can be carried away by blood or interstitial fluid[208-210]. Among them is the known pro-chondrogenic factor IGF-I[207, 209]. In our experiments, we did not specifically select muscle cells of different stages (myoblast and myocyte or myotube), which have different gene expression profiles[208, 211]. It is not clear whether IGF-I or other unknown factors are responsible for muscle cell-mediated cartilage regulation in our experimental settings. Our work is consistent with the study that showed that mouse muscle-derived stem cells were cocultured with human nucleus pulposus cells, proteoglycan expression of the nucleus pulposus cells were increased[212]. The fact that muscle cells secrete pro-chondrogenic factors is also consistent with the results from studying muscle-specific gene knockouts and muscle paralysis models. Although knocking out muscle-specific genes would affect the profile of muscle secreted factors[61, 184], muscle paralysis would inevitably lead to muscle atrophy and myocyte cell death[213], thereby also leading to altered muscle cell-derived biochemical signals.

3.4.2 Differential control of mRNA and protein expression in chondrocytes

The expression of cartilage ECM can be regulated at both the mRNA level and the protein level. It is known that cartilage gene expression is regulated by many factors at the transcriptional level. Transcription factors such as Sox9 family members, Nkx3.2, Groucho, PGC1a, Delta-EF1 or AP2, all control the transcription of cartilage matrix genes[142, 214-218]. We have found that muscle cells strongly promote cartilage matrix production at the

protein level. It is possible that muscle cells regulate cartilage ECM at the level of translational control, or at the level of matrix degradation and maintenance. Thus, it will be intriguing to investigate whether muscle cells affect the expression of cartilage degradation enzymes (such as MMPs and ADAMTs) or MMP inhibitors, TIMPs [219-221]. Future studies will focus on the mechanism by which muscle cells regulate cartilage gene expression. Identification of novel factors and regulatory pathways will undoubtedly have a positive impact on the understanding of skeletal diseases and the technology of cartilage regeneration.

3.5 Chapter 3 figures and legends

Figure 3.1

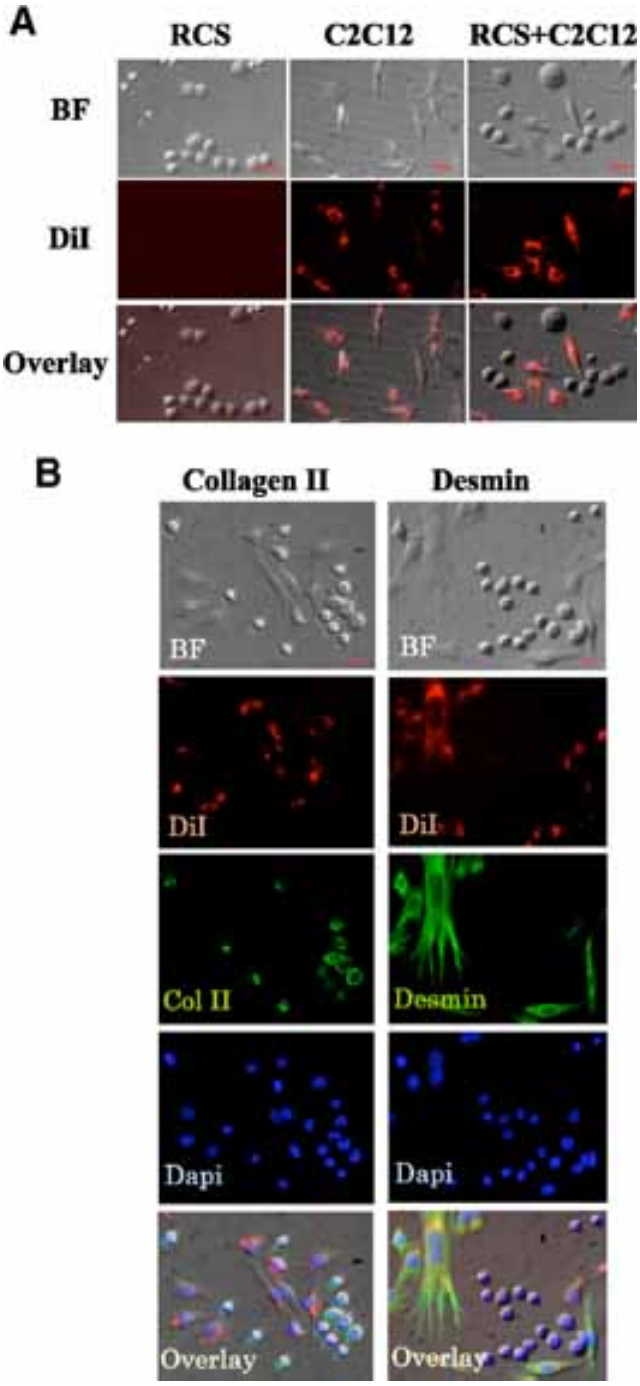

Fig. 1. RCS chondrocytes and C2C12 muscle cells maintain their phenotypes in 2D cocultures. (A) C2C12 cells were labeled with DiI (red), and exhibit elongated morphology in cocultures. RCS cells were unlabeled, and were round shaped. BF, bright-field. After coculturing for 2 days, both cell types maintained their cell morphology. Scale bars, 20 μm. (B) In cocultures, all RCS chondrocytes continued to express Collagen II (cartilage marker, green), while DiI-labeled C2C12 muscle cells continued to express Desmin (muscle marker, green). The specificity of the antibodies was confirmed by staining with secondary antibodies alone (see Supplemental Fig. 2). BF, bright-field. Dapi, nucleus staining. Overlay, merged images of Collagen II, Desmin, Dapi and Bright-field. Scale bars, 20 μm.

Figure 3.2

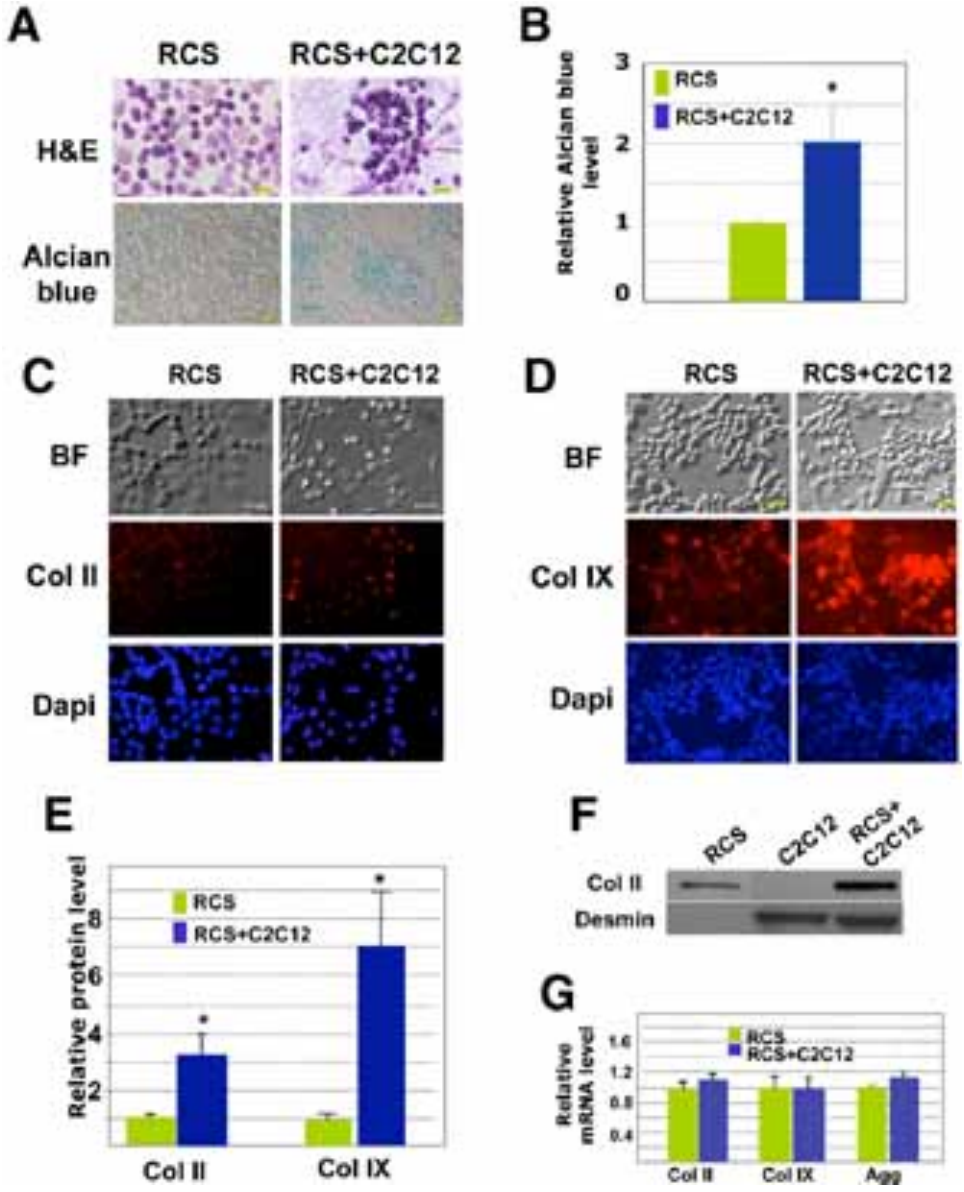
A
RCS
RCS+C2C12
H&E
Alcian blue
B
Relative Alcian blue level
RCS
RCS+C2C12
C
BF
Col II
Dapi
D
Col IX
E
Relative protein level
Col II
Col IX
F
C2C12
Desmin
G
Relative mRNA level
Agg

Fig. 2. C2C12 muscle cells enhance cartilage matrix production in RCS cells in 2D cocultures. (A) H&E and alcian blue staining after 4 days of culture. Cocultured chondrocytes showed stronger basophilic staining than chondrocytes cultured alone. No alcian blue staining was observed for C2C12 muscle cells cultured alone (data not shown). Scale bars, 20 μm. (B) Alcian blue quantification. Values shown are the absorbance values at 590 nm when dissolved in 4 M GuCl after being normalized to the total chondrocyte number. (C) Immunocytochemistry analysis of Col II (red). For a negative IgG control, see Supplementary Figure 2A. BF, bright-field. (D) Immunocytochemistry analysis of Col IX (red). BF, Bright-field. For a negative IgG control, see Supplementary Figure 2A. (E). Quantification of relative Col II and Col IX protein level based on immunofluorescent intensities (pixels) using the program “Image J.” Values were normalized to total chondrocyte number. *Indicates: $p<0.05$ in statistical analysis (n=4). (F) Western Blot analysis on cell lysates obtained from RCS cultures, C2C12 cultures, and RCS and C2C12 cocultures. As we were unable to locate an antibody that reacts specifically to rat GAPDH, but not mouse GAPDH, we normalized the protein loading to the number of RCS chondrocytes. Desmin, a muscle-specific protein, was only present in lysates of C2C12 cells. (G) qRT-PCR analysis of Col II, Col IX, and Aggrecan mRNA levels. Green, RCS alone; blue, RCS-C2C12 cocultures.

Figure 3.3

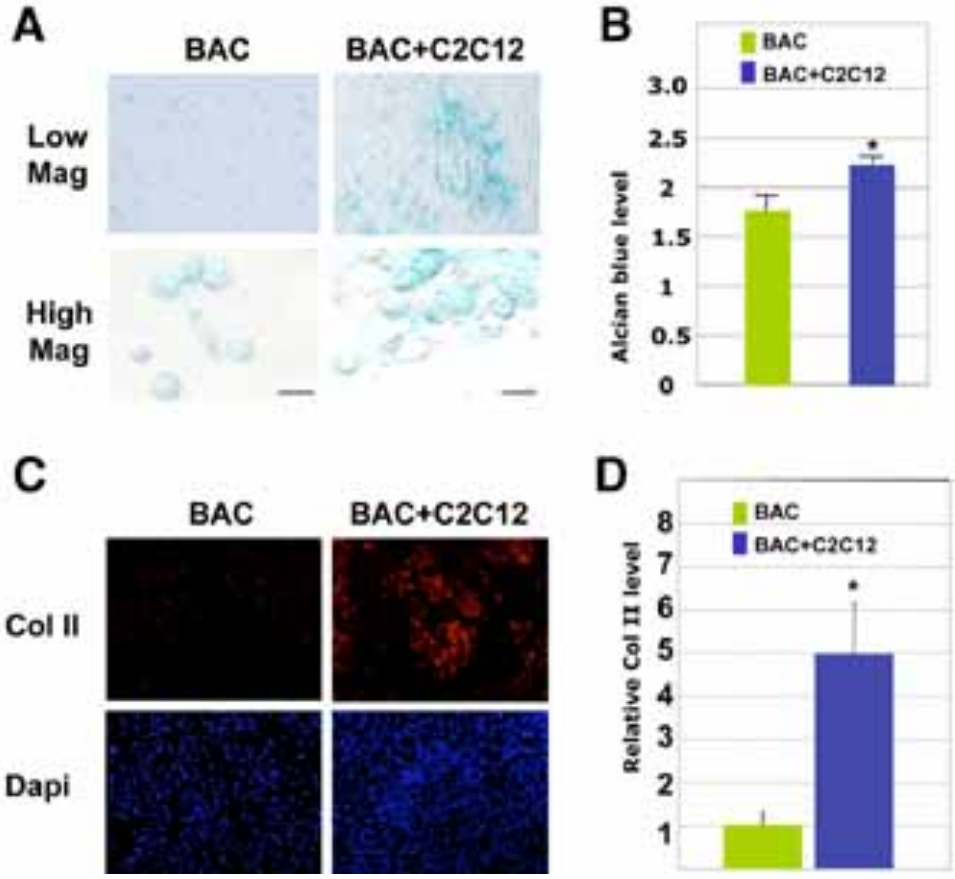
A
BAC
BAC+C2C12
Low Mag
High Mag
B
BAC
BAC+C2C12
Alcian blue level
3.0
2.5
2
1.5
1
0.5
0
*
C
BAC
BAC+C2C12
Col II
Dapi
D
BAC
BAC+C2C12
Relative Col II level
8
7
6
5
4
3
2
1
*

Fig. 3. C2C12 muscle cells promote cartilage matrix production in primary bovine articular chondrocytes (BAC) in 2D cultures. (A) Alcian blue staining after 4 days of culturing. Low mag (low magnification), 10×; high mag (high magnification, 40×). Some BAC cells had a dedifferentiated morphology when cultured alone. Scale bars, 20 μm. (B) Quantification of alcian blue staining from three culture replicates. Values shown are the absorbance values at 590 nm when dissolved in 4 M GuCl. *Denotes: $p<0.05$ in statistical analysis. (C) Immunocytochemistry analysis of Collagen II staining (red). Muscle cells were not positive for Col II. (D) Quantification of relative Collagen II protein level, using Image J. The values are fluorescent signal intensities of the entire microscopic views of BAC and BAC+C2C12 cells, and were not normalized to total numbers of chondrocytes. *Denotes: $p<0.05$ in statistical analysis (n=4).

Figure 3.4

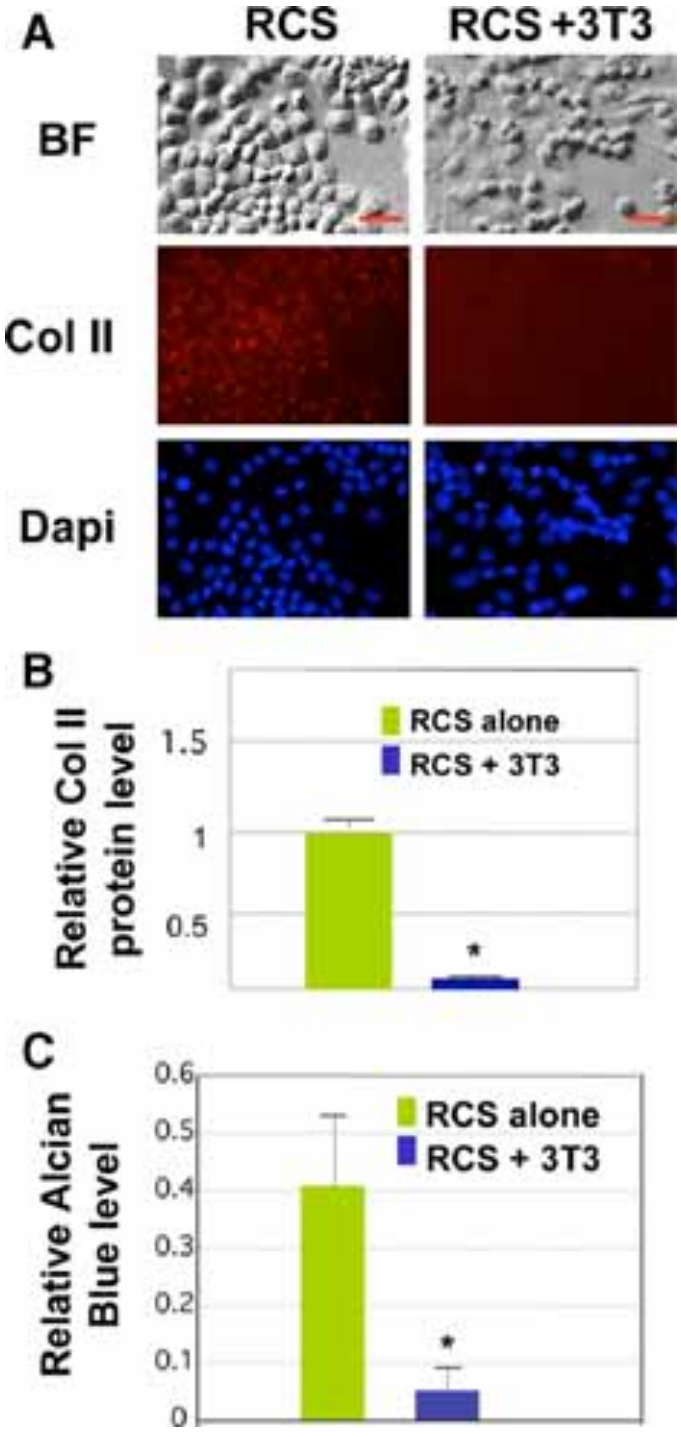
A
RCS
RCS +3T3
BF
Col II
Dapi
B
Relative Col II protein level
1.5
1
0.5
RCS alone
RCS + 3T3
*
C
Relative Alcian Blue level
0.6
0.5
0.4
0.3
0.2
0.1
0
RCS alone
RCS + 3T3
*

Fig. 4. Nonmuscle NIH3T3 cells do not promote cartilage matrix production in 2D cultures. (A) Collagen II immunostaining (red) after coculturing RCS chondrocytes with NIH3T3 cells for 4 days. Scale bars, 20 μm. (B) Quantification of Col II fluorescent intensity using the program Image J. Values are normalized to total number of chondrocytes. *Denotes: $p < 0.05$ in statistical analysis ($n = 4$). (C) Quantification of alcian blue staining from three culture replicates. Values shown here are the absolute values of absorbance reading at 590 nm when dissolved in 4 M GuCl. *Denotes: $p < 0.05$ in statistical analysis.

Figure 3.5

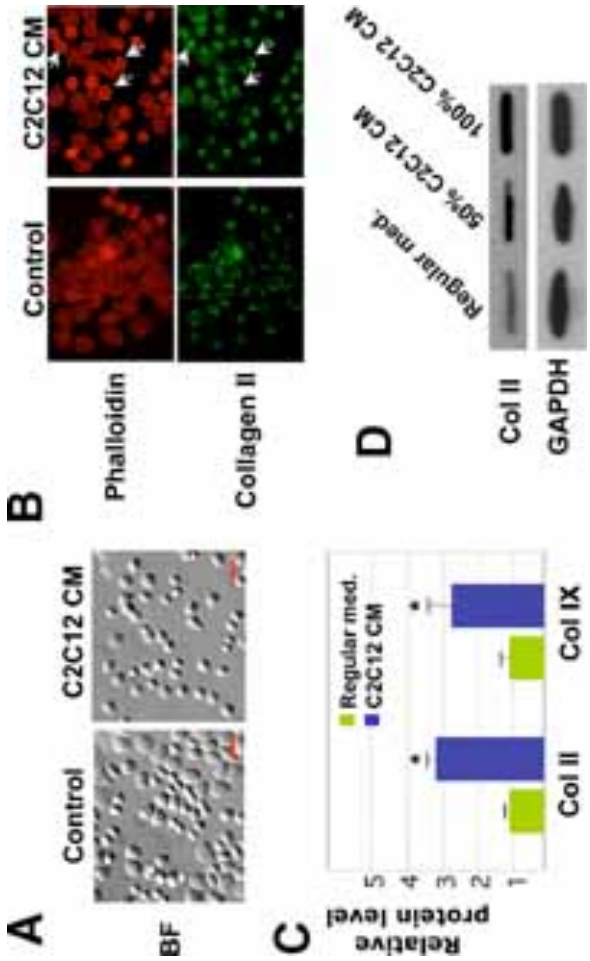
A
Control
C2C12 CM
BF
B
Control
C2C12 CM
Phalloidin
Collagen II
C
Regular med.
C2C12 CM
Relative protein level
Col II
Col IX
D
Regular med.
50% C2C12 CM
100% C2C12 CM
Col II
GAPDH

Fig. 5. The effect of muscle cell-conditioned medium on chondrocyte cell morphology and cartilage expression in 2D cultures. (A) Bright-field (BF) images of RCS chondrocytes cultured in regular medium or C2C12 muscle cell conditioned (CM) medium for 3 days. Cells cultured in C2C12 CM have a more rounded morphology. Scale bars, 20 μm. (B) Phalloidin (red) and collagen II (green) staining of RCS chondrocytes, indicating a change of cytoskeletal structures upon culturing in muscle cell-conditioned medium. Arrows: cells with rounder morphology. (C) Quantification of relative Col II and Col IX protein levels using the program Image J. The intensity values shown here were normalized to total cell numbers. *Denotes: $p < 0.05$ in statistical analysis ($n = 4$). (D) Western blot analysis on Collagen II protein expression in RCS chondrocytes grown in regular culture medium or medium containing 50 and 100% C2C12 conditioned medium. Medium was changed daily. GAPDH, loading control.

Figure 3.6

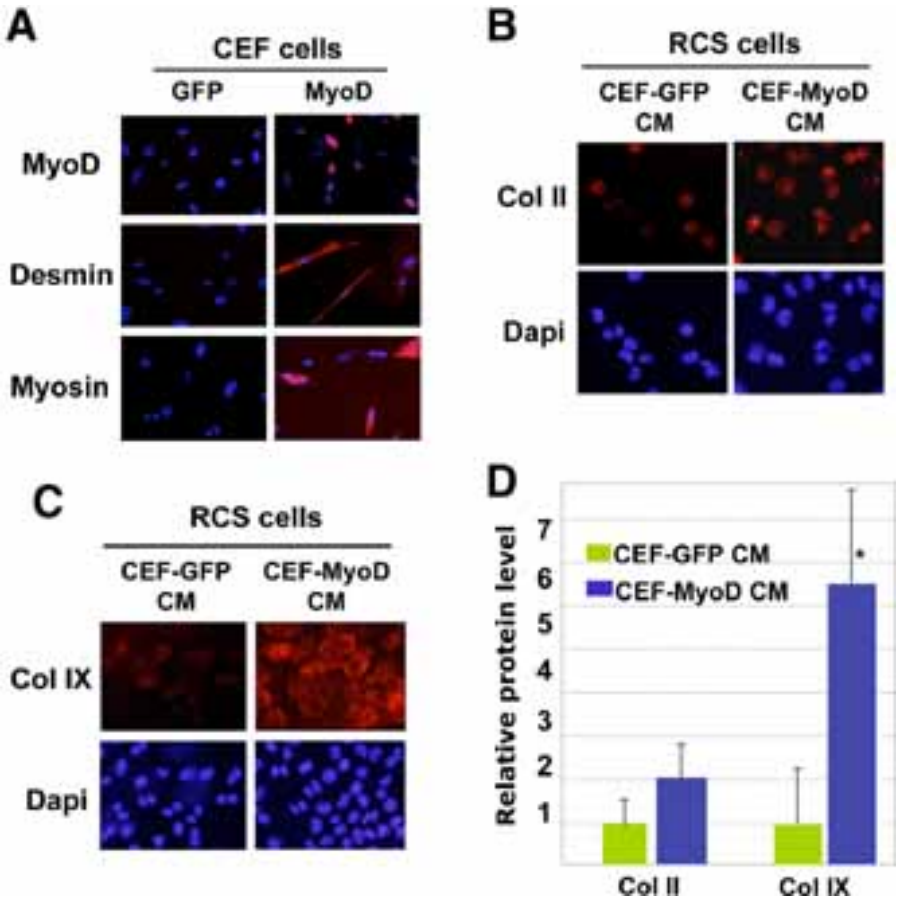
A
CEF cells
GFP
MyoD
MyoD
Desmin
Myosin
B
RCS cells
CEF-GFP CM
CEF-MyoD CM
Col II
Dapi
C
RCS cells
CEF-GFP CM
CEF-MyoD CM
Col IX
Dapi
D
Relative protein level
1
2
3
4
5
6
7
CEF-GFP CM
CEF-MyoD CM
*
Col II
Col IX

Fig. 6. Cells converted into muscle cells promote cartilage gene expression. (A) Chicken embryo fibroblasts (CEF) were infected with avian-specific retroviruses GFP (control) and MyoD. Three days after administration of MyoD virus into the medium, many CEFs were positive for MyoD (red), as well as muscle-specific markers Desmin and Myosin (red). Control, GFP virus infection. The muscle marker stainings were overlaid with Dapi staining. (B) Immunofluorescent analysis of Col II protein expression in RCS chondrocytes cultured in CEF–GFP and CEF–MyoD conditioned medium (CM) (3 days of culture). (C) Analysis of Col IX protein expression by immunostaining in RCS chondrocytes cultured in CEF–GFP or CEF–MyoD conditioned medium (CM) (3 days of culture). (D) Quantification of Col II and Col IX protein expression using the program Image J. Values were normalized to total chondrocyte number. *Denotes: $p < 0.05$ in statistical analysis ($n = 4$).

Figure 3.7

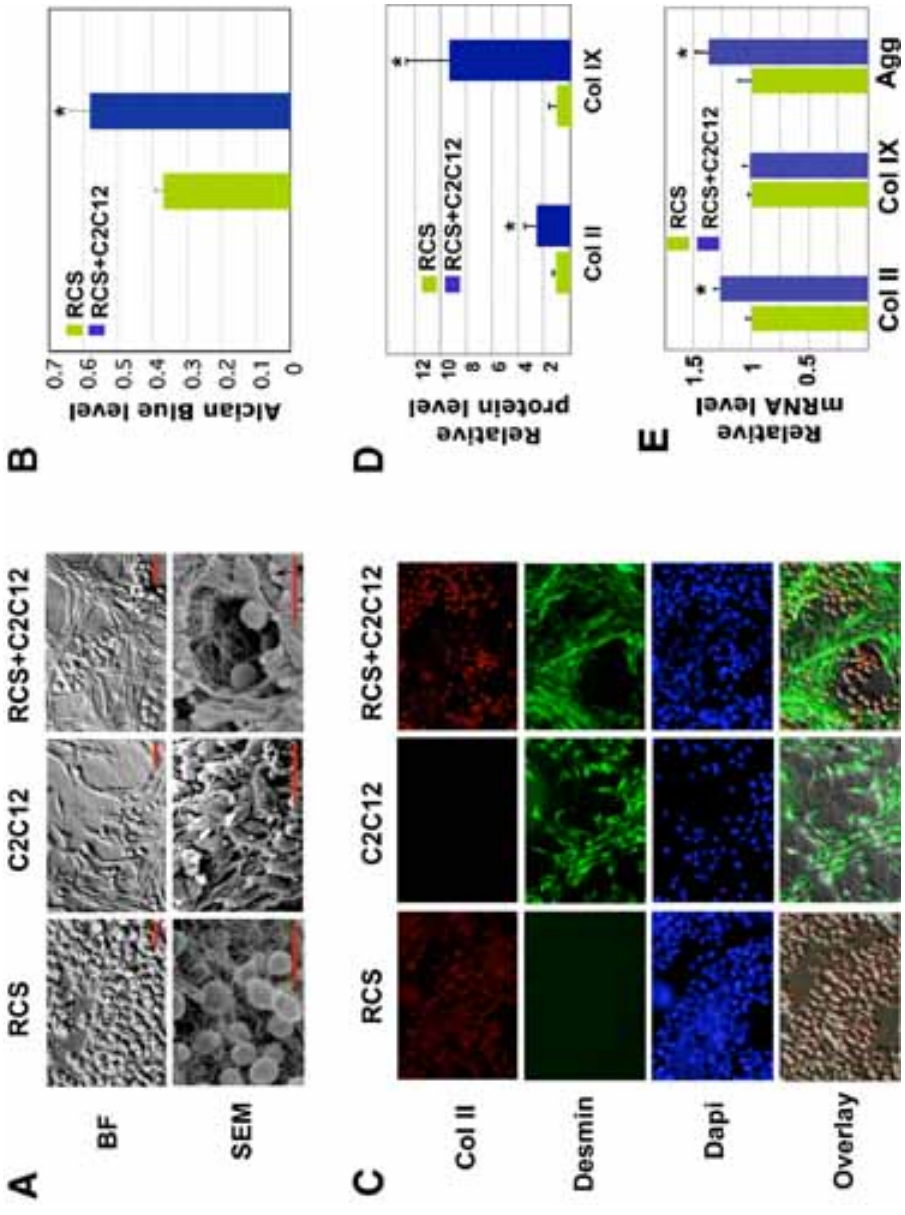
A
RCS
C2C12
RCS+C2C12
BF
SEM
B
Alcian Blue level
RCS
RCS+C2C12
C
RCS
C2C12
RCS+C2C12
Col II
Desmin
Dapi
Overlay
D
Relative protein level
RCS
RCS+C2C12
Col II
Col IX
E
Relative mRNA level
RCS
RCS+C2C12
Col II
Col IX
Agg

Fig. 7. Analysis of RCS chondrocytes and C2C12 muscle cells in 3D collagen gels. (A) Morphological analysis of 3D cultures using light (BF) and scanning electron microscopy (SEM). Scale bars, 20 μm. (B) Quantification of alcian blue staining with A590 nm spectrophotometer reading from three culture replicates. Values were normalized to the total chondrocyte number. No alcian blue staining was observed in C2C12 muscle cells cultured alone (data not shown). *Denotes: $p < 0.05$ in statistical analysis. (C) Immunocytochemistry analysis of collagen II (red) and Desmin (green) expression. (D) Quantification of Col II and Col IX protein levels in 3D cultures using the program Image J. The fluorescent intensities from confocal images were normalized to total chondrocyte number. *Denotes: $p < 0.05$ in statistical analysis ($n = 6$). (E) qRT-PCR analysis of Col II, Col IX, and Aggrecan mRNA levels in 3D cultures. Green, RCS alone; blue, RCS–C2C12 cocultures.

Supplemental Figure 3.1

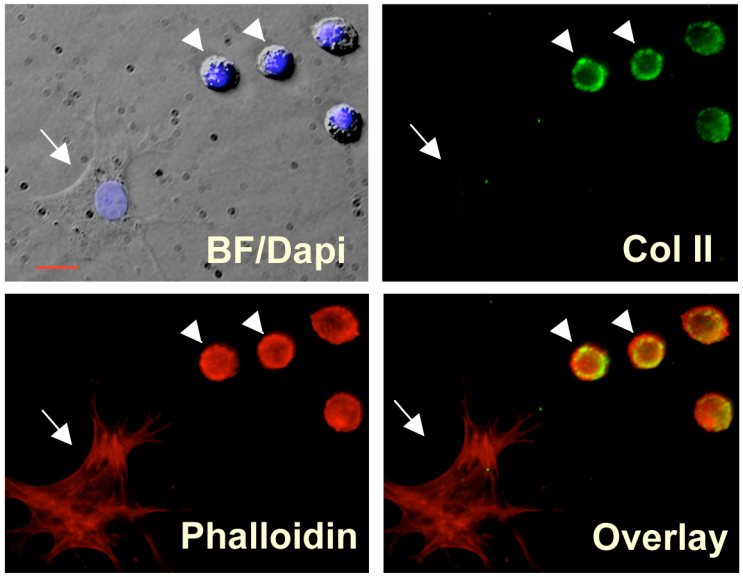

Fig. S1. Phalloidin staining indicating actin cytoskeletal structure in differentiated and de-differentiated primary bovine articular chondrocytes (BAC). Upon 2D culturing for an extended period of time (7 days), some BAC cells assume a fibroblast-like morphology (Bright field, BF and Dapi, blue) and actin structure (red), and have a diminished level of Collagen II protein (green), which is suggestive of de-differentiation (arrow). Round chondrocytes have a more compact actin structure and express significant levels of Collagen II (green), which is indicative of a differentiated state (arrowheads). Scale bar, 20 um.

Supplemental Figure 3.2

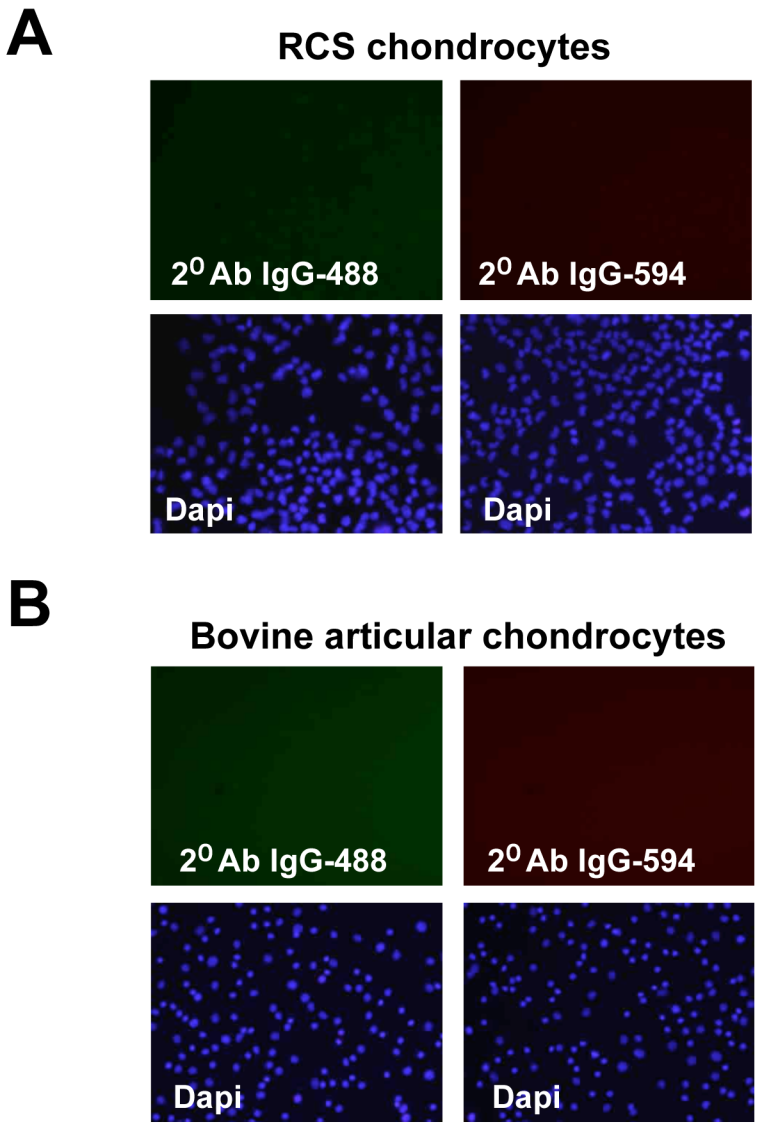

Fig. S2. Immunocytochemistry control experiments indicating the specificity of primary antibodies. (A) RCS chondrocytes were immunostained with goat anti-mouse secondary antibodies conjugated with Alexa 488 and Alexa 594. (B) Bovine articular chondrocytes (BAC) were immunostained with goat anti-mouse secondary antibodies conjugated with Alexa 488 and Alexa 594. No specific stainings were observed in either cell type.

CHAPTER 4: Muscle cells enhance resistance to pro-inflammatory cytokine-induced cartilage destruction

4.1 Introduction

Inflammation of the joint leads to chronic pain and swelling in arthritis[83]. Pro-inflammatory cytokines in the inflamed joint, most notably IL-1β and TNFα, disrupt the catabolic and anabolic balance of the cartilage cells (i.e. chondrocytes). This leads to the destruction of cartilage extracellular matrix (ECM), whose major protein components are collagens and proteoglycans[98]. Collagen type II is the major collagen in cartilage that makes up over 90% of collagen mass[83]. Other collagens, including collagen IX and XI, play important roles in stabilizing the structure of collagen type II [180]. Pro-inflammatory cytokine-induced destruction of collagen and other ECM is mediated by the signaling pathways of MAP kinases and NFκB[98]. These events subsequently lead to two effects on cartilage gene expression. One effect is the induction of metalloproteinases MMPs and ADAMTS, which leads to the degradation of collagen and aggrecan at the protein level. This effect is mediated by transcription factors such as AP-1 [98]. The other effect is the inhibition of transcription of cartilage matrix proteins such as collagen II, which reduces the cartilage matrix production at the mRNA level [98]. This effect is mediated by transcription factors EGR-1, ESE-1 and GADD45β, as well as by cyclooxygenase-2 (Cox-2) [222-224].

Under normal conditions, cartilage homeostasis is regulated by factors expressed within the cartilage tissue as well as tissues surrounding the cartilage [98]. Muscle is a tissue that lies immediately next to the developing cartilage in the embryo[31]. While cartilage does not directly contact muscle tissue in the adult joint (except at the temporomandibular

joint), it remains in close proximity to cartilage throughout life [225]. Multiple pieces of evidence suggest that muscle may regulate skeletal development. For example, when muscle in the chick embryo was paralyzed by botulinum toxin, which abolished muscle contraction and caused muscle atrophy, the embryo showed abnormal joint formation and shortened bones [183]. Mouse mutants that lack muscle-specific proteins such as dystrophin/utrophin or myogenin also exhibited skeletal abnormalities including a curved spine or a reduced skeletal size [61, 66]. Similarly, mouse knockouts of Pax7, a marker for muscle progenitor cells, exhibited reduced body sizes as well [35]. Consistent with the phenotype of these mouse mutants, short stature and scoliosis are common features of children with Duchenne Muscular Dystrophy [62, 63]. More recently, we found that chondrocytes cocultured with muscle cells exhibited increased expression of cartilage matrix proteins [226]. Since these muscle cell cultures do not contract, we believe that muscle cells provide biochemical signals to regulate cartilage gene expression[226].

In addition to regulating skeletal development, muscle has also been proposed to be an immunogenic organ [114]. Muscle produces a variety of cytokines (such as IL-6 and IL-15), matrix metalloproteinases (MMPs) and MMP inhibitors (TIMPs)[114-116]. Some of these factors have been shown to mediate exercise-associated anti-inflammatory responses [114]. Furthermore, patients with congenital myopathy showed deformation and fibrosis of the temporomandibular joint, and it has been suggested that reduced muscle strength may be a risk factor for knee arthritis[118, 119].

Despite the implication of muscle in regulating inflammation, the role of muscle cells on the response of chondrocytes to pro-inflammatory cytokines has never been reported. Here we show that chondrocytes cocultured with muscle cells are more resistant to cartilage destruction induced by pro-inflammatory cytokines, suggesting a novel role of muscle cells in regulating catabolic and anabolic processes in cartilage tissue.

4.2 Materials and methods

4.2.1 Cell culture

Murine myoblasts (C2C12) and NIH3T3 cells were purchased from American Type Culture Collection. RCS chondrocytes were a generous gift from Dr. Andrew Lassar (Harvard Medical School). The cells were seeded at a density of 10^4/well of 24 well plates. All cocultures were seeded at a ratio of 2:1 (RCS:C2C12). C2C12 muscle cell-conditioned medium was prepared as described [226]. Cells were cultured in DMEM with 10% FBS (Hyclone) and 1% pen/strep for 3 days before 3 days of IL-1β or TNFα (Peprotech) treatment.

4.2.2 Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde and incubated with primary antibodies overnight. The primary antibodies were: mouse anti-Collagen II (generous gift from Dr. Tom Linsenmayer, Tufts University); mouse anti-Collagen IX (D1–9, Dev. Stud. Hybr. Bank); rabbit anti-Desmin (Abcam Cat#12500). The secondary antibodies were: goat anti-mouse/rabbit conjugated with Alexa 488 (green) or 594 (red) (Invitrogen). Images were taken using the Olympus IX71 inverted microscope and Olympus DP70

digital camera. Relative protein levels were quantified by analyzing pixel intensities of fluorescent signals using the program Image J[187]. Values of pixel intensity were normalized to the total chondrocyte numbers, which were determined by their round cell morphology and collagen II protein expression. Three repeats were carried out for each experiment, and for each experiment 3–10 views were photographed for quantification. For statistical analysis, the mean and standard deviation were calculated. Statistically significant differences (i.e. $P<0.05$) were determined by one-way analysis of variance (ANOVA).

4.2.3 Western blot analysis

For Western Blot analysis, total protein lysates were obtained from confluent 6cm tissue culture plates that contained roughly 3×10^6 cells, following a standard protocol. The proteins were separated by SDS-PAGE and blotted using the BioRad apparatus. The membranes were hybridized with the following antibodies overnight: rabbit anti-Collagen II (Abcam, Ab34712), rabbit anti-Desmin (Abcam, Ab12500), mouse anti-Collagen IX (Chemicon/Millipore, MAB3304) and mouse anti-GAPDH (Abcam, Ab8245). After repeated washing, the membranes were hybridized with secondary antibodies of goat anti-mouse or anti-rabbit HRP-conjugated antibodies (Calbiochem). The signals were developed using ECL substrate (Pierce, cat# 32106), and films exposed to chemiluminescent signals were developed in Kodak M35A-X-OMAT processor.

4.2.4 RT-PCR analysis

Total RNA was isolated using the RNeasy mini kit (Qiagen) [226]. All PCR analyses were normalized based on GAPDH expression using the iQ5 Real Time PCR Detection System (BioRad). Rat primer sequences are (5' to 3'): GAPDH (NCBI#NM_017008), 1131-GTTGCTGAGGAGTCCCCA-1147 (Forw) and 1258-CCTATTCGAGAGAAGGGA-1241 (Rev); Col IIa1 (NCBI#NM_012929.2), 1972-AAGCAAGGTGACCAGGGTATTCCT-1995 (Forw) and 2255-TTCTCGCCAACATCACCTCTGTCT-2232 (Rev); Col IX (NCBI#NM_001108675), 1961-TCGTGGATGTGGTGCTGAAGATGA-1984 (Forw) and 2100-ATTGGGTCCCTGTTTGCCTGGATA-2083 (Rev); aggrecan (NCBI#NM_0221190.1), 1363-AAGGACTGTCTATCTGCACGCCAA-1386 (Forw) and 1487-TCACCACCCACTCCGAAGAAGTTT-1465 (Rev). MMP13 (NCBI#NM_133530.1), 1705-GCTGAAGAGTGATCATAA-1722 (Forw) and 1904-GCTATCTGTTAATGTGTGT-1886 (Rev). MMP9 (NCBI#NM-031055.1), 2869-AGCCCGTTTAAAGTGCATGTGTGC-2892 (Forw) and 2959-GAGTGTCCGAGGAAGATACTTGGT-2936 (Rev). ADAMTS4 (NCBI#XM_341154.1), 1818-AGCAGATGGTTCTTACGCCCTCAA-1841 (Forw) and 2012-AGACGTACATTCTGTGGGTTGCCA-1989 (Rev). NF-κB (NCBI#XM_342346), 3419-AAGGCCATCATATCGTTCCG-3438 (Forw) and 3619-CATACCCCAAGCCACACCGA-3600 (Rev). ESE-1 (NCBI#NM_001024768), 221-CCTGAACAACCAACACATGTCCC-243 (Forw) and 302-CTCAGTTCTGTCCCTTTGGGATCT-279 (Rev). COX-2 (NCBI#NM_017232.3) 1596-ACCTAGCACCTTCGGAGGA-1614 (Forw) and 1694-GAGGCAAAGGGACACCCTT-1676 (Rev). GADD45β (NCBI#NM_001008321), 2-AACCCGGGATCCGTGTCA-19 (Forw) and 187-GCAAAGCACTCGTCCAGATCC-167 (Rev).

4.3 Results

4.3.1 Chondrocytes cocultured with muscle cells maintained collagen protein expression upon pro-inflammatory cytokine challenge

We tested the effect of muscle cells on the response to pro-inflammatory cytokines in chondrocytes by coculturing these two cell types. We have previously used the same coculture system to evaluate the effect of muscle cells on cartilage matrix production [226]. In this system, we selected RCS cell line (rat chondrosarcoma cells), which is a commonly used chondrocyte cell line for studying cartilage gene expression [197]. For muscle cells, we selected C2C12 mouse muscle cell line, which is a widely used cell line for studying muscle differentiation [227]. RCS cells generally have a compact morphology and express cartilage marker collagen II, while C2C12 muscle cells have a fibroblast-like morphology and express muscle marker desmin (Fig.1A). In addition, C2C12 muscle cells do not express cartilage markers even when cocultured with chondrocytes (Fig.1A)[226]. This distinction between the two cell types allows us to evaluate protein expression in chondrocytes through immunocytochemistry.

We first assayed cartilage marker Collagen protein expression in chondrocytes treated with pro-inflammatory cytokines IL-1β and TNFα. We found that chondrocytes cultured alone showed reduced Collagen II and Collagen IX expression after the administration of 1ng/ml of IL-1β or 2ng/ml of TNFα (Fig.1B), which is consistent with prior reports [70]. Strikingly, Collagen II and Collagen IX protein expression remained high upon IL-1β or TNFα treatment in chondrocytes that were cocultured with muscle cells (Fig.1B). This result was further quantified using Image J, a program that

measures the fluorescent intensity of immunostained images [187] (Fig.1C and 1D). We performed Western Blot analysis and further confirmed that indeed chondrocytes cocultured with muscle cells expressed higher levels of collagen II and collagen IX proteins, which were maintained in the presence of IL-1β or TNFα (Fig. 1E). Since C2C12 cells do not express cartilage markers (Fig.1A and 1F)[226], our analysis suggests that muscle cells enhance the resistance of chondrocytes to pro-inflammatory cytokine-induced collagen reduction. We confirmed the specificity of this effect by coculturing chondrocytes with non-muscle cells NIH3T3 and found that coculturing with NIH3T3 cells did not lead to enhanced collagen II expression in chondrocytes upon IL1β treatment (S-Fig.1). Furthermore, we found that chondrocytes cultured in cell-free muscle cell-conditioned medium exhibited significantly enhanced resistance to pro-inflammatory cytokine-induced collagen destruction, suggesting that this effect is mediated by factors secreted by muscle cells (Fig.1F).

Increased resistance to IL-1β and TNFα-induced reduction of collagen II and collagen IX can be achieved by maintained mRNA production or by reduced protein degradation. To evaluate whether coculturing with muscle cells leads to maintained mRNA expression in chondrocytes treated with IL-1β or TNFα, we performed qRT-PCR analysis on collagen and aggrecan genes. We purposely chose cells from two different species so that the presence of mouse C2C12 cells would not interfere with our RT-PCR analysis of rat chondrocytes when we use rat-specific primers with GAPDH as a loading control. From this analysis, we found that coculturing with muscle cells did not alter the mRNA level of collagen II in the absence of IL-

1β and TNFα (Fig.2A and 2B). However, in the presence of increasing amounts of IL-1β (0.1-1ng/ml) or TNFα (0.5–2ng/ml), chondrocytes cocultured with C2C12 muscle cells exhibited significantly higher levels of collagen II mRNA, while control chondrocytes expressed a progressively reduced amount of collagen II mRNA (Fig.2A and 2B). In contrast to the results of collagen II, coculturing with muscle cells did not prevent IL-1β or TNFα-induced collagen IX mRNA downregulation (Fig.2C–2D). Since our earlier results indicated that collagen IX protein expression was maintained in co-cultured chondrocytes upon IL-1β or TNFα treatment (Fig.1D–1F), it suggests that muscle cells may regulate collagen IX expression primarily through a post-transcriptional mechanism. While we were unable to assay aggrecan protein expression due to the lack of a suitable antibody, we found that aggrecan mRNA was maintained at a higher level in cocultured chondrocytes than in chondrocytes cultured alone when treated with low levels of IL-1β or TNFα, although both cytokines did lead to significant reduction of aggrecan expression in cocultured chondrocytes (Fig.2E and 2F).

Taken together, we found that chondrocytes cocultured with muscle cells exhibited resistance to IL-1β and TNFα-induced collagen reduction. In addition, muscle cells may regulate cartilage gene expression at both the transcriptional level and the post-transcriptional level.

4.3.2 Coculturing with muscle cells leads to reduced expression of cartilage degrading enzymes

Cartilage degrading enzymes play important roles in regulating cartilage matrix expression at the post-transcriptional level [228]. Thus we

evaluated the expression of three such enzymes MMP13, MMP9 and ADAMTS4 [98]. We found that increasing amounts of IL-1β (from 0.1ng/ml to 1ng/ml) or TNFα (from 0.5 to 2ng/ml) led to a progressive increase in MMP13 expression in RCS chondrocytes cultured alone (Fig. 3A and 3B). However, IL-1β or TNFα (especially at low concentrations) did not induce a significant increase in MMP13 expression in chondrocytes cocultured with muscle cells (Fig.3A and 3B). Similarly, the expression level of other metalloproteinases MMP9 and ADAMTS-4 were also generally lower in chondrocytes co-cultured with muscle cells (Fig.3C–3F).

4.3.3 Coculturing with muscle cells reduces pro-inflammatory cytokine signaling in chondrocytes

We next evaluated whether reduced expression of cartilage degrading enzymes in chondrocytes cocultured with muscle cells correlates with reduced expression of downstream mediators of pro-inflammatory cytokines. As NFκB plays a key role in the signal transduction of pro-inflammatory cytokines[98], we evaluated the mRNA level of p105, which encodes a crucial NFκB subunit. We found that chondrocytes cocultured with muscle cells generally expressed lower levels of NFκB either in the absence or presence of IL-1β or TNFα (Fig.4A). To evaluate the activation of NFκB signaling, we assayed the expression of NFκB-induced factors. ESE1 (epithelial-specific ETS factor1) is a direct target of NFκB, and can inhibit collagen II expression by binding to its promoter [98]. Indeed, we found that ESE-1 expression in control chondrocytes was induced by both IL-1β and TNFα (Fig.4B). Interestingly, ESE-1 expression was significantly lower in cocultured chondrocytes treated with IL-1β or TNFα (Fig. 4B). Furthermore, Cox-2, a

factor induced by ESE-1 and mediates the IL-1β-induced inflammatory response, was also expressed at a lower level upon pro-inflammatory cytokine challenge in chondrocytes cocultured with muscle cells (Fig.4C). Finally, we assayed the expression of another NFκB-induced factor, GADD45β, which also inhibits collagen II mRNA expression [98, 224]. Similar to ESE-1 and Cox-2, GADD45β was expressed at a much lower level in chondrocytes cocultured with muscle cells upon IL-1β or TNFα challenge (Fig.4D). Therefore, we concluded that chondrocytes cocultured with muscle cells elicited an attenuated response to pro-inflammatory cytokines, leading to the reduced expression of cartilage-degrading enzymes, and the enhanced resistance to cytokine-induced reduction of cartilage matrix at both the mRNA and protein level.

4.4 Discussion

We observed a novel mechanism of muscle-mediated cartilage regulation. We found that in an inflammatory environment, coculturing with muscle cells significantly enhanced the expression of collagen II and collagen IX proteins in chondrocytes, while reducing the expression of cartilage-degrading enzymes. We suggest that this effect is achieved by inhibiting the expression of key components of the signaling pathways (such as NFκB) that mediate the activity of pro-inflammatory cytokines in chondrocytes. Since culturing in muscle cell-conditioned medium led to significantly enhanced resistance to pro-inflammatory cytokine-induced collagen destruction in chondrocytes, we suggest that the enhanced resistance to pro-inflammatory cytokine damage is conferred by factors secreted from the muscle cells alone, and is not due to the method of coculturing itself. Therefore, our work

indicates a potential role of muscle in regulating cartilage homeostasis in an inflammatory environment [119].

Several factors have shown antagonistic effects to pro-inflammatory cytokine-induced cartilage degradation. One such factor is TGFβ, which inhibited IL-1β-induced collagen II reduction and MMP13 induction, but did not inhibit the expression of aggrecanase ADAMTS-4 [229]. In contrast, in this study, the effect of chondrogenic factor BMP2 on IL-1β-induced cartilage destruction was minimal[229]. While IGF-I has been reported to inhibit pro-inflammatory cytokine-induced collagen degradation, often a large amount of IGF-I is required for this inhibitory activity [89-91]. In addition, components of cartilage ECM such as glucosamine and chondroitin sulfate were shown to be anti-inflammatory, as they inhibit NFκB signaling [92, 230]. Thus it will be of interest to examine the effect of muscle cells on the expression of glucosamine and chondroitin sulfate in chondrocytes.

While it is not yet clear which specific factors in muscle cells contribute to the anti-inflammatory effect we observed in our cocultures, proteomic analyses of muscle cells and muscle cell-conditioned medium have uncovered an array of muscle-derived proteins including IGFs and Wnts[113, 211, 231], which may be involved in the anti-inflammatory response. In this investigation, we evaluated our cultures two days after cytokine treatment, a timeline that did not allow us to analyze the expression of immediate early mediators of IL-1β (such as Egr-1 and c-Jun), whose induction occurs quickly (within one hour of IL-1β treatment) but subsequently tapers off over the course of one day[232]. Evaluation of early response genes that are involved in cartilage degradation upon treatment with pro-inflammatory cytokines in

our system will further our understanding on the mechanisms of muscle cell-mediated cartilage regulation[98, 233].

4.5 Chapter 4 figures and legends

Figure 4.1

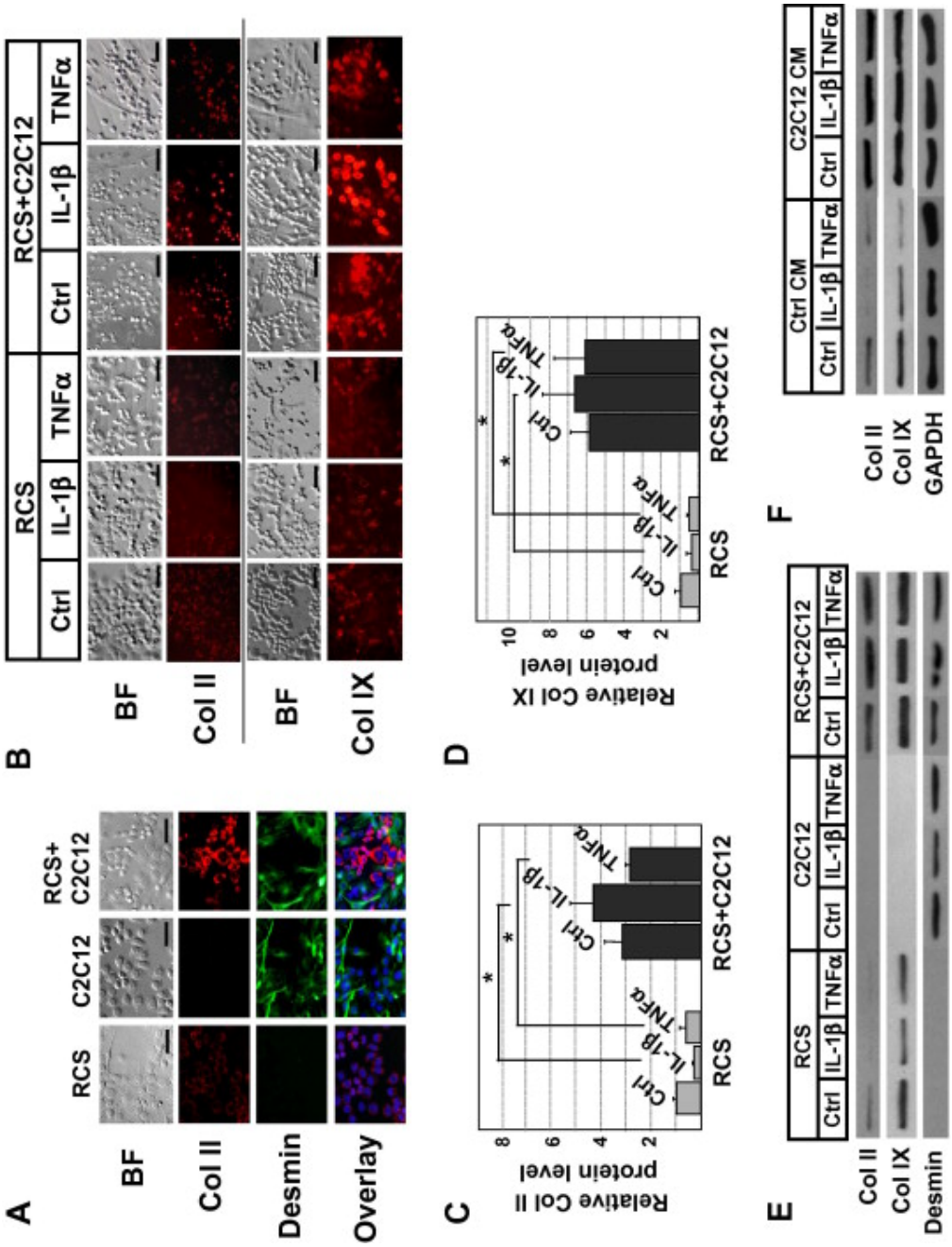

Fig. 1. Cartilage-specific collagen expression in muscle and cartilage cell cocultures upon IL-1β (1 ng/ml) or TNFα (2 ng/ml) treatment. (A) Immunocytochemistry analysis confirming that in cocultures, RCS chondrocytes and C2C12 muscle cells continue to express markers of cartilage (collagen II) and muscle (desmin), respectively. Overlay images of collagen II (red), desmin (green) and dapi (blue) indicate no overlapping expression of collagen II and desmin. (B) Immunocytochemistry analysis of collagen II and collagen IX proteins upon IL-1β or TNFα treatment. BF, bright field. (C and D) Quantification of relative collagen II and collagen IX protein levels based on immunofluorescent intensity (pixels) using the program "Image J". Values were normalized to total chondrocyte number. Gray bars, RCS alone. Black bars, RCS and C2C12 cocultures. (E) Western blot analysis of collagen II and collagen IX expression on rat chondrocyte lysates. As we were unable to obtain a rat-specific internal control for loading, we normalized protein loading to the number of chondrocytes. Desmin, but not collagen II or collagen IX, is only present in lysates of C2C12 cells. (F) Western blot analysis of collagen II and collagen IX expression in RCS chondrocytes cultured in C2C12 muscle cell-conditioned medium (C2C12 CM). Note: scale bars, 40 μm; samples with the same cytokine treatments were compared in statistical analysis, * denotes: $P < 0.05$.

Figure 4.2

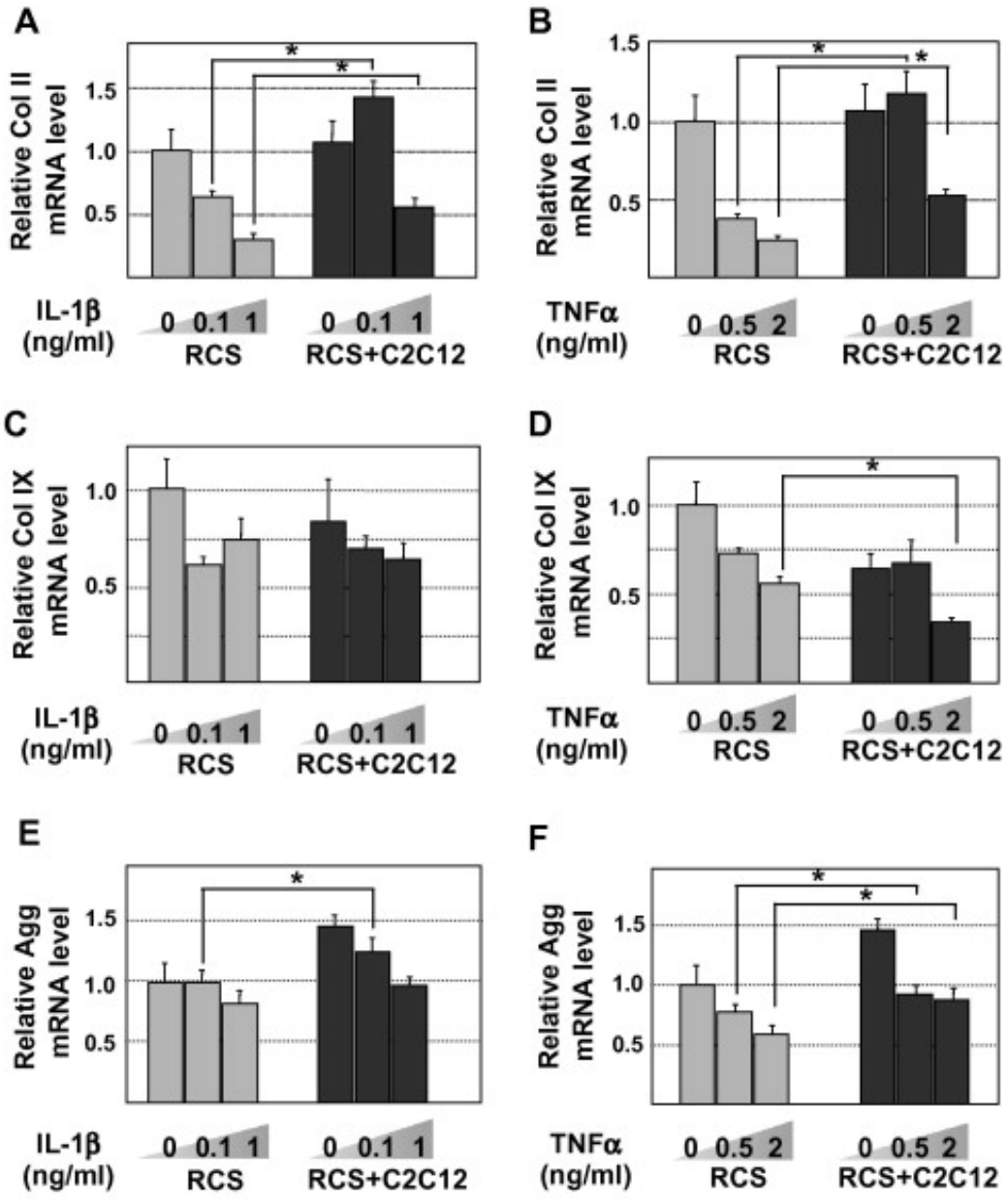
A
Relative Col II mRNA level
1.5
1.0
0.5
*
*
IL-1β (ng/ml)
0 0.1 1
RCS
0 0.1 1
RCS+C2C12
B
Relative Col II mRNA level
1.5
1.0
0.5
*
*
TNFα (ng/ml)
0 0.5 2
RCS
0 0.5 2
RCS+C2C12
C
Relative Col IX mRNA level
1.0
0.5
IL-1β (ng/ml)
0 0.1 1
RCS
0 0.1 1
RCS+C2C12
D
Relative Col IX mRNA level
1.0
0.5
*
TNFα (ng/ml)
0 0.5 2
RCS
0 0.5 2
RCS+C2C12
E
Relative Agg mRNA level
1.5
1.0
0.5
*
IL-1β (ng/ml)
0 0.1 1
RCS
0 0.1 1
RCS+C2C12
F
Relative Agg mRNA level
1.5
1.0
0.5
*
*
TNFα (ng/ml)
0 0.5 2
RCS
0 0.5 2
RCS+C2C12

Fig. 2. Quantitative RT-PCR analysis of Col II, Col IX and Aggrecan mRNA levels in chondrocytes. RCS and RCS-C2C12 cocultures were subject to two different levels of IL-1β (0.1 and 1 ng/ml) or TNFα (0.5 and 2 ng/ml). GAPDH was used for normalization. Solid bars, RCS culture alone. Gray bars, RCS alone. Black bars, RCS and C2C12 cocultures. Samples with the same cytokine treatments were compared in statistical analysis, * denotes: $P < 0.05$. (A and B) Collagen II levels upon IL-1β and TNFα treatment, respectively. (C and D) Collagen IX levels upon IL-1β and TNFα treatment, respectively. (E and F) Aggrecan levels upon IL-1β and TNFα treatment, respectively.

Figure 4.3

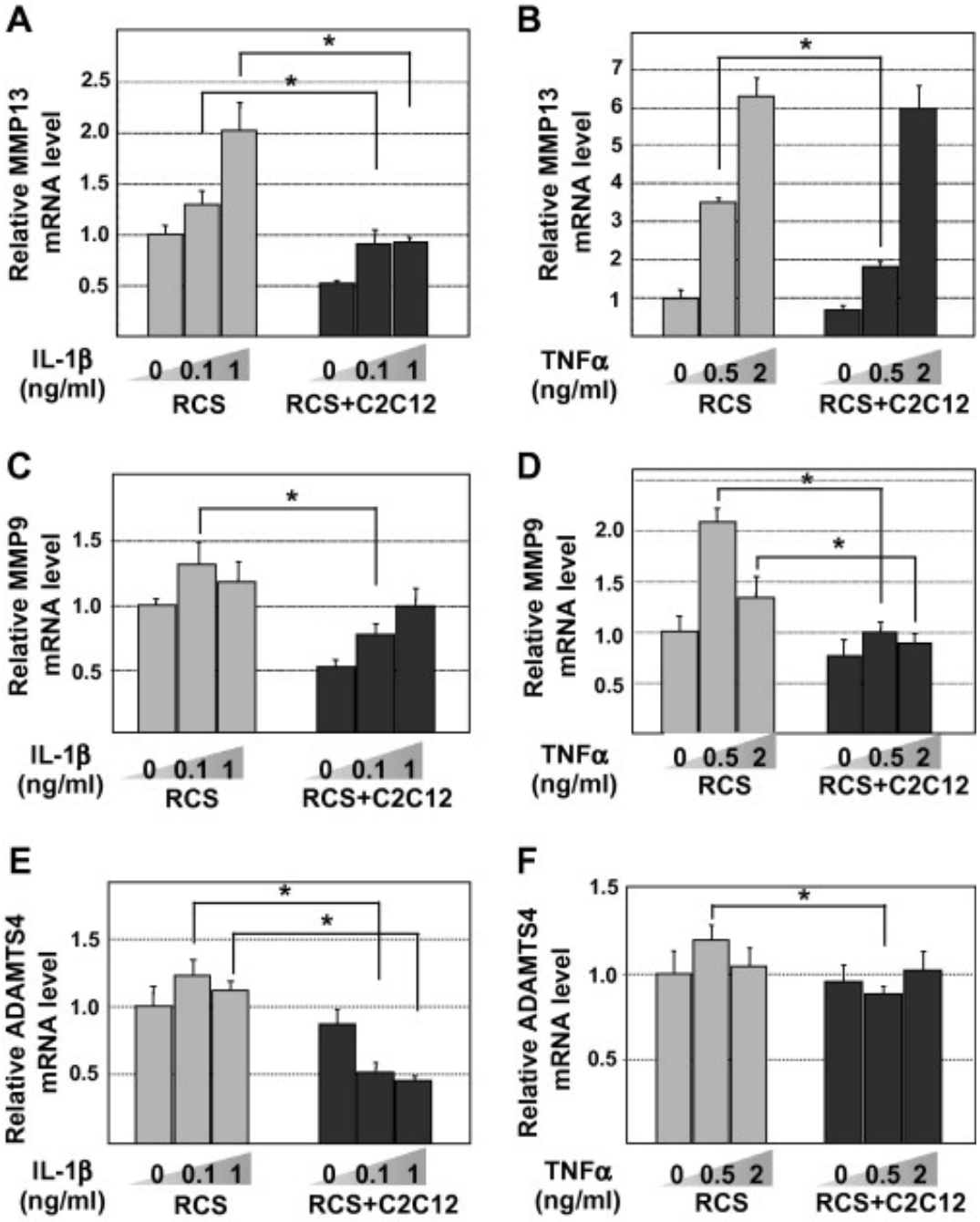
A
Relative MMP13 mRNA level
2.5
2.0
1.5
1.0
0.5
*
*
IL-1β (ng/ml)
0 0.1 1
0 0.1 1
RCS
RCS+C2C12
B
Relative MMP13 mRNA level
7
6
5
4
3
2
1
*
TNFα (ng/ml)
0 0.5 2
0 0.5 2
RCS
RCS+C2C12
C
Relative MMP9 mRNA level
1.5
1.0
0.5
*
IL-1β (ng/ml)
0 0.1 1
0 0.1 1
RCS
RCS+C2C12
D
Relative MMP9 mRNA level
2.0
1.5
1.0
0.5
*
*
TNFα (ng/ml)
0 0.5 2
0 0.5 2
RCS
RCS+C2C12
E
Relative ADAMTS4 mRNA level
1.5
1.0
0.5
*
*
IL-1β (ng/ml)
0 0.1 1
0 0.1 1
RCS
RCS+C2C12
F
Relative ADAMTS4 mRNA level
1.5
1.0
0.5
*
TNFα (ng/ml)
0 0.5 2
0 0.5 2
RCS
RCS+C2C12

Fig. 3. Quantitative RT-PCR analysis of MMP13, MMP9, and ADAMTS4 mRNA levels in chondrocytes. RCS and RCS-C2C12 cocultures were subject to two different levels of IL-1β (0.1 and 1 ng/ml) or TNFα (0.5 and 2 ng/ml). GAPDH was used for normalization. Gray bars, RCS alone. Black bars, RCS and C2C12 cocultures. Samples with the same cytokine treatments were compared in statistical analysis, * denotes: $P < 0.05$. (A and B), MMP13 levels upon IL-1β and TNFα treatment, respectively. (C and D) MMP9 levels upon IL-1β and TNFα treatment, respectively. (E and F) ADAMTS4 levels upon IL-1β and TNFα treatment, respectively.

Figure 4.4

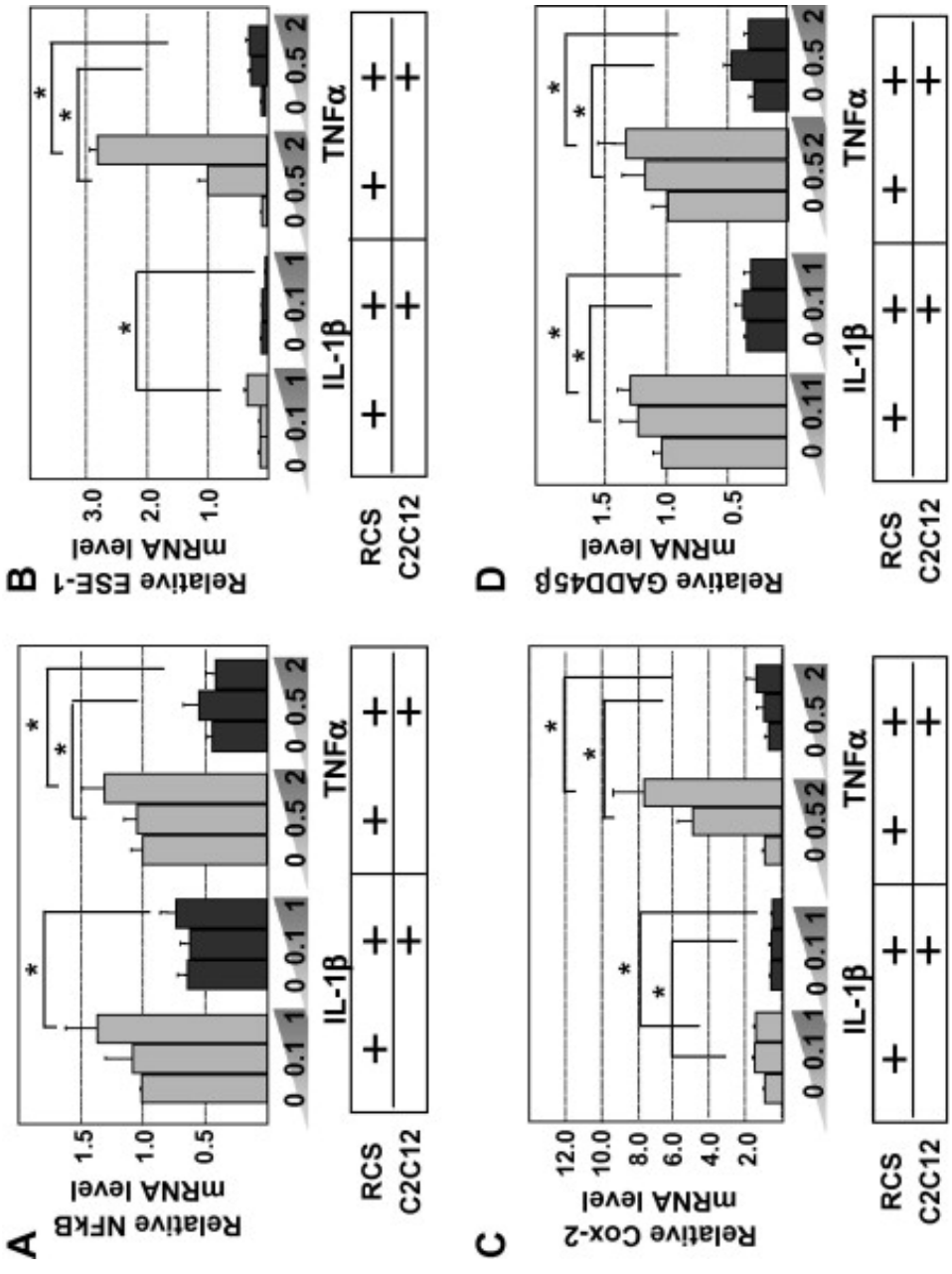
A
Relative NFkB mRNA level
1.5
1.0
0.5
0 0.1 1 0 0.1 1 0 0.5 2 0 0.5 2
IL-1β
TNFα
RCS
C2C12
B
Relative ESE-1 mRNA level
3.0
2.0
1.0
0 0.1 1 0 0.1 1 0 0.5 2 0 0.5 2
IL-1β
TNFα
RCS
C2C12
C
Relative Cox-2 mRNA level
12.0
10.0
8.0
6.0
4.0
2.0
0 0.1 1 0 0.1 1 0 0.5 2 0 0.5 2
IL-1β
TNFα
RCS
C2C12
D
Relative GADD45β mRNA level
1.5
1.0
0.5
0 0.11 0 0.1 1 0 0.52 0 0.5 2
IL-1β
TNFα
RCS
C2C12

Fig. 4. Quantitative RT-PCR analysis of NFκB, ESE-1, Cox-2, and GADD45β mRNA levels in chondrocytes. RCS and RCS-C2C12 cocultures were subject to two different levels of IL-1β (0.1 and 1 ng/ml) or TNFα (0.5 and 2 ng/ml). GAPDH was used for normalization. Gray bars, RCS alone. Black bars, RCS and C2C12 cocultures. Samples with the same cytokine treatments were compared in statistical analysis, * denotes: $P < 0.05$. (A). NFκB levels upon IL-1β and TNFα treatment, respectively. (B) ESE-1 levels upon IL-1β and TNFα treatment, respectively. (C) Cox-2 levels upon IL-1β and TNFα treatment, respectively. (D) GADD45β levels upon IL-1β and TNFα treatment, respectively.

CHAPTER 5: A molecular switch for chondrogenic differentiation of muscle progenitor cells

5.1 Introduction

Satellite cells are the tissue specific stem cells in the adult skeletal muscle. These cells lie beneath the basement membrane of the muscle fiber and are usually mitotically quiescent [31]. Upon injury or when challenged with a variety of mechanical or biochemical stimuli, satellite cells re-enter the cell cycle and give rise to differentiated myocytes, which form new muscle fibers or fuse with existing fibers, and contribute to muscle growth and repair [31]. Satellite cells from the trunk and the limb are derived from an embryonic population of progenitor cells in the somites, transient mesodermal structures that develop on either side of the neural tube [31]. These embryonic progenitor cells are characterized by the expression of transcription factors Pax3 and Pax7, which are important for muscle differentiation and survival [234] and for specifying the muscle satellite cell population responsible for postnatal growth [31, 147]. Upon activation, satellite cells rapidly initiate MyoD expression, which leads to the activation of myogenin, and terminally differentiated structural muscle genes such as myosin heavy chain (MHC) [31, 147]. Interestingly, recent data indicated that although MyoD is not expressed in the quiescent satellite cells in the adult, it is transiently expressed in the satellite cell progenitors in the embryo, suggesting that satellite cells are derived from committed embryonic precursors of myogenic lineage [235, 236].

Initially, satellite cells were considered to be unipotent stem cells with

the ability of generating a unique specialized phenotype, the skeletal muscle cells. However, satellite cells have hence been shown to have the ability to adopt alternative cell fates. One such alternative cell fate is the adipogenic fate, as Pax7(+) satellite cells isolated from single myofibers adopted adipogenic fate, in addition to muscle fate *in vitro* [237, 238]. Another alternative cell fate is the osteogenic fate, as muscle satellite cells have been shown to be induced by BMPs to differentiate into osteoblasts in culture [238-241].

Satellite cells have also been shown to have the capacity to form cartilage. *In vivo*, Pax7(+) satellite cells were found to contribute to cartilage growth in salamanders during limb regeneration after amputation [238, 242]. Furthermore, lineage-labeled satellite cells were found to express cartilage marker collagen II in a mouse model of fracture healing [243] [244]. Such satellite cells accumulate in the callus tissue of the fracture site, exhibit typical morphology of chondrocytes and participate in cartilage formation, which is an essential step in fracture healing [101, 245]. In fact, when a physical barrier (a cell impermeable membrane) was introduced between the muscle and fractured bone, subsequent fracture healing was significantly impaired [246]. In contrast, when isolated muscle was infected with BMP2, it served as a superior bridge for fracture repair [247]. *In vitro*, L6 myoblasts and C2C12 myoblasts were reported to differentiate into chondrocytes when treated with demineralized bone matrix or BMP2 [248-251]. These observations suggest that muscle satellite cells or myoblasts can undergo chondrogenic differentiation, and that this process may play an important role in cartilage and regeneration during fracture healing. However, the molecular

mechanisms by which muscle satellite cells adopt a cartilage fate still remain elusive. While TGFβ/BMP signaling was shown be important in this process, very little is known about how downstream intracellular factors regulate cell fate transition in muscle progenitor cells.

In this work, we have characterized the molecular events that lead to the adoption of cartilage cell fate in muscle satellite cells. We demonstrate that two transcription factors Nkx3.2 and Sox9 act downstream of TGFβ/BMP signaling to regulate the transition from myogenic fate to a chondrogenic fate. Nkx3.2 and Sox9 were both found to promote chondrogenesis in the satellite cells, but Nkx3.2 strongly inhibits the adoption of muscle cell fate while Sox9 only weakly inhibits myogenesis in satellite cells. A reverse function mutant of Nkx3.2 blocks the activity of Sox9, suggesting that Nkx3.2 is required for Sox9 to promote cartilage formation in satellite cells. In addition, we found that the muscle-determining factor Pax3 strongly inhibits chondrogenesis. To explore the *in vivo* significance of these factors, we used a mouse fracture healing model in a genetically modified reporter mouse where muscle progenitors were lineage-traced. We found that in the descendents of muscle progenitors that contributed to cartilage formation, Nkx3.2 and Sox9 were strongly induced, while Pax3 expression was strongly repressed. Together, our data suggest that the balance of Nkx3.2, Sox9 and Pax3 can act as a molecular switch during the chondrogenic differentiation of satellite cells, which may play an important role in the healing process *in vivo*.

5.2 Materials and methods

5.2.1 Satellite cell isolation

Chicken eggs were purchased from Hy-line Inc., Pennsylvania. Satellite cells were isolated from day 17 chicken pre-hatch embryos according to an established protocol [252]. Briefly, pectoral muscles were dissected and placed into sterile PBS, with penicillin/streptomycin, and then minced. Ground muscle was placed in a centrifuge tube and digested with pronase (1 mg/ml in PBS) in a 37 °C water bath for 40 min, with agitation every 10 min. Tubes were centrifuged for 4 min at 3000 rpm. The supernatant was discarded and replaced with PBS then vortexed briefly. Tubes were then centrifuged for 10 min at 1000 rpm for three times, and the supernatants from each cycle were saved and pooled into new sterile 50 ml centrifuge tubes. Supernatants were then passed through a 40 μM cell strainer (BD Biosciences, San Jose, CA), then centrifuged for 6 minutes at 3000 rpm and the resulting supernatants were discarded. The cell pellet was resuspended in medium: DMEM (Invitrogen, CA), 10% FBS (Hyclone, IL) and 1% penicillin/streptomycin (Invitrogen) and then cells were plated on tissue culture plates. Plates were incubated for 24 hours in a humidified 37 °C, 5% CO_2 incubator, and then washed with sterile PBS to remove non-adherent cells. All freshly isolated cells were confirmed to be positive for satellite cell specific markers, Pax3 and Pax7, before subsequent experiments were conducted.

5.2.2 Cell culture

Satellite cells were cultured in DMEM with 10% FBS (Hyclone, Logan, UT) and 1% antibiotic/mycotic (Invitrogen, CA). For chondrogenic induction, satellite cells were plated as high density micromass cultures in the presence of chondrogenic media: DMEM (Invitrogen), supplemented with ITS (insulin,

transferrin, selenate, Sigma Cat#12521), 0.1mM ascorbic acid (Sigma, MO), human serum albumin (HSA, Sigma), 10^{-7}M dexamethasone, 10 ng/ml TGFβ3 (R&D, MN) or BMP2 (R&D)) [253] [254]. Briefly, cells were split and resuspended as 10^5 cells/10 µl droplet. They were pipetted onto a plate and allowed to adhere in a 37 °C, 5% CO_2 incubator for approximately one hour before the addition of chondrogenic media. All cells were grown for 5 days before they were subject to histological and qRT-PCR analysis.

5.2.3 Virus production and infection of satellite cells

Avian-specific retroviruses (RCAS) were generated by first transfecting chick embryonic fibroblasts (CEF) with retroviral constructs encoding for the following genes: GFP, Nkx3.2HA, Sox9V5, Alkaline phosphatase (AP), Pax3HA, Nkx3.2ΔC-HA (deletion of C-terminus from aa219-278), or Nkx3.2ΔC-VP16 [214, 255, 256]. The viral supernatant was concentrated by ultracentrifugation (21,000rpm, 2 hrs), and titered by directly visualizing GFP expression (in the case of RCAS-GFP) or indirect immunocytochemistry using anti-GAG antibody (which recognizes the viral coat protein GAG). Viruses with titers of at least 10^8 particles/ml were used in all satellite cell cultures. For co-infection experiments, viruses of different coat proteins A- or B- were used. Retroviral infection of satellite cells was carried out by directly adding concentrated virus into growing cell cultures. High levels of expression were detectable 48 hrs post-infection, at which time the cells were split and used in subsequent experiments.

5.2.4 Luciferase assays

For the construction of the luciferase construct, a published murine Pax3 promoter sequence (1.5 kb) [257] was cloned into SmaI and NheI sites of the pGL3 luciferase vector (Promega, Wisconsin). Satellite cells were transfected with pGL3-Pax3 promoter construct or pGL3 control using Fugene6 according to the manufacturer's protocol. After 48 hrs, cells were processed using the Luciferase Assay System (Promega, Wisconsin). Briefly, cells were thoroughly lysed with lysis buffer using a freeze-thaw cycle. Supernatants were added to the luciferase assay reagent in a 96 well plate, then immediately read on a 1450 Microbeta Wallac Trilux plate reading luminescence counter (Perkin Elmer, MA).

5.2.5 Histological analyses

All samples were fixed with 4% paraformaldehyde (Sigma). For alcian blue staining, cryosections of satellite cell micromass cultures were pre-washed with 0.1N HCl then incubated with 1% (w/v) alcian blue (Sigma) overnight, followed by repeated washes with 0.1N HCl. Hematoxylin and eosin (H&E) (Sigma) staining was carried out according to standard protocol on cryosectioned mouse tissues. Staining for heat-inactivated alkaline phosphatase (HI-AP) on serial cryosectioned mouse tissue was achieved by incubating the slides at 75^0C for 50min, to eliminate endogenous AP activity, followed by application of NBT (100mg/ml in 70% dimethyl formamide) and BCIP (50mg/ml dimethyl formamide) (Invitrogen, CA).

For immunocytochemistry, the following primary antibodies were used: mouse anti-Collagen II (generous gift from Dr. Tom Linsenmayer, Tufts University), Rabbit anti-Col2 (Abcam, MA), Rabbit anti-Sox9 (Chemicon, MA),

Pax3 (Developmental Studies Hybridoma Bank (DSHB), IA), Pax7 (DSHB), Myosin Heavy Chain (MHC) (MF20 from DSHB), mouse anti-GAG (DSHB), Rabbit anti-HA (Sigma), Rabbit anti-V5 (Sigma), Rabbit anti-VP16 (Abcam). For immunohistochemistry of mouse tissues, cryosections were first subject to antigen retrieval by treating slides with 1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) for 5 min at room temperature prior to subsequent staining steps. For all other immunocytochemistry of cell culture, no antigen retrieval was used. All samples were first blocked with PBS with 0.1% Triton-X (Sigma) and 6% goat serum (Sigma), then incubated with primary antibodies overnight. After repeated washes with PBS with 0.1% Tween (PBST), cultures were incubated with secondary antibodies. For immunofluorescent staining, all secondary antibodies used were conjugated with Alexa 488 (green) or 594 (red) (Invitrogen, CA), and all cultures were counterstained with DAPI (Invitrogen, CA). For colorimetric immunostaining, secondary antibody was conjugated with biotin, and the signal was amplified using the Vectastain Elite ABC kit (Vector Laboratories, CA) and developed using DAB-peroxidase (Sigma).

5.2.6 RT-PCR analysis

RNA was isolated from all cell cultures using the RNeasy mini-kit from Qiagen (Chatsworth, CA), For RNA samples isolated from mouse tissue cryosections using laser capture microscopy (LCM), Qiagen MicroKit was used (Chatsworth, CA). cDNA was generated using MLV-reverse transcriptase (Invitrogen, CA) according to standard protocol. All quantitative PCRs were performed on the iQ5 Real-Time PCR Detection System (BioRad, Hercules, CA). All PCR analyses of *in vitro* experiments were normalized to

GAPDH. All PCR analyses from *in vivo* mouse LCM samples were normalized to the 18S RNA. Sequences for all primers are listed in Table 1.

5.2.7 Microscopy

Bright-field and fluorescent images from histological and immunocytochemistry analyses were taken under the Olympus IX71 inverted microscope using Olympus DP70 digital camera and associated software (Olympus, PA). Laser capture microscopy (LCM) was performed using the Arcturus PixCell IIe system (Tufts Imaging Facility, Center for Neuroscience Research) using the established protocol [258, 259]. Briefly, cryosectioned tissues were dehydrated and were overlaid with a thermoplastic membrane, which was mounted on an optically transparent cap (Arcturus Macro LCM caps, Applied Biosystem, CA). Target tissues were identified by comparisons with serial sections that were stained with heat-inactivated alkaline phosphatase (HI-AP). Upon laser activation, target cells were captured by focal melting of the membrane, then the captured tissue was immersed in a denaturation solution and was subsequently subject to RNA isolation.

5.2.8 Fracture creation in MyoD-cre Z/AP labeled mice

MyoD-cre Z/AP reporter mice were bred by the crossing of the MyoD-cre [235] and Z/AP [260] lines. The *MyoD*-Cre mouse line was a gift from Dr. David Goldhamer (University of Connecticut, Storrs, USA). The Z/AP line was supplied by Prof. Patrick Tam (Children's Medical Research Institute, Westmead, NSW, Australia) with permission from Prof. Andras Nagy (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada). The cross strain labels all MyoD(+) lineage cells to permanently express the heat-resistant

human placental alkaline phosphatase (hPLAP). Midshaft tibial fractures were generated in anaesthetized MyoD-cre Z/AP mice and littermate controls by manual three point using a previously published model [261]. Tissue specimens were harvested from mice at 1 week endpoint were used for enzymatic and immunohistochemical staining. Animal experimentation was approved by two committees: the Children's Medical Research Institute Animal Ethics Committee at the Children's Hospital at Westmead (protocol no. K248) and the South West Area Health Service Animal Ethics Committee (protocol no. 4102), New South Wales, Australia.

5.2.9 Statistical Analysis

For statistical analysis, the mean and standard deviation were calculated. Statistically significant differences (i.e., $p<0.05$) were determined by one-factor analysis of variance (ANOVA) with post hoc Tukey test using the statistics software SYSTAT12 (Systat, Chicago, IL, USA).

5.3 Results

5.3.1 Isolated muscle satellite cells can be differentiated into chondrocytes at the expense of normal muscle cell differentiation

To study the induced chondrogenic differentiation of myogenic cells, muscle satellite cells were grown as micromass cultures in chondrogenic medium. Satellite cells were isolated from the pectoralis muscles of late stage chicken embryos, which give rise to muscle cells that are phenotypically similar to those from adult muscle [262]. The identity of muscle satellite cells was confirmed by immunocytochemistry analysis, which indicated that our cells were ≥95% positive for satellite cells markers Pax3 and Pax7 at day 0

(D0) (Fig. 1A). This result was further confirmed by qRT-PCR analysis for Pax3 and Pax7 (Fig.1B). We then cultured the satellite cells in three-dimensional (3D) micromass cultures in the presence of the standard chondrogenic media containing TGFβ3 [253, 254]. This culture system has been well established to differentiate embryonic progenitor cells or bone marrow-derived mesenchymal stem cells into cartilage [253, 254, 263]. Our immunocytochemistry and RT-PCR analyses indicated that upon treatment with chondrogenic medium, there was a dramatic reduction of Pax3 and Pax7, as well as committed myoblast marker MyoD, and differentiated myocyte marker myosin heavy chain (MHC) (Fig. 1C and 1D). Concurrently, treatment with chondrogenic medium resulted in the induction of cartilage-specific protein collagen II, as shown by immunocytochemistry (Fig. 1E), as well as glycosaminoglycans as shown by alcian blue staining (Fig. 1E). This was further confirmed by qRT-PCR analysis, which showed that chondrogenic medium treatment led to the induction of transcription factors Nkx3.2 and Sox9, as well as cartilage matrix markers collagen II and aggrecan (Fig. 1F). Replacing TGFβ with BMP2 in the chondrogenic medium led to similar results (data not shown). Taken together, these data clearly demonstrate that muscle satellite cells can be driven to adopt a cartilage phenotype *in vitro* at the expense of the default muscle cell fate.

5.3.2 Pax3 inhibits the adoption of cartilage cell fate by muscle satellite cells

We next investigated the roles of intracellular factors on the chondrogenic differentiation of satellite cells. Upon chondrogenic differentiation of muscle satellite cells, muscle fate determining factor Pax3 expression was strongly downregulated (Fig.1C and 1D). Therefore, we

hypothesized that Pax3 would negatively regulate the differentiation of satellite cells to chondrocytes. To test this hypothesis, we infected muscle satellite cells with a Pax3-expressing retrovirus, and cultured these cells in 3D micromass in chondrogenic medium. Indeed, forced expression of Pax3 in chondrogenic medium caused a significant decrease in expression of cartilage markers collagen II and aggrecan as compared to controls (Fig. 2A,B), while leading to an increase in the expression of muscle markers MyoD, myogenin, and MHC (Fig. 2C). Therefore, our results indicate that Pax3 inhibits chondrogenesis in muscle satellite cells, and suggest that for muscle satellite cells to differentiate into chondrocytes, Pax3 expression must first be inhibited.

5.3.3 Nkx3.2 and Sox9 inhibit muscle gene expression in muscle satellite cells

Next we assessed whether factors induced by chondrogenic media were capable of inhibiting the default muscle fate of satellite cells. Sox9 and Nkx3.2 are two factors induced by TGFβ-containing chondrogenic medium (Fig.1F), and have been shown to promote cartilage formation during embryogenesis [50, 59, 264-267]. However, it has not been determined whether these factors might influence chondrogenic differentiation of muscle satellite cells. We found that forced expression of Nkx3.2 encoding retrovirus in muscle satellite cells strongly inhibited Pax3 expression, as Nkx3.2-expressing cells express less Pax3 as confirmed by immunostaining (Fig.3A) and qRT-PCR analysis (Fig.3B). Sox9, on the other hand, only weakly inhibited Pax3 expression, suggesting that Nkx3.2 is a more potent inhibitor of muscle cell fate in satellite cells (Fig. 3A and 3B). Similarly, Nkx3.2

inhibited the expression of satellite cell marker Pax7 as well as MHC, a marker for differentiated myocytes (Fig .3C-3F). As these satellite cells were already expressing a high level of Pax3 and Pax7 upon isolation, the inhibition of Pax3 and Pax7 at the protein level was not as dramatic as that of MHC (Fig.3). While the effect of Sox9 on muscle gene expression was not clearly visible in the less quantitative analysis of immunocytochemistry (Fig. 3A, 3C and 3E), RT-PCR analysis clearly indicates that Sox9 consistently exhibited a significant though weak activity in inhibiting Pax3, Pax7 and MHC expression in muscle satellite cells (Fig.3B, 3D and 3F). Combinatorial treatment of Nkx3.2 and Sox9 led to inhibition of muscle cell fate similar to that of Nkx3.2 alone, suggesting that there is no synergy between these two factors and that they may not act independently in this process (Fig. 3). Together, these data indicate that Nkx3.2, and to a much lesser extent, Sox9, are cartilage-associated transcription factors that can prevent satellite cells from adopting their default muscle fate.

5.3.4 The C-terminus of Nkx3.2 is required for the inhibition of muscle cell fate in muscle satellite cells

Given that Nkx3.2 strongly inhibited the muscle fate in satellite cells, we next determined if this effect was specific by using two Nkx3.2 mutants. In the Nkx3.2-ΔC mutant, 58 amino acids from the C-terminus were deleted. During somite differentiation in the embryo, Nkx3.2 was found to behave as a transcriptional repressor and the C-terminus was determined to be transcriptional repression domain for promoting cartilage formation [267] [255, 268]. However, it is not clear whether this domain is also required for inhibiting muscle cell fate in satellite cells. The other mutant we used was a

reverse function mutant of Nkx3.2 whose C-terminus domain had been replaced by a VP16 constitutive activation domain (Nkx3.2ΔC-VP16). We found that while wild type Nkx3.2 infection led to a significantly reduced Pax3, Pax7 and MHC expression in satellite cells, Nkx3.2-C-terminus deletion mutant (Nkx3.2-ΔC-HA) did not inhibit the expression of Pax3 at the protein or mRNA level (Fig. 4A and 4B). Significantly, Nkx3.2ΔC-VP16 induced the expression of Pax3 (Fig.4B). Nkx3.2 fusion with the VP16 activation domain led to the opposite phenotype as wild type Nkx3.2, suggesting that Nkx3.2 acts as a transcriptional repressor in inhibiting Pax3 expression. Similar to the analysis on Pax3 expression, Nkx3.2-ΔC mutant did not inhibit Pax7 expression (Fig 4C and 4D), suggesting that the C-terminus of Nkx3.2 is required for Pax7 repression as well. Interestingly, deletion of the C-terminus of Nkx3.2 alleviated, but did not completely abolish Nkx3.2-mediated MHC repression (Fig. 4E and 4F), suggesting Nkx3.2 may inhibit MHC expression through additional domains other than the C-terminus. While replacing the C-terminus with a VP16 activation domain led to significantly enhanced expression of Pax3 and MHC, it did not lead to increased Pax7 expression in the satellite cells (Fig. 4B, 4D and 4F). The effect of Nkx3.2ΔC-VP16 on Pax7 expression could be due to the intricate interaction of Pax7 with other myogenic factors, such as Pax3 and Myogenin, which were both shown to inhibit Pax7 expression [161, 269].

5.3.5 Nkx3.2 inhibits Pax3 promoter activity

Since Nkx3.2 acts as a repressor to strongly inhibit Pax3 expression, we hypothesized that Nkx3.2 may inhibit Pax3 at the promoter level. A mouse

Pax3 promoter sequence was previously identified from LacZ reporter analysis in transgenic mice which indicated that this promoter recapitulated endogenous Pax3 expression in the trunk [257]. Thus, we generated a luciferase reporter that contains this murine Pax3 promoter sequence to investigate whether Nkx3.2 acts directly on the Pax3 promoter to inhibit its expression (Fig. 5A). For comparison, we also evaluated the effect of GFP and Sox9 on the Pax3 promoter. We first infected satellite cells with retroviruses that express GFP, Sox9V5, Nkx3.2-HA, Nkx3.2ΔC-HA, or Nkx3.2ΔC-VP16, and confirmed the efficiency of viral infection by performing immunocytochemistry (Fig. 5B). We then transfected the Pax3 promoter luciferase construct into these satellite cells. We found that while Sox9 showed a moderate but significant reduction in Pax3 promoter activity, Nkx3.2 dramatically inhibited Pax3 promoter activity (Fig.5C). C-terminal deletion mutant of Nkx3.2 (Nkx3.2-ΔC) showed essentially no effect on the Pax3 promoter as compared to control GFP-infected cells (Fig. 5B). Intriguingly, the Nkx3.2 reverse function mutant (Nkx3.2-ΔC-VP16) activated the Pax3 promoter by two folds (Fig.5C). Therefore, our promoter study results are consistent with our analysis of endogenous Pax3 mRNA and protein expression in satellite cells, and led us to conclude that Nkx3.2 and Sox9 may inhibit muscle gene expression by inhibiting the Pax3 promoter.

5.3.6 Treatment with either Nkx3.2 or Sox9 can induce cartilage gene expression in muscle satellite cells

As the differentiation of muscle satellite cells into chondrocytes involves not only the repression of muscle cell fate but also the initiation of chondrogenesis, we next examined the roles of Nkx3.2 and Sox9 in the

induction of cartilage genes in muscle satellite cells. We tested if either of these factors could induce chondrogenesis by infecting the satellite cells with viruses expressing Sox9 and/or Nkx3.2. We found that either Nkx3.2 and Sox9 infection alone could induce cartilage marker Collagen II expression; and that the combination of these two factors had an additive effect, showing a more intense Collagen II staining upon Nkx3.2 and Sox9 co-infection (Fig.6A). This was further confirmed by qRT-PCR analysis (Fig. 6B). Interestingly, while Sox9 alone could induce Aggrecan expression, Nkx3.2 alone could not (Fig. 6C). However, when combined with Sox9, Nkx3.2 led to a synergistic induction of Aggrecan expression (Fig. 6C). Thus it is possible that Nkx3.2 and Sox9 regulate the expression of these two cartilage matrix components differently. Together, these data suggest that Nkx3.2 and Sox9 promote chondrogenic differentiation of muscle satellite cells.

5.3.7 A reverse function mutant of Nkx3.2 blocks the ability of Sox9 to induce chondrogenesis and inhibit myogenesis in muscle satellite cells

Since both Nkx3.2 and Sox9 can promote the chondrogenic differentiation of satellite cells, we investigated the relationship between these factors. We found that indeed, Nkx3.2 and Sox9 induced each other's expression in satellite cells (Fig. 7A and 7B). Sox9 is known to strongly induce chondrogenesis by directly activating the promoters of cartilage matrix genes [52, 265, 270]. Our data support this notion and further demonstrate that Sox9 has a weak activity in inhibiting myogenesis. Because Nkx3.2 exerts a stronger inhibitory activity on Pax3, the myogenic factor that can inhibit chondrogenesis, we asked whether the activity of Sox9 on chondrogenesis and myogenesis in satellite cells may be attributed to the

induction of Nkx3.2. Therefore, we made use of the reverse function mutant of Nkx3.2 (Nkx3.2-ΔC-VP16), to evaluate whether this mutant, acting in a dominant-negative manner, would compromise the ability of Sox9.

Strikingly, we found that while Sox9-treated satellite cells dramatically upregulated collagen II expression, Nkx3.2ΔC-VP16 co-infection with Sox9 led to a dramatic reduction in this cartilage matrix protein (Fig.7C). This is further confirmed by our RT-PCR analysis that showed diminished expression of collagen II mRNA as well as aggrecan mRNA upon Nkx3.2ΔC-VP16 and Sox9 co-infection (Fig.7D and 7E). We next asked whether Nkx3.2 is required for the weak inhibitory activity of Sox9 on muscle gene expression. Indeed, we found that co-infection of Nkx3.2ΔC-VP16 with Sox9 completely abolished the ability of Sox9 to inhibit Pax3 and MHC expression (Fig. 7F and 7G). This suggests that Sox9 is unable to induce chondrogenesis in the absence of the activity of Nkx3.2, and that Sox9 may inhibit the myogenic program in the satellite cells through the induction of Nkx3.2.

5.3.8 Nkx3.2 and Sox9 are induced in the muscle progenitor cells that contribute to cartilage formation and fracture repair in an in vivo mouse model of bone fracture healing

To establish the *in vivo* significance of Nkx3.2 and Sox9 in the chondrogenic differentiation of muscle satellite cells, we evaluated the expression of these factors in the myogenic progenitor cells that give rise to chondrocytes during the process of bone fracture healing. The MyoD-cre:Z/AP mouse was generated by crossing two transgenic lines as described in Materials and Methods (Fig. 8A). The MyoD-driven Cre mouse line allows Cre to be expressed in all muscle progenitor cells, including satellite cells

[235]. Upon Cre-mediated recombination, the Z/AP line permanently expresses the human placental alkaline phosphatase (hPLAP) reporter gene in affected cells (Fig. 8A). The heat-stable property of hPLAP enabled us to distinguish the expression of this reporter from that of endogenous alkaline phosphatase, which is abundantly expressed in bone cells [240]. Therefore, in this MyoD-Cre+:Z/AP+ mouse, all satellite cells and their progenitors express heat-stable alkaline phosphatase, which is marked by purple stainings from enzymatic reactions (Fig. 8A). When the MyoD-Cre+:Z/AP+ mouse was subject to open tibial midshaft fractures, muscle progenitor cells or their descendants were abundantly seen in the fracture callus region (Fig. 8B). This callus region is known to undergo cartilage and bone differentiation during the process of fracture healing [104, 271]. In addition to contributing to the fracture callus, it is evident that hPLAP-(+) cells also marked the muscle next to the bone, and did not mark any of the endogenous osteocytes within the bone (Fig. 8B), confirming the validity of this method.

To evaluate whether Sox9 and Nkx3.2 were induced in the muscle progenitor cells, we performed immunohistochemistry (IHC) on sections of the fracture region (Fig. 8C). We found that Sox9 was strongly expressed in the cells in the fracture callus, which correlated with the induction of cartilage marker collagen II expression (Fig. 8C). Due to the lack of a suitable antibody against Nkx3.2, we were unable to perform IHC to evaluate Nkx3.2 protein expression. Thus, to evaluate Nkx3.2 expression and to confirm that Nkx3.2 and Sox9 are indeed induced in the muscle progenitor cells that give rise to chondrocytes in the fracture healing process, we performed laser capture microscopy (LCM). We isolated muscle progenitor cells by LCM with reference to alkaline phosphatase (HI-AP) staining, and compared the

expression of muscle and cartilage from the these cells with those of neighboring muscle cells (Fig. 8D). We found that muscle progenitor cells in the fracture callus strongly expressed Nkx3.2 and Sox9, as well as Collagen II, as compared with cells in the muscle (Fig. 8D). In the meantime, these cells downregulated expression of muscle markers Pax3, Pax7 and MHC (Fig. 8D). These data thus suggest that Nkx3.2 and Sox9 are indeed expressed in the muscle progenitor cells that contribute to cartilage formation during fracture healing, which is consistent with the notion that they promote chondrogenic differentiation of satellite cells.

5.4 Discussion

In this work we show that the muscle satellite cells that normally undergo myogenesis can be converted to express cartilage matrix proteins *in vitro* upon treatment with chondrogenic medium containing TGFβ or BMP2. In the meantime, the myogenic program is repressed, suggesting that muscle satellite cells have undergone chondrogenic differentiation. Furthermore, we demonstrate that muscle-determining factor Pax3 strongly inhibits chondrogenesis in these cells, while Nkx3.2 and Sox9 act downstream of TGFβ or BMP to promote this cell fate transition. Importantly, our data suggest that Nkx3.2 is required for Sox9 to inhibit myogenesis and induce chondrogenesis. Finally, in the muscle progenitor cells that give rise to chondrocytes in an *in vivo* model of fracture healing, Nkx3.2 and Sox9 are significantly upregulated while Pax3 is significantly downregulated. These events correlated with the induction of cartilage matrix protein collagen II in lineage-traced muscle progenitor cells, suggesting that the balance of Pax3,

Nkx3.2 and Sox9 may play an important role in the cell fate switch from muscle to cartilage, which may be important for fracture healing.

Multiple other progenitor cell populations are present in the muscle that can be instructed to adopt alternative cell fates. While muscle satellite cells reside underneath the basal lamina of the myocytes [147], a fibrocyte or adipocyte population (FAP) has been identified in the interstitial spaces of the muscle fibers [272, 273]. These cells do not express muscle satellite cell marker Pax7 or SM/C-2.6, but are positive for Sca1, Tie-2 and PDGFR-1a [272, 273]. While this FAP population can give rise to adipocytes, it cannot be induced to become myogenic or chondrogenic. On the other hand, a Sca-1-negative (also lin-negative) population (i.e. the double-negative (DN) population) in the muscle was found to be capable of differentiating into cartilage and bone, but incapable of differentiating into myocytes [272]. Another progenitor population is the muscle-derived stem cell population (MDSC) [274, 275]. Unlike muscle satellite cells or the FAP population, these MDSCs reside within the basal lamina [275]. MDSCs are also positive for Sca-1 and negative for Pax7, and have the ability to give rise to muscle, cartilage or bone cells [275]. As the FAP and MDSC population do not express Pax3 and Pax7, we believe that the cells used in our experiments do not belong to the above populations. However, it is plausible that similar molecular mechanisms are involved in the chondrogenic differentiation of FAP or MDSC cells.

It has been established by the Goldhamer laboratory that the MyoD(+) progenitors permanently labels muscle satellite cells as well as their

derivatives in the mature muscle fibers [235]. While Goldhamer and colleagues showed that these muscle progenitor cells did not give rise to non-myogenic adipocytes [276], it was not clear whether they have the capacity to adopt a chondrogenic or osteogenic fate. In our study, we evaluated the expression of Nkx3.2, Sox9 and Pax3 in the muscle progenitors that contribute to cartilage formation during bone healing during fracture repair. We demonstrate that muscle progenitor cells adopt a cartilage cell fate upon chondrogenic stimulation *in vitro*, as well as during open fracture healing *in vivo*. However, we did not did not distinguish which specific subpopulations of satellite cells are more likely to undergo chondrogenesis [234]. It is also not clear whether these muscle progenitor cells have undergone de-differentiation/re-differentiation or *bone fide* transdifferentiation in our *in vitro* cell culture or *in vivo* fracture healing models. While there was a significant amount of Pax3 and Pax7 protein expression at the beginning of culturing, Pax3 and Pax7 became gradually diminished upon Nkx3.2 and Sox9 viral infection, concurrently with the induction of cartilage genes, which should be consistent with a transdifferentiation process. Msx1 is correlated with muscle cell dedifferentiation [277, 278]. However, msx1 is also highly expressed in chondrocytes and is induced by BMP/TGFβ signaling. Thus, although we observed a significant induction of msx1 expression upon chondrogenic differentiation in the satellite cells (data not shown), it does not indicate whether the satellite cells have undergone dedifferentiation. Regardless, our data support that muscle progenitor cells that normally would undergo myogenesis, can be redirected to adopt a cartilage cell fate *in vitro* and *in vivo*.

In this study, we have evaluated cartilage gene expression in the muscle progenitor cells that contribute to fracture healing [279]. However, other cell types located in the vicinity of bone may also participate in cartilage and bone formation. Elegant grafting experiments using LacZ-positive donor mice and Lac-Z-negative recipients revealed that cells from the perichondrium, the fibrous covering of the bone, differentiate into chondrocytes and osteocytes during fracture repair [280]. Cells associated with blood vessels, such as pericytes, have also been shown to have the ability to differentiate into chondrocytes [281]. Cells that are positive for Tie-2, an endothelial cell marker, while not yet shown to be recruited to the fracture callus, are known to contribute to cartilage and bone formation during heterotopic ossification [282, 283]. Because of the diverse cell types that participate in cartilage formation during fracture healing, it is likely that these different types of cells use different signaling mechanisms when undergoing chondrogenic differentiation. It is known that TGFβ, BMP, PTH, as well as Wnt signaling are all activated during fracture healing, and downstream molecules such as Smad, prostaglandin, Cox-2 and β-catenin regulate this process [104, 271, 284-293]. Our work demonstrates that transcription factors Pax3, Nkx3.2 and Sox9 regulate chondrogenic differentiation of muscle progenitor cells. However, it is unclear whether Nkx3.2 and Sox9 also participate in the chondrogenic differentiation of other cell types, such as perichondrial or endothelial cells, and how these different cell types coordinate their signaling events during fracture healing. The understanding of such signaling processes in different cell types may help to accelerate fracture healing.

Pax3, Nkx3.2 and Sox9 are all known to play important roles during development. In embryogenesis, Pax3 is expressed in the dermomyotome of the somite, which gives rise to muscle cell precursors [32]. Pax3 mutant mice exhibit somite truncations with loss of hypaxial dermomyotome, and absence of limb muscle [147]. Our data support the role of Pax3 in promoting myogenesis in muscle satellite cells [129]. Furthermore, our data shows that Pax3 has an additional function of inhibiting chondrogenic differentiation of muscle satellite cells. In the double knockout of Pax3 and its paralogue Pax7, significant cell death takes place, leading to the loss of most muscle fibers [147]. In addition, Pax3 and Pax7 double mutant cells were found in the forming rib [147], suggesting that they may have adopted a cartilage fate, a result consistent with our findings [30, 214]. While Pax3 acts as a transcriptional activator to promote myogenesis [146], it also has a transcriptional repressor domain that is important for the development of melanocytes [294-298]. It will be of interest to determine whether Pax3 inhibits chondrogenesis by acting as a transcriptional repressor or activator in the satellite cells. It will be also of interest to investigate whether other myogenic factors play inhibitory roles in chondrogenic differentiation.

We also discovered a novel function for Sox9 in this study. Sox9 is the master regulator of chondrogenesis, as no cartilage formation takes place in the absence of Sox9 [50]. Sox9 acts as a transcriptional activator in chondrogenic precursor cells by binding to the promoters of cartilage-specific matrix genes collagen II and aggrecan [52, 265, 270]. We found that Sox9 strongly induced collagen II and aggrecan expression in the muscle satellite

cells, which normally are non-chondrogenic precursors, consistent with its activity in the somite [214]. In the meantime, Sox9 also significantly, although weakly, inhibited the expression of early muscle lineage marker Pax3 and Pax7, as well as myosin heavy chain (MHC). It has been reported that Sox9 is expressed in the satellite cells, and has the ability to inhibit α-sarcoglycan expression in the C2C12 myoblast cell line [299] and the myogenin promoter in 10T1/2 cells [300]. Our data are consistent with these reports. While Sox9 may be expressed in satellite cells, it is apparent from our work and others that Sox9 is much more strongly expressed in chondrocytes, and that ectopic expression of Sox9 leads to chondrogenic differentiation and maintenance of the chondrocyte phenotype [301, 302].

Our data suggest that Nkx3.2 plays a central role in the chondrogenic differentiation of satellite cells, and that its activity is required for Sox9 to promote chondrogenesis and inhibit myogenesis. Like Sox9, Nkx3.2 is expressed in the cartilage precursors in the embryo, and promotes cartilage cell fate in the somites [214, 255]. Nkx3.2 null mice exhibit reduced cartilage formation including a downregulation of Sox9 expression [59, 303, 304]. Inactivating mutations of Nkx3.2 in human lead to spondylo-megaepiphyseal-metaphyseal dysplasia (SMMD), a disease that causes abnormalities of the vertebral bodies, limbs and joints [305]. Here we show that Nkx3.2 is activated in the muscle satellite cells during chondrogenic differentiation *in vitro* as well as in the adult fracture healing process *in vivo*, suggesting that Nkx3.2 may also be involved in a cell fate determination process at a stage later than early embryogenesis. Furthermore, we show that Nkx3.2 acts as a transcriptional repressor to inhibit Pax3 promoter activity. While there are

consensus Nkx3.2 binding sites on the Pax3 promoter, we have not determined whether Nkx3.2 binds to the Pax3 promoter [257]. Interestingly, Nkx3.2 has also been shown to act as a repressor to inhibit osteogenic determining factor Runx2, suggesting Nkx3.2 may be used to inhibit other non-cartilage cell fates [168]. We have also uncovered a pivotal role for Nkx3.2 in the induction of chondrogenic genes. We found that without the repressing activity of Nkx3.2, Sox9, despite its ability to bind to collagen II and aggrecan promoters, was unable to activate those genes or inhibit myogenesis. Additionally, Nkx3.2 potentiates the ability of Sox9 to induce aggrecan expression, which may be due to its repression of chondrogenic inhibitor Pax3. In all, our findings indicate that the intricate balance of Pax3, Nkx3.2 and Sox9 controls the determination of cartilage and muscle cell fate in muscle satellite cells, and may play important roles in regulating the process of fracture healing. As these factors are also involved in early embryonic cell fate determination, our work supports the notion that healing recapitulates development. Understanding these signaling events may eventually allow us to harness these mechanisms of chondrogenic differentiation to enhance fracture healing.

5.5 Chapter 5 figures, tables and legends

Figure 5.1

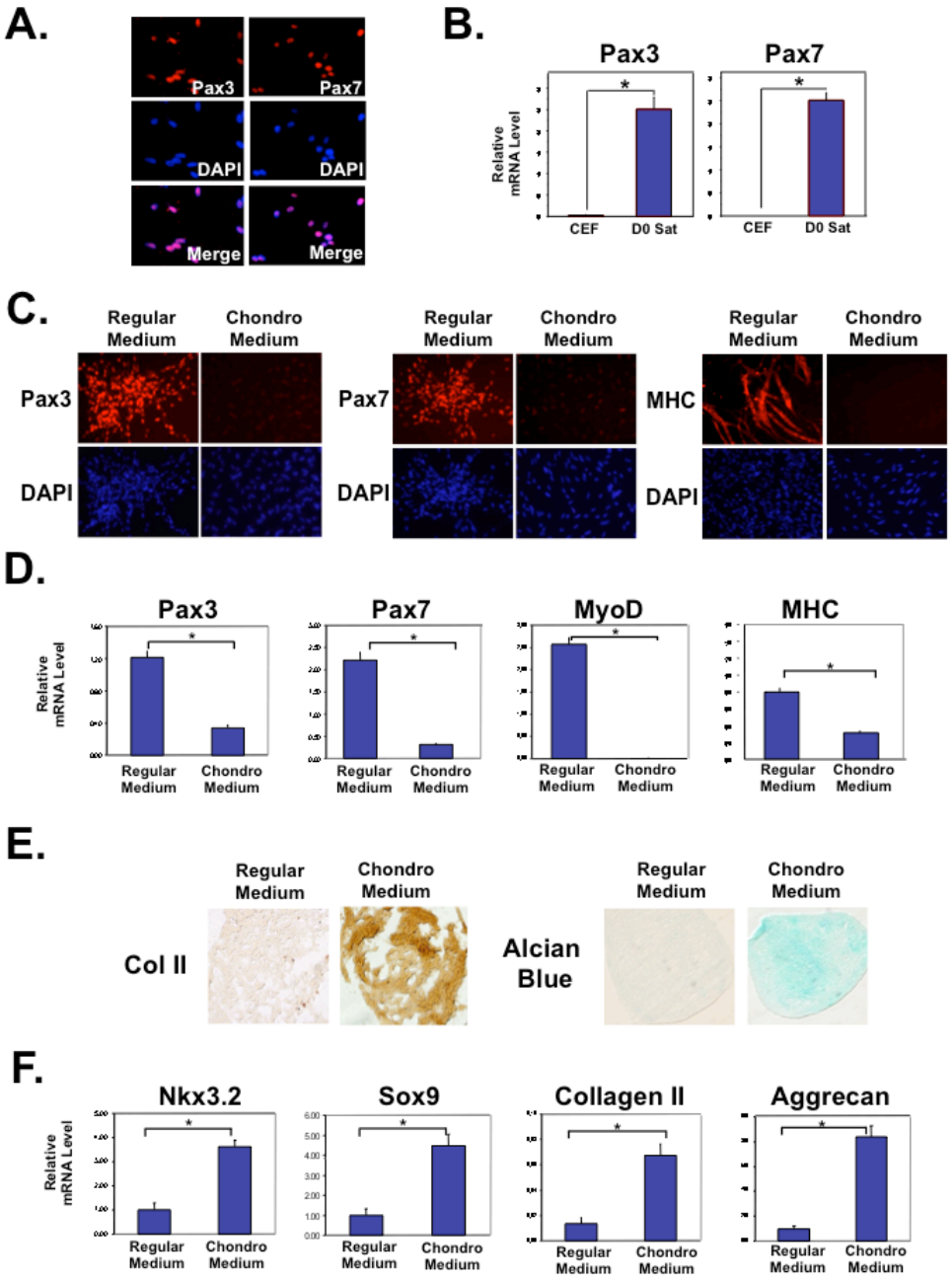

Fig. 1. Isolated muscle satellite cell can be redirected toward a cartilage phenotype at the expense of the default muscle phenotype. (A) Immunocytochemistry analysis indicating that isolated chicken muscle progenitor cells at day 0 are over 95% positive for Pax3 and Pax7 (red staining). Merged images of Pax3 and Pax7 with DAPI staining. (B) qRT-PCR analysis showing that isolated satellite cell markers express Pax3 and Pax7 at day 0, while chicken embryonic fibroblasts (CEFs) do not express these markers. (C) Immunocytochemistry results indicating a dramatic downregulation of Pax3, Pax7, and MHC in response to chondrogenic stimuli. (D) qRT-PCR analysis showing decreased expression of muscle markers Pax3, Pax7, MyoD, and MHC in chondrogenic media as compared to those cultured in regular media. (E) Immunocytochemistry analysis on sectioned micromass cultures showing increased collagen II protein expression upon chondrogenic media treatment. Alcian Blue staining indicates increased glycosaminoglycan levels when cultured in chondrogenic media as compared to those cultured in regular media. (F) qRT-PCR analysis showing increased expression of cartilage markers Nkx3.2 , Sox9, collagen II, and aggrecan in chondrogenic media as compared to those cultured in regular media. All PCR results were normalized to GAPDH. “*” denotes $p<0.05$ in statistical analysis.

Figure 5.2

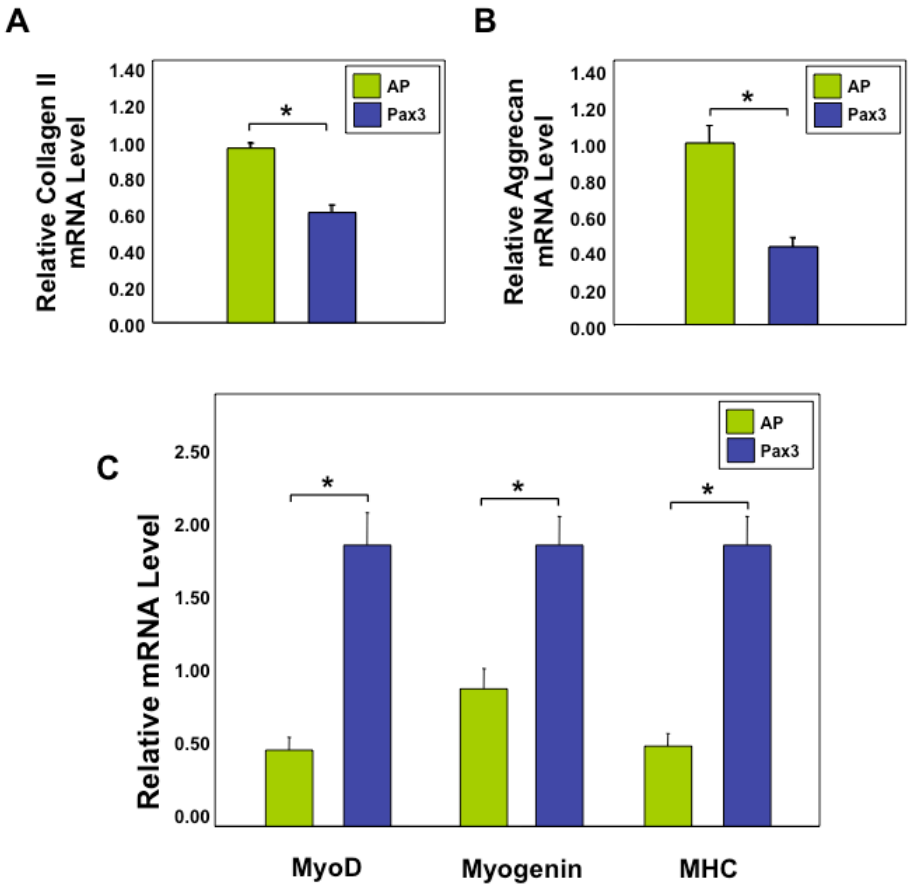
A
Relative Collagen II mRNA Level
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
AP
Pax3
*
B
Relative Aggrecan mRNA Level
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
AP
Pax3
*
C
Relative mRNA Level
2.50
2.00
1.50
1.00
0.50
0.00
AP
Pax3
*
*
*
MyoD
Myogenin
MHC

Fig. 2. Viral infection with Pax3 in muscle satellite cells inhibits chondrogenesis while maintaining muscle gene expression. qRT-PCR analysis of satellite cells infected with RCAS-A-Pax3 or RCAS-A-AP (alkaline phosphatase, control virus) and cultured in 3D micromasses in chondrogenic media. (A) Collagen II mRNA, (B) Aggrecan mRNA, and (C) MyoD, Myogenin, MHC mRNA expression. All PCR results were normalized to GAPDH. "*" denotes $p<0.05$ in statistical analysis.

Figure 5.3

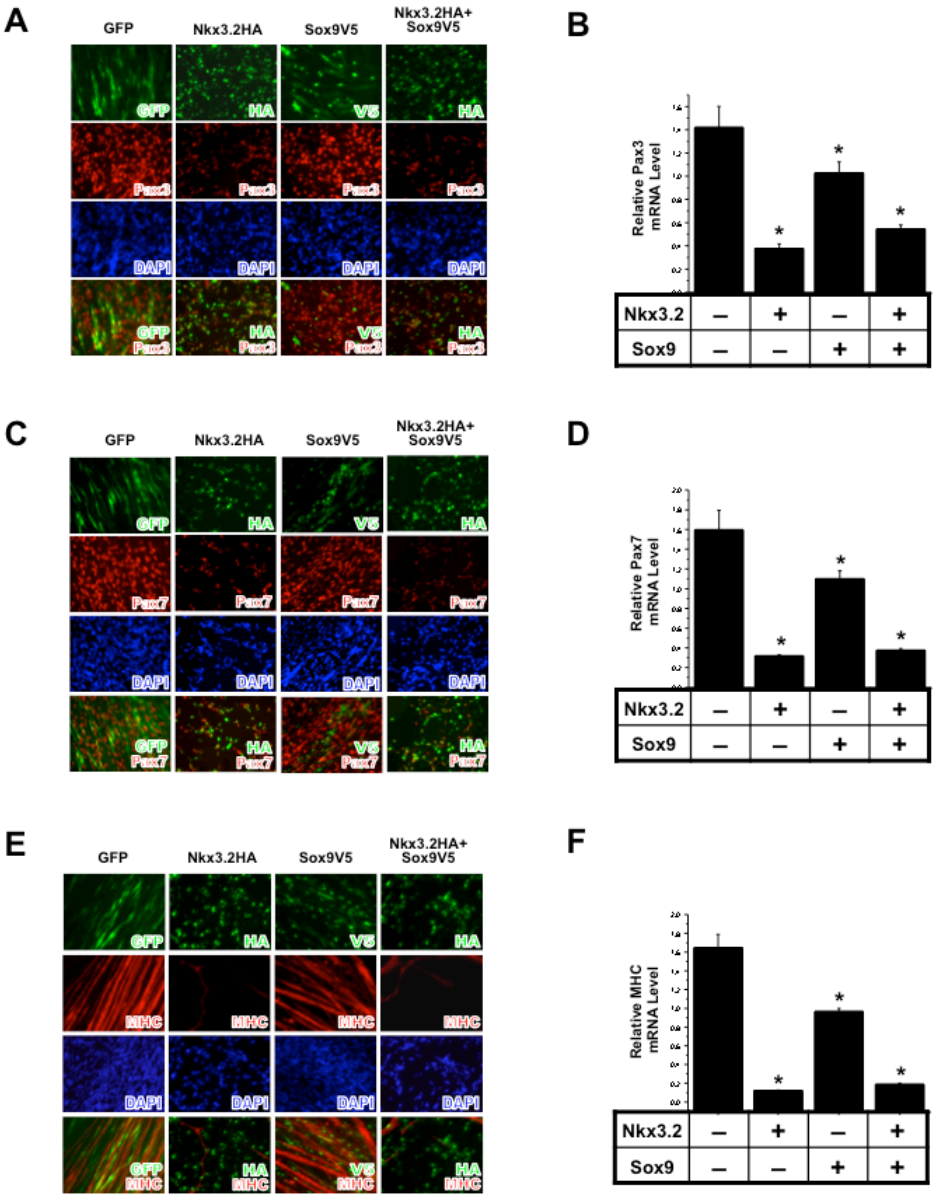
A
GFP
Nkx3.2HA
Sox9V5
Nkx3.2HA+ Sox9V5
B
Relative Pax3 mRNA Level
Nkx3.2 – + – +
Sox9 – – + +
C
D
Relative Pax7 mRNA Level
E
F
Relative MHC mRNA Level

Fig. 3. Nkx3.2 and Sox9 inhibit muscle-specific gene expression in muscle satellite cells. Muscle satellite cells were infected with RCAS viruses encoding GFP, Nkx3.2HA, Sox9V5 or Nkx3.2HA+Sox9V5. Immunocytochemistry and qRT-PCR analyses were performed. Virus staining images were overlaid with Pax3, Pax7 and MHC images, and DAPI stains cell nuclei. For all qRT-PCR, GAPDH was used for normalization. (A) Pax3 immunostaining results. (B) Pax3 mRNA expression. (C) Pax7 immunostaining results. (D) Pax7 mRNA expression. (E) MHC immunostaining results. (F) MHC mRNA expression. "*" denotes $p<0.05$ in statistical analysis.

Figure 5.4

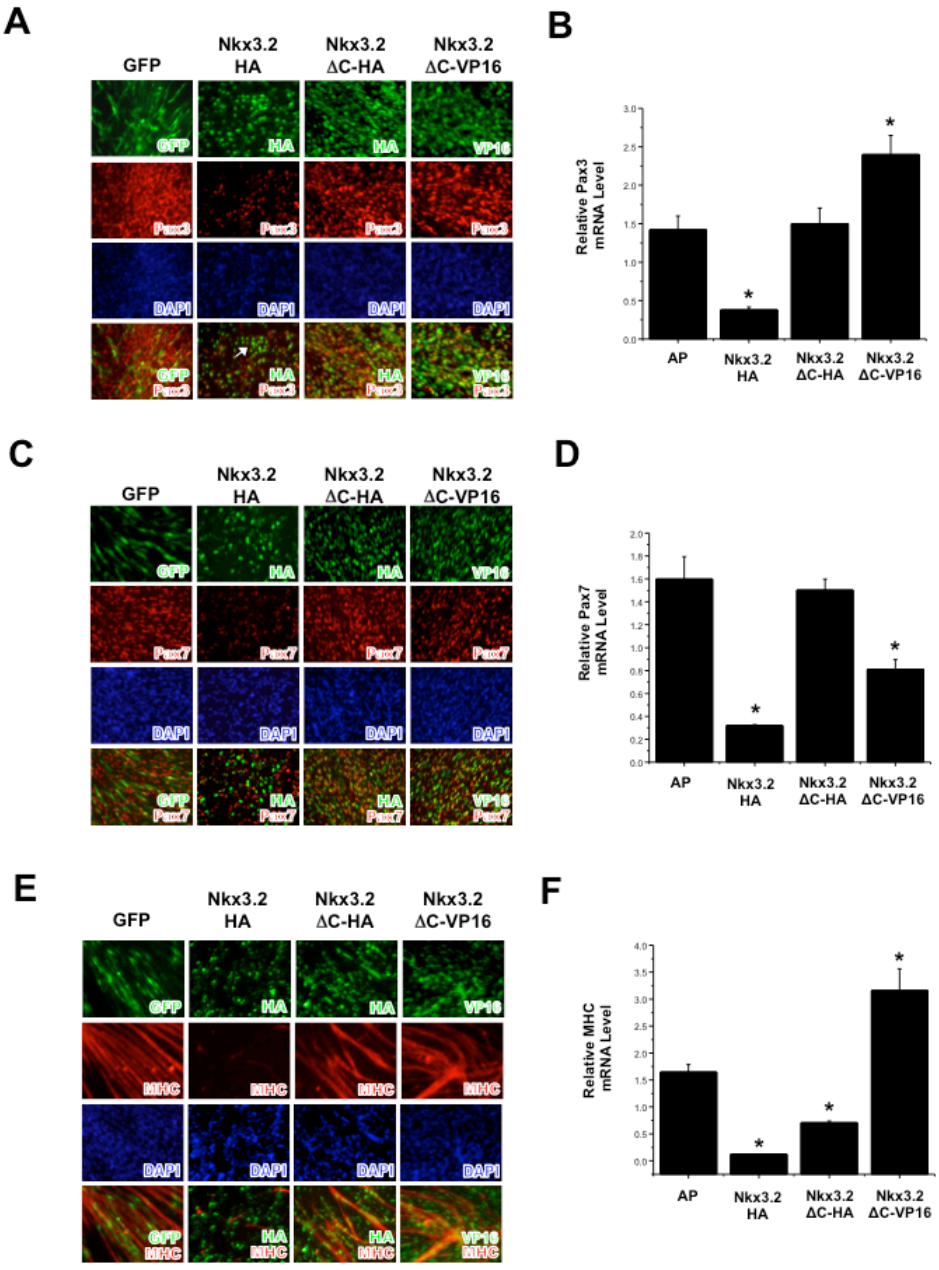
A
GFP
Nkx3.2 HA
Nkx3.2 ΔC-HA
Nkx3.2 ΔC-VP16
GFP
HA
HA
VP16
Pax3
DAPI
B
Relative Pax3 mRNA Level
AP
Nkx3.2 HA
Nkx3.2 ΔC-HA
Nkx3.2 ΔC-VP16
C
Pax7
D
Relative Pax7 mRNA Level
E
MHC
F
Relative MHC mRNA Level

Fig. 4. The C-terminus of Nkx3.2 is required to inhibit muscle cell fate in muscle satellite cells. Muscle satellite cells were infected with RCAS viruses encoding GFP, Nkx3.2HA, Nkx3.2-ΔCHA or Nkx3.2-ΔC-VP16. Immunocytochemistry and qRT-PCR analyses were performed. Virus staining images were overlaid with Pax3, Pax7 and MHC images, and DAPI stains cell nuclei. For all qRT-PCR, GAPDH was used for normalization. (A) Pax3 immunostaining results. (B) Pax3 mRNA expression. (C) Pax7 immunostaining results. (D) Pax7 mRNA expression. (E) MHC immunostaining results. (F) MHC mRNA expression. "*" denotes $p<0.05$ in statistical analysis.

Figure 5.5

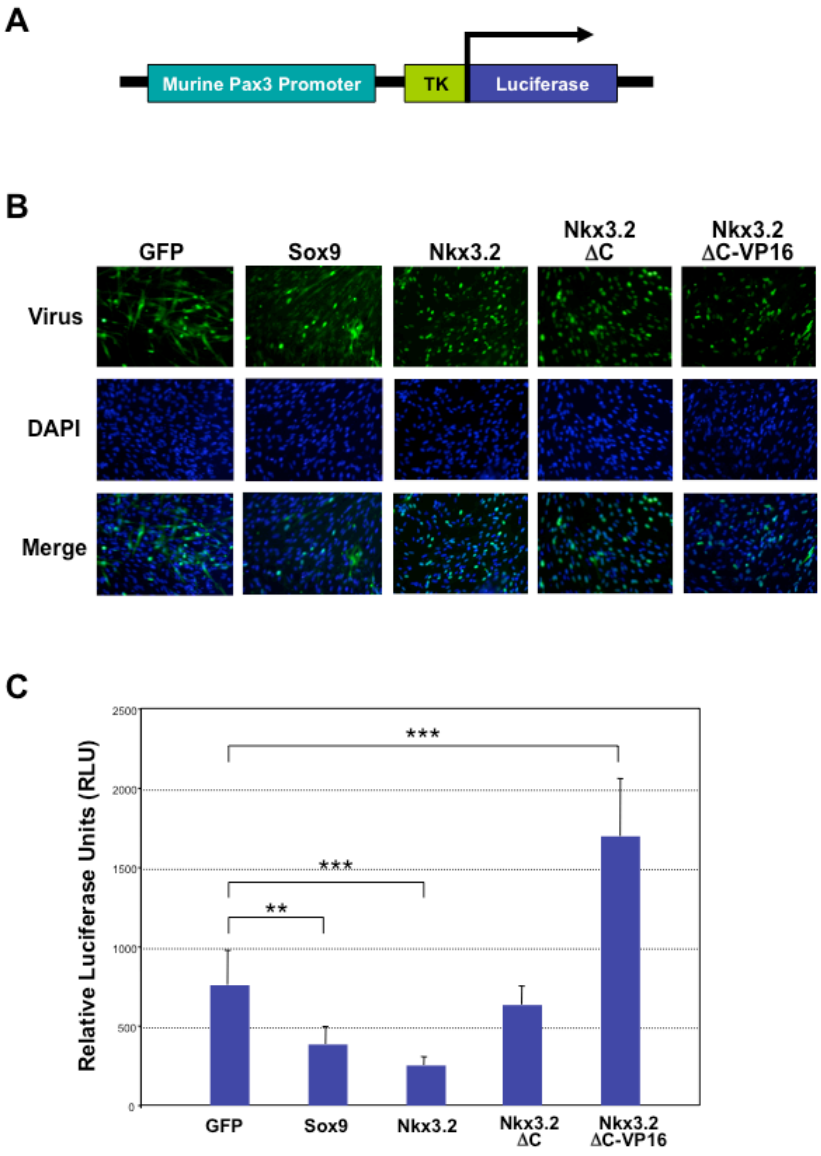
A
Murine Pax3 Promoter
TK
Luciferase
B
GFP
Sox9
Nkx3.2
Nkx3.2 ΔC
Nkx3.2 ΔC-VP16
Virus
DAPI
Merge
C
Relative Luciferase Units (RLU)
2500
2000
1500
1000
500
0
***
***
**
GFP
Sox9
Nkx3.2
Nkx3.2 ΔC
Nkx3.2 ΔC-VP16

Fig. 5. Nkx3.2 and Sox9 inhibit mouse Pax3 promoter activity. (A) Schematic diagram of the mouse Pax3 promoter luciferase construct. (B) Immunocytochemistry analysis showing equal infection efficiencies of all viruses (GFP, Sox9, Nkx3.2, Nkx3.2-ΔC-HA, Nkx3.2-ΔCVP16). (C) Luciferase analysis on satellite cells infected with all viruses (GFP, Sox9, Nkx3.2, Nkx3.2-ΔC-HA, Nkx3.2-ΔC-VP16) and transfected with the Pax3 luciferase construct. A control luciferase vector was used for normalization. "*" denotes $p<0.05$ in statistical analysis.

Figure 5.6

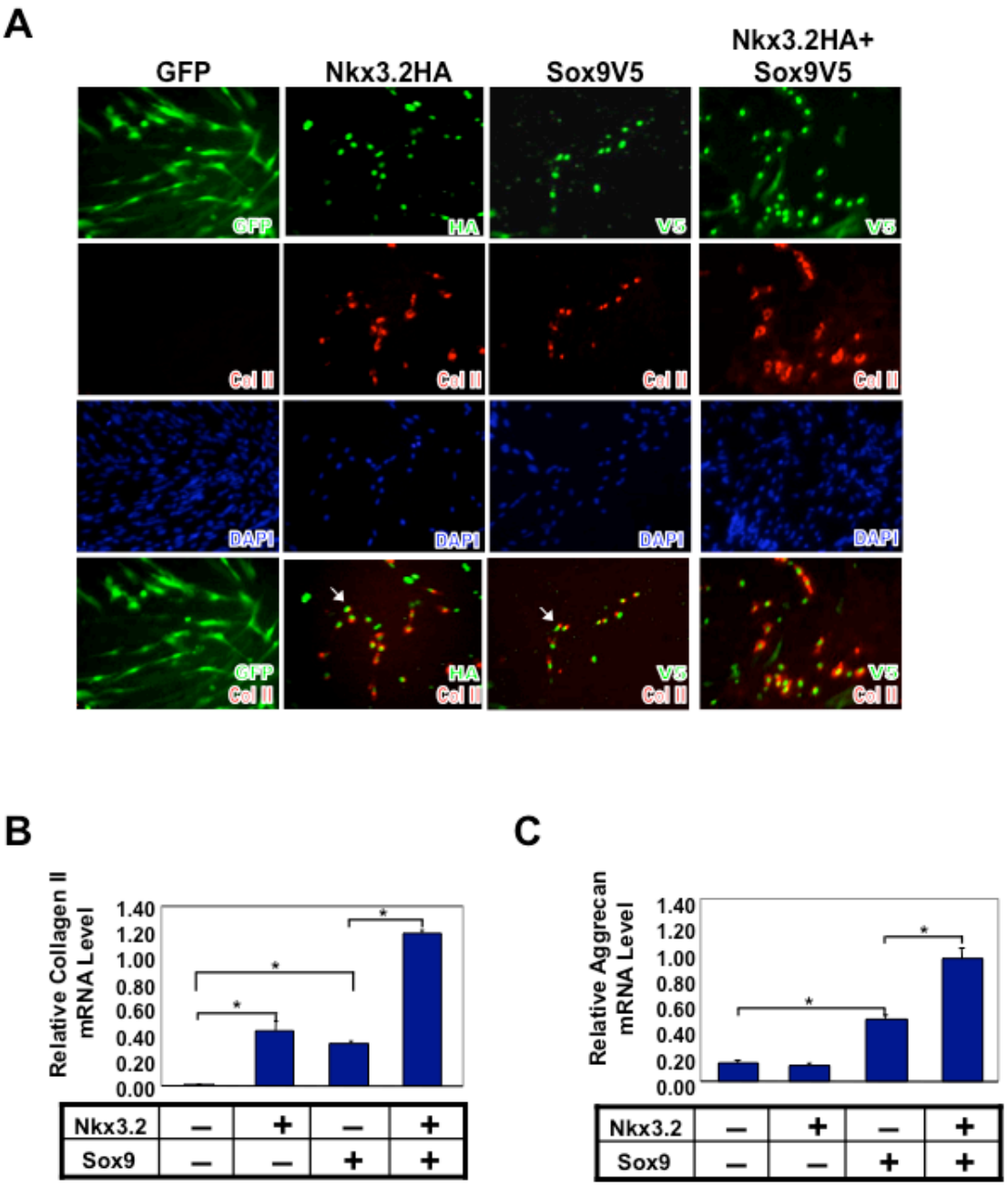
A
GFP
Nkx3.2HA
Sox9V5
Nkx3.2HA+
Sox9V5
GFP
HA
V5
V5
Col II
Col II
Col II
Col II
DAPI
DAPI
DAPI
DAPI
GFP
Col II
HA
Col II
V5
Col II
V5
Col II
B
Relative Collagen II
mRNA Level
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
*
*
Nkx3.2 – + – +
Sox9 – – + +
C
Relative Aggrecan
mRNA Level
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
*
Nkx3.2 – + – +
Sox9 – – + +

Fig. 6. Nkx3.2 and Sox9 can induce muscle satellite cells to express cartilage markers collagen II and aggrecan. Muscle satellite cells were infected with RCAS viruses encoding GFP, Nkx3.2HA, Nkx3.2-ΔC-HA or Nkx3.2-ΔC-VP16. Immunocytochemistry and qRT-PCR analyses were performed. For all qRT-PCR, GAPDH was used for normalization. (A) Immunocytochemistry analysis of Collagen II protein expression in satellite cells. Virus staining images were overlaid with collagen II images, and DAPI stains cell nuclei. (B) qRT-PCR analysis of collagen II mRNA and (C) aggrecan mRNA expression. "*" denotes $p<0.05$ in statistical analysis.

Figure 5.7

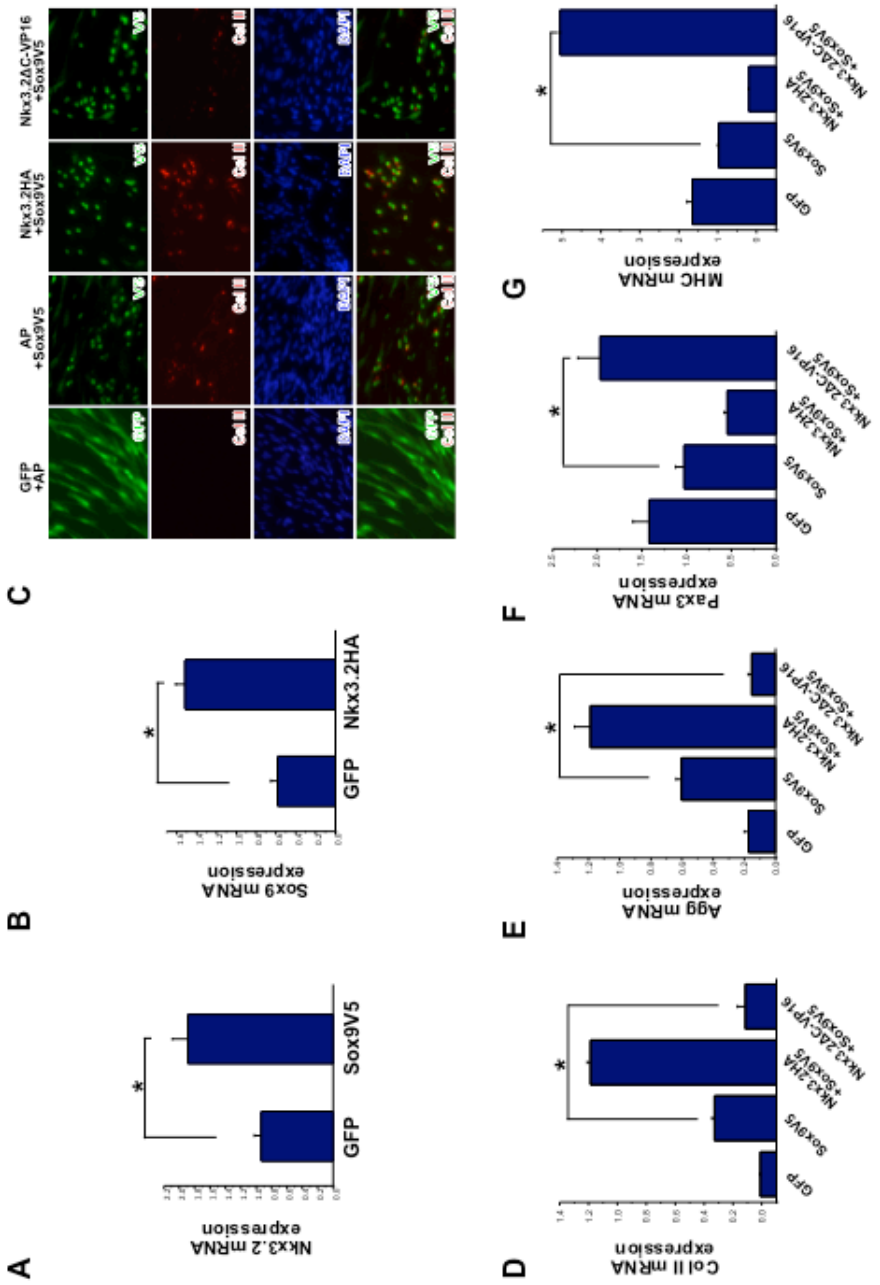
A
Nkx3.2 mRNA expression
GFP
Sox9V5
B
Sox9 mRNA expression
GFP
Nkx3.2HA
C
GFP +AP
AP +Sox9V5
Nkx3.2HA +Sox9V5
Nkx3.2ΔC-VP16 +Sox9V5
Col II
DAPI
V5
D
Col II mRNA expression
E
Agg mRNA expression
F
Pax3 mRNA expression
G
MHC mRNA expression
GFP
Sox9V5
Nkx3.2HA +Sox9V5
Nkx3.2ΔC-VP16 +Sox9V5

Fig. 7. Nkx3.2 is required for Sox9 to activate a cartilage program and to inhibit the muscle program in muscle satellite cells. For all qRT-PCR, GAPDH was used for normalization. (A) qRT-PCR analysis of Nkx3.2 expression in satellite cells infected with control RCAS-GFP virus or RCAS-Sox9V5 virus. (B) qRT-PCR analysis of Sox9 expression in satellite cells infected with control RCAS-GFP virus or RCAS-Nkx3.2HA virus. (C)-(G) Muscle satellite cells were co-infected with the following combination of viruses: RCAS-A-GFP + RCAS-B-AP (alkaline phosphatase); RCAS-A-AP + RCAS-B-Sox9V5; RCAS-A-Nkx3.2HA + RCAS-B-Sox9V5; RCAS-A-Nkx3.2-ΔC-VP16 + RCAS-B-Sox9V5. These infected cells were subject to immunocytochemistry analysis and qRT-PCR. (C) Immunocytochemistry analysis of Collagen II protein expression in satellite cells. Virus staining images were overlaid with Collagen II images, and DAPI stains cell nuclei. (D) Collagen II mRNA expression. (E). Aggrecan mRNA expression. (F). Pax3 mRNA expression. (G). MHC mRNA expression. “*” denotes $p<0.05$ in statistical analysis.

Figure 5.8

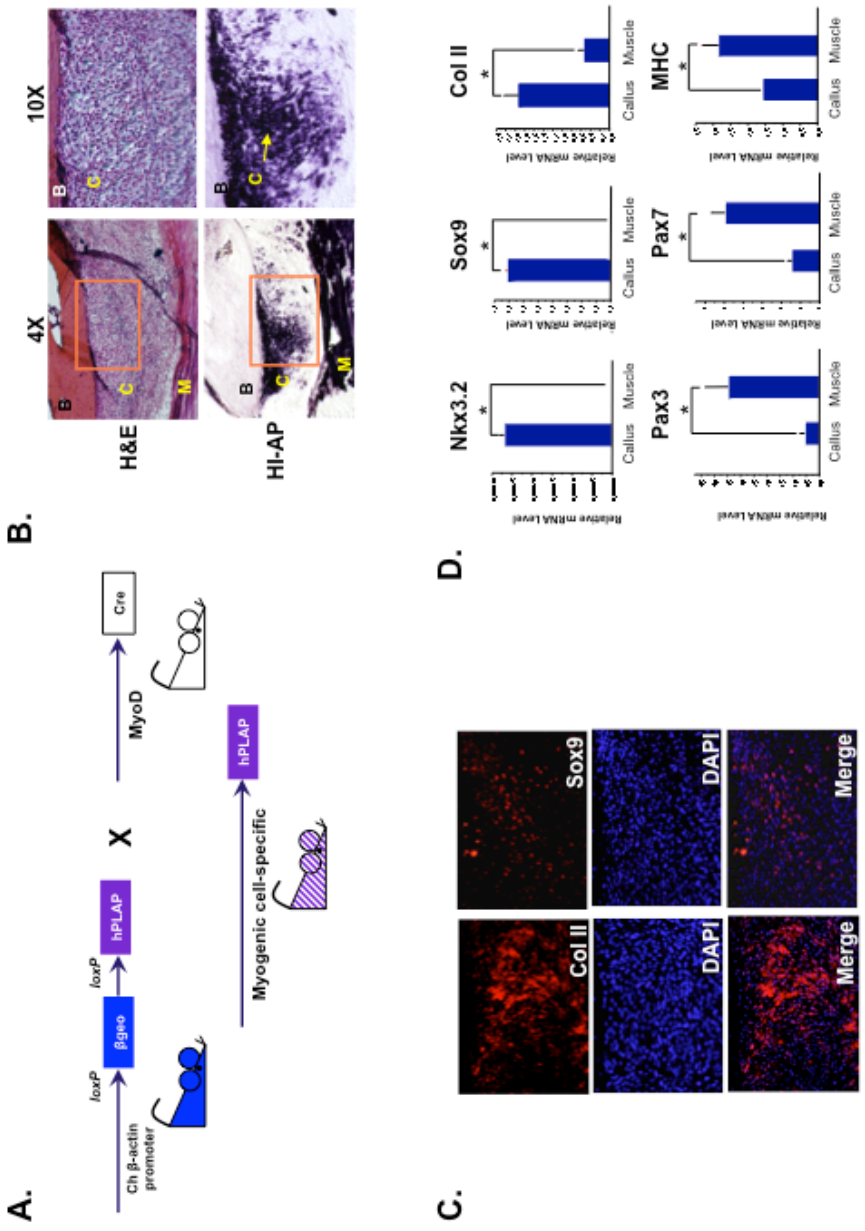
A.
loxP
Ch β-actin promoter
βgeo
loxP
hPLAP
X
MyoD
Cre
Myogenic cell-specific
hPLAP
B.
4X
10X
H&E
HI-AP
B
C
M
C.
Col II
Sox9
DAPI
Merge
D.
Nkx3.2
Sox9
Pax3
Pax7
Col II
MHC
Relative mRNA Level
Callus
Muscle
*

Fig. 8. Nkx3.2 and Sox9 are induced in the muscle progenitor cells that contribute to cartilage formation in an in vivo mouse model of fracture healing. (A) Schematic diagram illustrating the generation of the transgenic mice in which MyoD+ lineage cells are labeled with heat-resistant alkaline phosphatase (hPLAP). (B) H&E histological analysis showing the fracture callus site 1 week post-fracture. Muscle progenitor cells were identified by assaying for heat-resistant alkaline phosphatase (arrow). Left panels, low magnification (4X); right panels, high magnification of the boxed areas from the left panels (10X). B, bone. C, callus, M, muscle. (C) Immunohistochemistry analyses of collagen II and Sox9 expression in the fracture callus. DAPI staining images were overlaid with collagen II and Sox9 staining images. (D) Laser Capture Microscopy (LCM) analysis of muscle progenitor cells in the fracture callus and in the neighboring muscle. qRT-PCR analyses were performed for the following genes: Nkx3.2, Sox9, collagen II, Pax3, Pax7 and MHC. For all qRT-PCR, 18S RNA was used for normalization. “*” denotes $p<0.05$ in statistical analysis.

Table 5.1

Species	Gene & Accession No.	Forward Sequence	Reverse Sequence
Chicken	GAPDH NM_204305.1	5'- CCT GCT GCC TAG GGA AGC -3'	5'- CAG ATC AGT TTC TAT CAG CCT CT -3'
Chicken	Collagen II NM_204426.1	5'- GCA CAA CTT CTG CAC TGA ACG GAT -3'	5'- TCA CAC CTG CCA GAT TGA TTC CCA -3'
Chicken	Aggrecan XM_001232949.1	5'- AGT GAC AAC CCA GTC AGT TGC AGA -3'	5'- AGA AGC GCT CCC ACC AAA GTC TAT -3'
Chicken	Nkx3.2 NM_204137.1	5'- TGC AGC CCT CCT CAC AAG TGT AAT -3'	5'- CGG GCT GCT TAC ACA CAT TCA CAA -3'
Chicken	Sox9 NM_204281.1	5'- GTC TCT GCC GGC TTT ACT TCT TGT -3'	5'- TGC GAG AAA GCG GCA CAG GG -3'
Chicken	Pax3 NM_204269.1	5'- TTC AGG TTT GGT TTA GCA ACC GCC -3'	5'- TAC TGC TTG GAT CAG ACA CGG CTT -3'
Chicken	Pax7 NM_205065.1	5'- AGC CGT GTG CTA CGC ATC AAA TTC -3'	5'- TTC CTC TTC AAA GGC AGG TCT GGT -3'
Chicken	MyoD NM_204214.1	5'- ACG ACA GCA GCT ACT ACA CGG AAT -3'	5'- TCT CCA CAA TGC TTG AGA GGC AGT -3'
Chicken	Myogenin NM_204184.1	5'- TGA AAC CGC CCA AAT CCT TTC CCA -3'	5'- CGA AGA GCA ACT TGG AAA CAG CCA -3'
Chicken	Myosin Heavy Chain (MHC) NM_204228.1	5'- GCA GAA TTT CAG AAG ATG CGC CGT -3'	5'- TGA CTC GTT GCA GGT TGT CGA TCT -3'
Mouse	18S NR_003278.2	5'- TCA ACT TTC GAT GGT AGT CGC CGT -3'	5'- TCC TTG GAT GTG GTA GCC GTT TCT -3'
Mouse	Collagen II NM_001113515.2	5'- ACA TAG GGC CTG TCT GCT TCT TGT -3'	5'- TGA CTG CGG TTG GAA AGT GTT TGG -3'
Mouse	Nkx3.2 NM_007524.3	5'- TCA GAA CCG TCG CTA CAA GAC CAA -3'	5'- CAG CAC CTT TAC GGC CAC TTT CTT -3'
Mouse	Sox9 NM_011448.4	5'- AGG TTT CAG ATG CAG TGA GGA GCA -3'	5'- ACA TAC AGT CCA GGC AGA CCC AAA -3'
Mouse	Pax3 NM_008781.4	5'- TAC CAG CCC ACG TCT ATT CCA CAA -3'	5'- TTT GGT GTA CAG TGC TCG GAG GAA -3'
Mouse	Pax7 NM_011039.2	5'- TTC AAA GGA GGA GAC TGT TGG GCT -3'	5'- TGT GGA GGA GGA TGC ATT TGG TCT -3'
Mouse	Myosin Heavy Chain (MHC) X57377.1	5'- AGG CTT ACA AGC AAA TGG CAA GGG -3'	5'- ACC GCA TGG CAT ACT TAG CAG AGA -3'

Table 1. Primer sequences used for all qRT-PCR analyses.

CHAPTER 6: General discussion

6.1 Cartilage and muscle cell fate determination in the embryo: Nkx3.2, Sox9, Pax3

6.1.1 Impact of our research on the understanding of somite cell fate determination

Our research has had a great impact on the field of developmental biology. It has provided a deeper understanding of the specification of cell fate during embryonic development, by further elucidating the role of Sonic Hedgehog (Shh) in patterning the nascent somite. Briefly, we determined that Shh behaves like a morphogen, which is a signaling molecule that guides the specification of cell type of an immature population depending on its location from the source and the resulting concentration of the molecule[8]. In our work, we demonstrated that altering the levels of Shh in somite cultures (while in the presence of competing factor Wnt3a) alters the cell fate, with relatively low levels corresponding to an increase in dermomyotome gene expression (as indicated by Pax3 and -7 expression), intermediate levels promoting the myotome fate (Myf5 and MyoD expression), and high levels inducing sclerotome markers (Nkx3.2, Sox9 and Pax1). Further, we demonstrated that expression of these target genes generates well-defined borders demarcating the different somitic cell fates, and that overexpression of those same target genes in ectopic locations is sufficient to alter cell fate after the gradient has patterned the tissue.

More specifically, Pax3 is initially expressed both in the dorsal neural tube, and throughout the entirety of the presomitic mesoderm. The latter expression domain then becomes restricted to the ventral somite as a consequence of a high concentration of localized Shh[11, 12]. Although Sox9

is co-expressed with Pax3 in the pre-somitic mesoderm, it is not until the appearance of Nkx3.2 expression that Sox9 becomes restricted to the ventral somite. Ectopically expressing Nkx3.2 in the dorsal domain of the somite inhibited Pax3, Pax7 and MHC expression in vivo. Similar experiments using Sox9 caused reduced MHC expression, but not Pax3 or Pax7. A proposed explanation for this is that we have also shown that Sox9 can induce expression of Nkx3.2, which potently inhibits both early and late muscle marker expression[58, 60]. We suspect that Sox9 inhibits MHC at least partially through the induction of Nkx3.2. By the time Sox9 induces Nkx3.2, Pax3/7 dermomyotomal expression has already been stabilized, but the subsequent differentiation into myotomal fate can still be prevented thereby explaining the inhibition of downstream MHC expression.

Previous work has shown that knocking out Pax3 and Pax7 caused death of dermomyotomal cells or the re-differentiation of these cells into Col2-positive cartilage cells[129, 306]. Our data suggest that Pax3 or -7 expression induces a competence for the dermomyotomal and/or myotomal cell fate, which ultimately prevents sclerotome-specific expression. In contrast, Nkx3.2 confers a chondrogenic capacity for sclerotome cells thereby limiting their capacity to adopt the dermomyotomal/myotomal cell fate. This mutually repressive activity of Pax3 and Nkx3.2 ensures that each cell will adopt only one cell type. For example, Pax3 and Nkx3.2 mutually repress each other's expression – an evolutionary safeguard against the adoption of multiple cell fates by one cell. In 10T1/2 cells, Lengner et. al. also found that Nkx3.2 induces Sox9 expression during chondrogenic differentiation[168].

Furthermore, in Nkx3.2 knockout mice, Sox9 expression is significantly decreased[59].

Many aspects of muscle and cartilage cell fate specification during somite development have been studied, however, our work is among the few that describe how the interaction of dermomyotomal cells and sclerotomal cells and their respective intrinsic transcription factors can specify cell fate. The effects of other intrinsic factors that serve to pattern the somite have also been described. Definition of the roles of other intrinsic factors in patterning the early embryo. Meox1 knockouts show a marked reduction in embryonic Nkx3.2 expression, but showed no effect on the expression of muscle markers Pax3 and MyoD, nor does it affect the expression of other sclerotomal markers Pax1 and Pax9[307]. Meox1 seems to be required for the maintenance but not the induction of Nkx3.2 expression. Furthermore, these mutants had altered sclerotomal compartments (which was more pronounced in the rostral older somites), which ultimately suggests a role for this factor in sclerotome maintenance as well as the resegmentation of the vertebrae and IVD that occurs during formation of the axial skeleton[307].

Furthermore, other studies have examined the compartmentalization of somites, and the way in which somite domains affect one another. For example, more recent work in chick embryos has shown that Slit1, a sclerotomal factor, affects the differentiation of migrating myoblasts in the neighboring myotome using a RhoA dependent mechanism[308]. Further embryonic studies can be executed to understand other aspects of somite patterning.

6.1.2 Other extracellular factors that affect somite cell fate determination

Similar studies have been conducted to understand somite patterning by other secreted factors, including Notch, BMP and FGF signals. For example, one study analyzed mutant mouse embryos deficient in either of the two BMP antagonists Noggin (Nog) or Gremlin (Grem1) to ascertain the role of BMP signaling on sclerotome specification. In both types of mutant embryos there was a dramatic loss in Pax1 and -9 expression, and furthermore double knockouts of both genes fail to initate sclerotome formation at all. Conversely, Pax3 and Myf5 expression is relatively normal, suggesting that dermomyotomal induction does not require the activity of BMP signaling. This also suggests that Nog and Grem1 are required to inhibit local BMP signals to allow for Shh to elicit its initial activity in patterning the ventral sclerotome. In the absence of BMP antagonists, BMP can cause these cells surrounding the notochord to either stop proliferating, prematurely differentiate or to undergo apoptosis. The author conclude that Nog and Grem1 are required to control the levels of BMP in the microenvironment to allow for Shh to initiate sclerotome formation[309].

Additional studies have examined the roles of other well known signaling molecules. For example, fibroblast growth factor (FGF) secreted from the myotomal compartment induces the formation of scleraxis (Scx) positive tendon progenitors cells in between the myotome and the adjacent sclerotome. It was then determined that transcription factors Pea3 and Erm are necessary and sufficient to induce Scx expression[310], and that their

coordinated expression patterns are responsible for carving out the domain of the syndetome[311]

6.1.3 Extracellular factors pattern other neighboring tissues and embryonic structures

Similar research has been conducted to examine the extracellular signals that pattern neighboring embryonic tissues and structures. Our research is in accordance with similar work in developmental biology as well as other scientific fields. [158-160, 312-315] Other work has demonstrated that the mechanisms elucidated in our studies are not exclusive to the somite. For example, the neural tube is also patterned by extrinsic signals ultimately causing restriction of Pax genes to the dorsal neural tube, and Nkx expression confined to the ventral domain. Shh has been shown to pattern the ventral neural tube.[176] We detail the mechanisms by which Shh patterns the somite into dermomyotomal, myotomal and sclerotomal cell fates. Our results are in congruence with those from similar zebrafish studies [139] as well as other studies that say ectopic Shh essentially promotes a switch from dermomyotomal to myotomal cell fate by inducing premature differentiation of Pax3-positive cells[141].

Extracellular morphogen-like signals not only pattern the very primitive structures in very early embryonic stages, they also function throughout the entirety of the development process. Recent work by Sohn et. al. describes a thorough gene expression analysis to understand the intricate patterning of TGFβ that helps to direct the development of the vertebrae and intervertebral discs (IVD). Briefly, they found that in conditional knockouts of Tgfbr2, cells of

the IVD take on the phenotype of vertebral cells, suggesting that TGFβ is required for the proper development of the IVD[316].

6.1.4 Implications of our findings on the development of other tissues

Another area of potential future research might be in understanding the patterning of the gut. It has been speculated that smooth muscle cells of the early esophagus transdifferentiate to become skeletal muscle cells. Studies using three separate kinds of transgenic mice in which cells expressing skeletal muscle factor Myf5, MyoD or Myogenin were labeled with lacZ indicated that the expression of skeletal muscle markers began in smooth muscle cells of the upper esophagus[317] Of additional interest is that Nkx3.2 has been shown to be expressed in smooth muscle cells of the gut mesenchyme[318]. Perhaps a similar mechanism of mutually repressive cell fates regulated by Pax3 and Nkx3.2 occurs during the development of this structure as well.

Further studies could also address the significance of Pax3 and Sox9 co-expression in various tissues such as the pre-somitic mesoderm and neural crest cells. Are these cells initially equipped with these two potential and generally quite potent triggers so that they maintain the capacity to form either dermomyotome or sclerotome? Nkx3.2 expression does not arise until the establishment of somite domains, at which time Pax3 and Sox9 then become restricted to their respective compartments. Perhaps like with sclerotomal specification, there exists a molecular factor in the ventral domain that acts as a switch to specify dermomyotomal fate. As with our studies, identification of such a factor will not only aid in the understanding of

embryonic patterning, but could potentially yield interesting insights into those adult mechanisms that recapitulate mechanisms during development.

6.1.5 Studying the embryonic regulation of Nkx3.2 may help understand human diseases in which Nkx3.2 expression is altered

From a more translational and clinical perspective, our research can also be applied to the understanding of human diseases. Homozygous inactivating mutations of Nkx3.2 have been correlated to the disease spondylo-megaepiphyseal-metaphyseal dysplasia (SMMD), a disease characterized by short stature, disproportionally long limbs, and impaired ossification of the vertebral bodies. Interestingly, in the embryo, Bapx1 is more highly expressed in the vertebrae than in the intervertebral discs [55, 169, 319], and Bapx1−/− mice exhibit lethal skeletal dysplasia, with malformed vertebrae lacking ossification centers.(9). Nkx3.2 has been correlated to another disease known as Oculo-Auriculo-Vertebral Spectrum or OAVS. While not caused by or associated with a direct mutation of the Nkx3.2 gene, (OAVS) does cause abnormal Nkx3.2 expression in these patients. This disease is the second most common cranial birth defect. OAVS is characterized by hypoplasia of certain craniofacial structures such as the maxillary and temporal bones, as well as undersized (microtia) or complete absence (anotia) of ears[320-322]. In addition to these symptoms, patients often exhibit spinal defects such as fused vertebrae and spina bifida[320]. Nkx3.2 also shows embryonic expression in a portion of the mandible known as Meckel's cartilage[323]. These same Nkx3.2 knockout mice also exhibit defects in patterning of the middle ear, resulting in misshapen ossicle bones [56, 59, 323]. Studying the regulation of Nkx3.2 in the embryo will ultimately help to understand human diseases in which Nkx3.2 expression is altered

[305, 324].

6.2 Effect of muscle on cartilage homeostasis and response to inflammatory stimuli

6.2.1 Impact of our research on treating cartilage-related disease

Other aspects of our work will likely have a significant impact with regard to arthritis treatment and cartilage tissue engineering. While previous work elucidated some of the mechanisms involved in the specification of muscle and cartilage cell fates, we also wanted to understand the relationship that exists between those two differentiated cell types. There are multiple lines of evidence to suggest that muscle tissue may impact the development of neighboring cartilage tissue. For example, mouse mutants in which muscle-specific markers are knocked out exhibit dramatically reduced skeletal elements[35, 66]. To begin to address this issue, we first examined the effect of muscle on cartilage matrix production. We demonstrated that chondrocytes co-cultured with muscle cells had increased cartilage-specific Collagens II and IX, as well as increased glycosaminoglycan production as indicated by alcian blue staining. Because we saw a similar increase in cartilage matrix production when culturing chondrocytes in media conditioned by muscle cells, we propose that muscle cells elicit their pro-chondrogenic effect by way of muscle-secreted factors. Interestingly, other studies of muscle-cartilage co-cultures have yielded similar results. For example, culturing mouse muscle derived stem cells with human nucleus pulposus cells increased proteoglycan production[212]. Perhaps these types of studies will inspire future research to further elucidate the mechanisms involved

therein, and to harness this approach for cartilage tissue engineering and potential clinical applications.

As we found muscle to have a significant positive effect on cartilage matrix production, we then wanted to determine whether muscle and/or muscle secreted factors would similarly have positive effects on other aspects of cartilage metabolism. Arthritis is caused by pro-inflammatory cytokines present in the joint that serve to destroy articular cartilage matrix. Many studies have shown that culturing chondrocytes in the presence of pro-inflammatory cytokines IL-1β and/or TNFα will cause degradation of cartilage matrix proteins Col2, Col9 and Aggrecan. Similarly, in our experiments we have shown that either IL-1β or TNFα causes a decrease in expression of these markers. Strikingly, co-culturing with muscle cells actually attenuates the response of chondrocytes to inflammation by reducing the expression of degradative enzymes and ultimately preventing this decrease in cartilage matrix production.

Maintenance of this balance of anabolic and catabolic activity is a tightly regulated process. Cartilage homeostasis is crucial for the biomechanical strength of the extracellular matrix and ultimately the survival of the articular chondrocytes. It is likely that this muscle-mediated anti-inflammatory activity is actually due to the actions of one or more muscle-secreted factors, as conditioned media appears to elicit similar effects to those of muscle-cartilage co-cultures. One proposed mechanism is that this factor or factors inhibits downstream effectors of IL-1β and/or TNFα. For example, it has been shown that activation of the canonical nuclear factor-κB (NF-κB) pathway by the heterodimerization of p50 and p65 greatly

contributes to the severity of the inflammatory phenotype in arthritis, and it has been proposed that inhibiting this pathway could provide a potential treatment strategy for treating the disease[325]. Therefore, further elucidating these types of pathways and the potential ways in which muscle-specific factors may interact will likely provide insight into therapeutic targets for treating arthritis.

Ours is not the first report of extracellular factors with anti-inflammatory properties. Various factors have been shown to exhibit anti-inflammatory activity with respect to cartilage metabolism. For example, TGFβ has been shown to inhibit the IL-1β-induced upregulation of MMP13 and subsequent reduction collagen II, however it had no effect on aggrecanase ADAMTS4. Additionally, similar properties have been attributed to IGF-I, however, in order to achieve such an effect required unreasonably high concentrations[326-328].

6.2.2 Other neighboring tissues affect cartilage metabolism

Ours is also not the first study to examine the effect of neighboring tissues on cartilage metabolism. Interaction of cartilage with other neighboring tissues, including synovium, adipose tissue and bone, all modulate cartilage matrix production and inflammatory response. Studies in which human articular chondrocytes (AC) were cultured in media conditioned by synovial cells from patients with OA, caused an increase in 3H thymidine incorporation indicative of increased proliferation which may help to explain the formation the fibrous scar-like tissue known as the pannus that often grows on the articular surface of diseased joints. [329] Angiogenesis often

occurs in patients with OA, and this process exacerbates the disease. Synovial fibroblasts have been shown to secrete factors that stimulate angiogenesis such as VEGF. [330]

However, adipose tissue has been shown to secrete inflammatory mediators termed adipokines, including interleukin (IL)-1, IL-6, IL-8, monocyte chemotactic protein 1, leptin, adiponectin and others. The infrapatellar fat pad (IPFP) is located close to both the synovium and the articular cartilage, and has been shown to have effects on both of these tissues. The IPFP aids in the maintenance of synovial fluid and the distribution of mechanical force in the joint [331-334].

6.2.3 Myokines: muscle-secreted factors

We demonstrated that muscle may affect cartilage development and metabolism via the secretion of various biochemical molecules. Skeletal muscle has been described as an “endocrine organ” as it secretes a variety of factors termed “myokines.” Some myokines already identified include such factors as IL-6, IL-8, IL-15, BDNF, LIF, FGF21 and Follistatin-like-1 [335-340]. Muscle has been found to secrete a number of growth factors such as IGF-I, a factor which already has a proposed role in preventing cartilage degradation. The potential of one of those muscle secreted factors, IL-6, has even been proposed to have anti-inflammatory effects. IL-6 inhibits LPS-induced TNFα in vitro in cultured human monocytes[341] and IL-6 knockout mice have markedly increased levels of TNFα[342]. In human studies, infusion of the blood with IL-6 attenuates TNFα production brought on by infection with E.coli endotoxin, presumably in part by causing an increase in

IL-1 receptor agonist (IL-1ra) which is thought to mitigate the downstream effects of TNFα signaling [343-345].

These types of proteomic analyses of muscle cells and their secreted factors have revealed a number of muscle-derived proteins such as IGFs and Wnts [32–34], which could potentially exhibit anti-inflammatory activity. We did not explicitly identify and characterize which molecule or molecules might be eliciting these effects, however we did perform a cytokine array on the media conditioned by the C2C12 myoblast cell line. There were a number of potential factors upregulated in muscle-conditioned media relative to controls. One such highly expressed factor of particular interest was IGF-II, based on my cytokine array analysis. We are currently working to understand the effect of IGF-II in muscle cell mediated cartilage regulation. It will be important to investigate which muscle cells elicit what type of effects. It is possible that muscle cells at various stages of differentiation secrete different factors. Further analysis of the proteins secreted by satellite cells, myoblasts, myocytes and mature muscle fibers may help to identify additional pro-cartilage factors secreted by muscle (IGF-II). Analyze secretory profiles of muscle cells at various stages of differentiation, and during disease states. It is likelys that diseased muscle has an altered expression of secreted factors. For example, patients with centronuclear myopathy, a disease of adult onset muscular dystrophy, often exhibit arthritis of the cartilage of the temporomandibular joint (TMJ) [118]. Given the close proximity of muscle and cartilage in the TMJ, this suggests that perhaps these diseased muscle cells have an altered secretory profile that negatively regulates cartilage metabolism in the TMJ.

6.2.4 Muscle cell based therapy has potential for treatment of other diseases

While we described a novel mechanism of muscle-mediated cartilage regulation that could be harnessed to treat arthritis, it seems possible then that these secreted factors and myokines could also play a role in other pathways of inflammation. Factors derived from serum and muscle cells from mice post-exercise inhibited proliferation and induced apoptotic marker caspase 3 in a breast cancer cell line [346], suggesting the muscle derived factors may ultimately be utilized to treat other diseases in which inflammation plays a central role, such as cancer, atherosclerosis, neurodegeneration[343, 347]. Muscle cell-based therapy not only useful for the treatment of arthritis but potentially for other diseases.

Understanding the mechanism by which muscle cell proteins elicit these effects will be crucial in their development as a potential therapeutic. Our results indicate that muscle cells promote the production of cartilage matrix mostly at the level of post-transcriptional control – perhaps by stabilization of the extracellular matrix proteins, by prevention of the induction of degradative enzymes or apoptotic factors, or perhaps some combination of those types of mechanisms. Another avenue of research will be clearly elucidating the pro-chondrogenic activites of muscle. Including the identification and characterization of those factors secreted by muscle, and subsequently their downstream targets and signaling pathways.

6.2.5 Potential muscle cell based strategies to enhance cartilage tissue engineering

Muscles can be harnessed not only because they enhance cartilage matrix production and inflammatory cytokine resistance, but also to try to combat other obstacles in treating cartilage injury and disease – namely host integration. In addition to the aforementioned myokines, muscles have also been shown to secrete enzymes associated with increased migration. For example, muscle cells treated with bFGF were found to secrete MMP1 and cathepsin L by the muscle cells. Furthermore, conditioned media from those muscle cells was found to enhance the migration of endothelial cells[348]. In addition, we have preliminary data to suggest that C2C12 muscle cells appear to enhance the ability of bovine articular chondrocytes to integrate into host cartilage in an ex vivo model (data not shown). During repair, muscle satellite cells continuously re-fuse with existing muscle fibers. Chondrocytes lack the ability to properly merge with existing cartilage tissue. In this regard, muscle cells may provide a mechanism that could be extrapolated for the purpose of more efficiently securing engineered cartilage into the host site.

6.3 Repair recapitulates development: a novel role for Nkx3.2 and Sox9 in cartilage formation during fracture healing

6.3.1 Studies of embryonic cartilage and bone development help to understand similar mechanisms that occur in adult regeneration and repair

It has been well documented that a number of processes that occur during development are recapitulated in regenerative processes in the adult. For example, bone fracture healing is thought to occur by processes that parallel those that occur during endochondral ossification in the embryo [99, 349]. Briefly, post-fracture a hematoma forms as a result of injury to the

periosteum. This inury also causes an influx of inflammatory cells and cytokines[350]. There is a proliferation of localized mesenchymal cells and fibroblasts from the bone marrow, periosteum and neighboring soft tissues [351-353], accompanied by an overall decrease in oxygen tension, which creates a hypoxic environment. These proliferating cells then undergo a transition into osteoprogenitor cells due to the presence of high levels of morphogens such as TGFβ and various BMPs[354-357]. These cells become chondrocytes thereby forming the initial fracture callus. The ECM of the cartilage within the callus then becomes calcifed bony tissue[353, 358-360], which ultimately becomes vascularized and remodeled to form the fully repaired mature endochondral bone.

Studies in development have helped to understand processes in differentiation of adult tissues. Development of the embryo occurs via a series of very tightly coordinated processes of differentiation, during which specific tissue types are patterned. In addition, cells found within those defined tissue types specialize even further to allow for both fully differentiated mature cells as well as less-specified stem and/or progrenitor cells that serve as a reserve population that exists to ultimately repair injured or damaged tissue in the adult. Determination of cell fate is driven by multiple factors, both extracellular and cell-autonomous. As such, we utilized knowledge gained during our previous studies of embryonic development, to generate hypotheses for similar mechanisms that might be involved in repair of adult tissues. We found a way to extrapolate our embryo system into an adult setting by studying the conversion of muscle-derived cells into cartilage cells.

6.3.2 Extracellular signals involved in embryonic patterning and fracture healing elicit cell fate changes

Many cells of one type are driven to become cells of another via the use of extracellular morphogen-like factors such as TGFβ and BMPs. TGF-β family members have been shown to cause cells of one type to switch to acquire characteristics of a completely different lineage or differentiation pathway. While TGFβ has been shown to promote the adoption of cartilage cell fate, it has also been shown to inhibit differentiation of osteoblasts, myoblasts and adipocytes, but instead stimulates their proliferation[361]. In that sense, TGFβ can elicit differing effects on various cell types.

Culturing myoblasts in the presence of BMPs forces them down the osteogenic pathway at the expense of the default muscle pathway[241]. This is thought to occur via the expression of transcription factor Id1, as Id1 has been shown to inhibit downstream muscle specific transcription factors, causing them to be degraded[362-364]. These results and others clearly demonstrate that cells pre-destined to become one cell type can be re-directed to become other distinct cell types under the influence of TGFβ and BMP signaling. Similar research has utilized BMPs to re-direct adipocytes into osteoblasts. This presents an attractive mechanism for cell-based clinical therapies for bone repair, as adipocytes are often in large abundance. Interestingly, adipose stromal cells have already been shown efficacy in repairing calvarial bone defects in both mouse models[365] and in humans[366]. BMP signaling in combination with retinoic acid treatment has been shown to induce various fat cells to become fully mature osteoblasts [367, 368].

While much is known with regard to the extracellular signals that are involved in the transition of cells into cartilage and bone tissue such as BMP[355, 369-371] or TGFβ[104], much less is known about the downstream regulation of those signaling molecules, and how this cell fate transition actually takes place. In our previous work, we identified a role for Nkx3.2, a transcriptional repressor, in inhibiting muscle cell fate during early embryonic patterning of the somite[214]. In our studies, we found that Nkx3.2 and Sox9 are two key transcription factors involved in the conversion of muscle cells to cartilage cells.

6.3.3 Impact of our research on the understanding of muscle-to-cartilage cell fate transition

Our data serve to more fully characterize this role of Nkx3.2 in inhibiting muscle cell formation. We demonstrate that Nkx3.2 is sufficient to inhibit the expression of satellite cell marker Pax3/7 as well as downstream skeletal muscle markers, MyoD, Myogenin and MHC. We also show that in our model as is previously described in previous studies [58, 214], Nkx3.2 and Sox9 induce reciprocal expression of the other factor. A phenomenon that likely explains then why either factor is sufficient to induce cartilage marker expression in these muscle satellite cell cultures. Furthermore, we go on to show that blocking the function of Nkx3.2 by way of a reverse function mutant construct of Nkx3.2, prevents muscle cells from becoming cartilage suggesting that the inhibition of myogenesis by Nkx3.2 is required for Sox9 to initiate chondrogenesis of muscle cells.

Additionally, our research not only provides mechanistic detail regarding the switch from muscle to cartilage, we also examine an in vivo

model in which this type of transition is likely to take place – during fracture repair. While the contribution of other cell types such as those from the periosteum[280, 352, 372] and bone marrow[280] has been well characterized, our work is one of the first to clearly identify a role for muscle cells in endochondral bone fracture healing in vivo in a mammalian system. In open – non-union fractures in which there is severe damage to the main stem cell source, the periosteum, that other neighboring contributors must be recruited in large quantities to faciliate the repair of the critically damaged tissue. In those muscle progenitor cells that ultimately become the cartilage cells in the fracture callus of the healing bone, in a mouse model of open fracture healing, we [279] both Nkx3.2 and Sox9 are highly upregulated while muscle cell marker Pax3 is downregulated. This then suggests that the balance of these factors ultimately confers cell fate determination between muscle and cartilage.

We show that muscle progenitors can adopt a cartilage fate under chondrogenic conditions in vitro, and in vivo during open fracture repair. While these findings have broad implications, it is important to note that our studies focused on the transition of muscle progenitors to cartilage cells, and not the conclusive identification of which muscle progenitors are explicitly involved in this process. Multiple studies have shown that various stem or progenitor cell subtypes can undergo chondrogenic differentiation[239, 373, 374]. However, other studies have more fully characterized which muscle-derived cells have the capacity to form various cell types, and more specifically which are more likely to exhibit chondrogenic potential. For example, a subpopulation of mesenchymal progenitors in muscle tissue

(distinct from satellite cells) have recently been shown to have a predisposition to form fat rather than muscle[272, 273]. Clonal isolation of a subpopulation of CD34+Bcl-2+ muscle derived cells were capable of fully differentiating into both myoblasts and osteoblasts in vitro and in vivo[275]. Muscle derived stem cells enriched for the cell surface markers Stem cell antigen-1 (Sca-1) and cluster of differentiation 34 (CD34) have been described as earlier muscle progenitors that have a greater capacity to become cartilage tissue relative to other progenitors such as satellite cells[375]. In general, the literature varies on the competence for muscle-derived cells to become cartilage, and most of this variation lies with respect to the isolation of these cells, their initial myogenic capacity, as well as the characterization of cell surface markers[275, 276, 376, 377], Further research can explore whether our Nkx3.2-mediated muscle-to-cartilage transition applies to all muscle-derived cells or perhaps only a defined subset.

In summary, we define a function of Nkx3.2 in the chondrogenic differentiation of muscle satellite cells in vitro and an implication of this same function of Nkx3.2 in the chondrogenesis of muscle progenitors in an adult model of fracture healing in vivo, thereby proposing that Nkx3.2 plays a similar role of muscle cell inhibitor in both the embryo and the adult.[288, 289, 299, 300, 302, 378]

6.3.4 Mechanisms by which cells adopt different cell fates

Cell differentiation from one cell type to another can occur through a variety of processes. One mechanism is by dedifferentiation. Dedifferentiation is the process by which a cell essentially travels backward in its differentiation

pathway, giving it more plasticity thereby allowing it to proliferate and re-differentiate back into its original cell type, or alternatively be easily re-directed along a different pathway. One example of cells becoming dedifferentiated for the purpose of repopulating their own respective tissue is in the repopulation of muscle fibers by satellite cells. Myostatin is a transcription factor induced by TGFβ signaling. Myoblasts and satellite cells isolated from myostatin deficient mice proliferate and differentiate significantly faster than those from wildtype controls, suggesting that myostatin inhibits muscle cell proliferation and differentiation [379, 380]. Cells can also de-differentiate then become re-directed down alternative cell fates. For example, upon limb amputation in the newt, a blastema forms, which is a population of de-differentiated cells which ultimately re-differentiate to regenerate all the tissue types of the limb [381].

Another process by which cells adopt alternative cell fates is transdifferentiation. Transdifferentiation is the process by which one cell type becomes another, without the formation of a de-differentiated intermediate cell type. The mechanisms involved in transdifferentiation have not been fully elucidated, however it seems plausible that some of these transcription factors have the ability to cause a cell to take a step backward in its respective lineage, then allowing it to be re-directed along a different pathway. Or perhaps expression of these factors leads to some type of epigenetic regulation that allows for these cellular changes to occur[382]. Transdifferentiated cells should be assayed for expression of markers of their original phenotype in order to ensure that actual re-differentiation has taken place. A very stringent measure would be to compare expression levels and

functionality of the newly transdifferentiated cells with those of cells from native tissues. If truly differentiated, cells should exhibit most if not all qualities of the tissue being regenerated. These cells can also be tested to determine if they can be incorporated into the natural environment of that tissue. It is also important to make certain that the new cells are stable. Some reports have indicated that transdifferentiated cells often exhibit incomplete programming that can have detrimental effects. For example, fibroblasts converted into melanocyte-like cells by overexpressing Mitf expressed a number of melanocyte markers, but also retained expression of fibroblast markers.[383]. Thus, the expression of markers from the original cell may ultimately cause multiple severe problems upon transplantation.

In recent years there has been a surge to develop methods of regenerating tissues using patient specific cells. Reasons for this being that the demand for transplants incredibly outweighs the supply, and furthermore those organs and tissues transplanted into patients from outside donors are often rejected. Mesenchymal stem cells can be harvested from bone marrow and subsequently utilized for various applications including cartilage tissue engineering. Any even more recently, somatic cells such as fibroblasts have been harvested and induced to acquire the characteristic of embryonic stem (ES) cells. These cells are known as induced pluripotent stem cells, or iPS cells. Over the years, the techniques for generating iPS cells have been streamlined to achieve higher feasibility and efficiency. The most well-characterized method involves the use of four transcription factors: Klf4, Sox2, Oct4 and c-myc. Introducing these combined factors ultimately produces cells that meet the criteria of ES cells, such as the ability to form

cell types of all germ layers. Perhaps for the regeneration of certain tissues it may not require that isolated cells de-differentiate back into a multipotent state. It seems plausible that cells of closely related lineages could switch cell fates by a more convenient method. Furthermore, iPS cells that are subsequently re-differentiated into various cell lineages, often do not completely assume the identity of the new cell type, but instead revert back to an ES-like state, thereby increasing the likelihood of tumor formation. Like ES cells, iPS cells have been shown to form teratomas in nude mice.

6.3.5 Other intrinsic factors that are sufficient to elicit cell fate changes

Ours is not the first study to demonstrate the conversion of one cell type to another via the use of transcription factors. The identification of cell lineage-directing transcription factors is based on previous developmental studies that define and characterize roles for those factors in the embryo. The most promising factors being those that when deleted cause severe defects in a tissue or organ and/or the complete absence of a specific cell type. Multiple studies have illustrated a cell fate switch simply by overexpressing one of more of these types of transcription factors. For example, NIH-3T3 cells treated with microphthalmia-associated transcription factor (MITF) take on a melanocyte phenotype[383], mouse glial cells have been shown to transition into neurons when expressing paired box gene 6 (Pax6)[384], and mouse fibroblasts[385] and chondroblasts[386] have been shown to transform into myoblasts upon ectopic expression of bHLH factor MyoD. In other cases, this cell type conversion requires the usage of multiple transcription factors to elicit a response. For example, postnatal cardiac and dermal fibroblasts can be converted into cardiomyocytes by combining the

cell-intrinsic factors GATA binding protein 4 (Gata4), myocyte enhancer factor 2C, and T-box (Tbx5) [387]. This generation of specific cells via the use of several factors is also applicable to human cells. Dermal fibroblasts from humans have been utilized to generate blood cells without first going through a pluripotent intermediate state by expressing Oct4 along with various hematopoietic transcription factors in the presence of cytokines. These fibroblast-derived cells were capable of differentiating into most blood cell lineages and had the ability to engraft in vivo[388].

Interestingly, a method for inducing chondrogenesis in cells by using intrinsic factors has been developed. Fibroblasts isolated from mouse dermis have recently been shown to have the capacity to become chondrocytes via the combined expression of factors Klf4, c-Myc and Sox9 in vitro, in the absence of going through a pluripotent intermediate. However, while some induced chondrocytes were able to generate stable cartilage tissue, others formed tumors when injected into nude mice subcutaneously[389, 390]. While this method is similar to ours in the sense that they utilized transcription factors, their approach utilizes the cancer-associated gene c-myc, and would likely not be practical for clinical use.

Cartilage repair still poses a challenge clinically, due to the limited number of available autologous chondrocytes as well as their phenotypic instability when cultured in vitro. Autologous stem cells can be isolated and differentiated, however, this cartilage can those too ultimately lack structural and mechanical integrity required for healthy cartilage tissue. Our virally induced method of generating stable chondrocytes for tissue engineering from monolayer cultures could also potentially provide a key solution to these

problems as it would circumvent the need for large numbers of cells for 3D cultures as well as the need for constant exposure to extracellular chondrogenic factors that could potentially have off-target effects on nearby tissues.

6.3.6 Nkx3.2 as a mediator of cell fate determination in other cell types

Our method works because Nkx3.2 inhibits muscle cell fate thereby allowing for the induction of cartilage cell fate by Sox9. This is not the first description of this phenomenon. Nkx3.2 has also been shown to inhibit Runx2 by directly binding and inhibiting its promoter. Runx2 is a transcription factor present in both hypertrophic chondrocytes as well as osteoblasts[391]. Thus, the inhibition of Runx2 by Nkx3.2 is believed to prevent premature differentiation of hypertrophic cartilage during endochondral ossification. Because Nkx3.2 now has an established role in inhibiting both bone and now muscle cell fate, perhaps then it may be involved in the inhibition of other non-cartilage cell fates[168, 256, 392-394].

Hypoxia, or a lowered concentration of oxygen, is crucial for the proper induction and maintenance of the cartilage phenotype. Interestingly, chondrogenic Sox transcription factors have not been implicated in this process, which suggests that other pathways may be involved. Work by Kawato et. al., very recently characterized a role for Nkx3.2 in this process [394]. C3H10T1/2 mesenchymal cells were cultured in BMP2-containing chondrogenic media, under either normoxic (20% O2) of hypoxic (5% O2) conditions, and it was found that Nkx3.2 was highly induced while Runx2 was significantly reduced under hypoxia, and that the expression of Sox9 was not

significantly affected by the altered O2 environment. Furthermore, they found that knocking down endogenous Nkx3.2 expression in these cultures resulted in a decrease in glycosaminoglycan production and a concomitant increase in Runx2 expression, which suggests that the suppression of Runx2 by Nkx3.2 is a critical step in the chondrogenic differentiation of mesenchymal cells under hypoxic conditions[394].

As alluded to previously, it will be interesting to see if similar molecular “switches” exist to turn off one potent activator in favor of another expressed in the same cell. Dermal fibroblasts are known to express Runx2, however they do not ultimately become osteoblasts. This is at least in part regulated by TGFβ signaling, as dermal fibroblasts lacking Smad3 spontaneously undergo Runx2-mediated osteoblast differentiation [395]. It is likely that in these cells a similar mechanism exists in which multiple factors are concurrently expressed in a cell type – i.e. Pax3 and Sox9 in same cells, and it takes Nkx3.2 to drive the specific cell fate decision. Like with neural crest cells and presomitic mesoderm there are adult non-cartilage cells in which Sox9 is expressed, implying that some mechanism exists to prevent Sox9, a potent activator of Col2 and Agg gene promoters, from inducing chondrogenesis. Sox9 is expressed at some level in many mesenchymal stem-like progenitors including neural crest cells[396-398], melanocytes[399-402], satellite cells[300]. These cells maintain their phenotype despite this basal Sox9 expression through the concurrent expression of competing transcription factors that serve to prevent Sox9 from activating cartilage matrix genes. Neural crest cells like satellite cells also co-express Pax7[403] however, despite Sox9 being a potent activator of chondrogenic genes, these

cells do not become cartilage. Pax3 expressed in melanocytes[402] Prostate cells[404]

Conversely, it will also be interesting to study the mechanism by which Pax3 prevents the adoption of cartilage cell fate. While our results implicated a role for Pax3 in the prevention of chondrogenesis of muscle satellite cells, the molecular mechanism by which this occurs is not fully elucidated. It may be that Pax3 simply drives myogenesis, and that overexpression of Pax3 is sufficient to prevent cell re-programming. It will be interesting to examine whether other downstream muscle specific factors such as MyoD or myogenin can yield similar results.

6.3.7 Nkx3.2 in fracture healing

While ours is the first to identify a role for Nkx3.2 in the process of mammalian fracture healing, interestingly, Nkx3.2 has been implicated in the process of tail regeneration in the newt. During tail regeneration, Nkx3.2 is expressed throughout the wound, specifically in the epidermis, regenerating muscle, neural tube, spinal ganglia and the cartilage rod. This wide distribution of Nkx3.2 expression across multiple tissue types during the regrowth of the newt tail is quite interesting, especially given that the newt is one of the few vertebrates in which the adult CNS has the ability to regenerate. Further studies to elucidate the role of Nkx3.2 in this model system may reveal additional functions for this factor in this unique regenerative process that may also have implications in higher vertebrates. [405]

It will be of great interest to more thoroughly study the activity of Nkx3.2 in cartilage and bone generation and repair. Overexpression of Nkx3.2 in both chicken and mouse embryos results in ectopic cartilage formation, rib fusion and polydactyly [406], suggesting that gain-of-function of Nkx3.2 is sufficient to promote cartilage formation and even complete skeletal elements in the case of superfluous rib and metacarpal bone formation. It will be interesting to study if overexpressing Nkx3.2 in an adult in vivo model would yield similar effects, and if this mechanism could ultimately be harnessed for the purpose of enhancing fracture healing.

6.4 Concluding remarks

In summary, the relationship between muscle and cartilage is an intricate one. While this work has identified multiple novel mechanisms with regard to cartilage development, homeostasis, and regeneration, the need for further study in these areas still exists. Further understanding of these mechanisms will not only provide new insights, but it may ultimately allow for the development of more efficient strategies to enhance cartilage tissue engineering and fracture healing.

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