Osteoarthritis and Cartilage (2000) **8,** 309–334
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doi:10.1053/joca.1999.0306, available online at http://www.idealibrary.com on IDEAL®
1063–4584/00/050309+26 $35.00/0

Osteoarthritis and Cartilage
Journal of the OsteoArthritis Research Society International

Cellular interactions and signaling in cartilage development

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Summary

The long bones of the developing skeleton, such as those of the limb, arise from the process of endochondral ossification, where cartilage serves as the initial anlage element and is later replaced by bone. One of the earliest events of embryonic limb development is cellular condensation, whereby pre-cartilage mesenchymal cells aggregate as a result of specific cell–cell interactions, a requisite step in the chondrogenic pathway. In this review an extensive examination of historical and recent literature pertaining to limb development and mesenchymal condensation has been undertaken. Topics reviewed include limb initiation and axial induction, mesenchymal condensation and its regulation by various adhesion molecules, and regulation of chondrocyte differentiation and limb patterning. The complexity of limb development is exemplified by the involvement of multiple growth factors and morphogens such as Wnts, transforming growth factor-beta and fibroblast growth factors, as well as condensation events mediated by both cell-cell (neural cadherin and neural cell adhesion molecule) and cell–matrix adhesion (fibronectin, proteoglycans and collagens), as well as numerous intracellular signaling pathways transduced by integrins, mitogen activated protein kinases, protein kinase C, lipid metabolites and cyclic adenosine monophosphate. Furthermore, information pertaining to limb patterning and the functional importance of Hox genes and various other signaling molecules such as *radical fringe, engrailed,* Sox-9, and the Hedgehog family is reviewed. The exquisite three-dimensional structure of the vertebrate limb represents the culmination of these highly orchestrated and strictly regulated events. Understanding the development of cartilage should provide insights into mechanisms underlying the biology of both normal and pathologic (e.g. osteoarthritis) adult cartilage. © 2000 OsteoArthritis Research Society International

Key words: Endochondral ossification, Cartilage, Condensation, Growth factors, Cell adhesion, Signaling.

Introduction

One of the earliest overt morphogenetic events of embryonic development is cartilage formation. Chondrogenesis, the first step in endochondral ossification, ultimately gives rise to skeletal tissues, which afford structure, motility and protection to the developing vertebrate animal. Cartilage development involves a series of multifaceted and strictly regulated events, encompassing condensation of mesenchymal chondroprogenitor cells, differentiation into chondrocytes, and the patterning of chondrifying tissues into skeletal structures. For a comprehensive analysis of the formation of the three-dimensional vertebrate limb, this review summarizes the molecular events contributing to the above processes, specifically cell–cell and cell–matrix interactions, extra- and intracellular signaling pathways, and regulation of gene expression.

It is noteworthy that while the embryonic limb has served as a classic model for the study of cartilage development, the cartilage anlage is eventually replaced by invading osteoblasts to form bone. Understanding how this 'transient' cartilage is formed and more importantly, the control of its maturation program, should also shed light on the development and maintenance of the 'permanent' articular cartilage found within joints.

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Supported in part by NIH grants (ES07005, DE16864, AR44501, and T32ES07282).

Endochondral ossification

The formation of skeletal elements is a highly organized, complex process that begins with the migration of undifferentiated mesenchymal cells to areas destined to become bone. These cells undergo a 'condensation' step[1–4] that is the result of an increase in cell packing, i.e. an increase in cells/unit area or volume, without an increase in cell proliferation.[5,6] This process forms the cartilaginous anlagen of the future skeletal elements. It is at this point that the position, shape and number of the future skeletal elements are established. The undifferentiated mesenchymal cells produce an extracellular matrix (ECM) rich in collagen type I,[7–9] hyaluronan,[10] tenascin[11–14] and fibronectin.[7,15] The formation of pre-cartilaginous condensations and the differentiation of mesenchymal cells into chondrocytes marks a change in the ECM composition. The chondrocytes begin producing cartilage specific collagen type II,[16–20] collagen types IX[20–22] and XI, Gla protein,[23–25] the large chondroitin sulfate rich proteoglycan, aggrecan,[26–29] and link protein,[30] while the expression of collagen type I is turned off.[31] The chondrocytes become encased in their ECM and acquire a characteristic rounded morphology. After further differentiation and hypertrophy, the chondrocytes express collagen type X and decrease the expression of collagen type II. The cartilage is then vascularized by the invasion of blood vessels from the perichondrium. Osteoblasts are transported into the cartilage by the blood vessels and begin replacing the cartilage with mineralized bone. This process of chondrogenesis, hypertrophy and mineralization, followed by bone formation, is termed endochondral ossification.[32] The skeletal components of the axis, pelvis, and limbs form by endochondral ossification, while the

majority of the bones of the face and skull form via intramembranous ossification, the direct conversion of undifferentiated mesenchymal cells into bone.

Embryonic limb as a classic experimental model

The body plan is established in the early embryo by precise coordination of cell migration, proliferation and differentiation. The embryonic limb possesses two signaling centers, the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), which produce signals responsible for directing the proximo–distal outgrowth and anterior–posterior patterning of the limb skeletal elements, respectively. The embryonic limb has served for years as the model system for studying pattern formation and chondrogenesis, and this review will summarize the current knowledge of mesenchymal cell pre-cartilaginous condensation using the developing embryonic limb as its model system (see ref. 33 for a presentation of current techniques used in studying limb mesenchymal chondrogenesis).

Origin and characterization of chondroprogenitor cells

The cells that contribute to the skeletal elements of the embryonic limb are derived from the lateral plate mesoderm. These cells migrate into the limb field and undergo a phenomenon termed 'pre-cartilaginous condensation', first described by Fell.[1] These condensations are easily visualized, using standard light or transmission electron microscopy, and appear as closely packed mesenchymal cells in the chondrogenic regions when compared to the surrounding nonchondrogenic mesenchyme. Another method of visualizing cellular condensations is by taking advantage of a characteristic specific to condensing, pre-cartilage mesenchymal cells. These cells bind the lectin peanut (*Arachis hypogaea*) agglutinin (PNA) that recognizes the glycosyl terminal of the disaccharide Gal(β1,3)GalNAc,[39–40] thereby demarcating cellular condensations during the development of skeletal tissues.[41] Evidence suggests that PNA binds to some cell-surface component(s) of the condensing mesenchymal cells.[36,42] After chondrogenic differentiation, these cells are no longer able to bind PNA, further illustrating the utility of PNA binding as a method for identifying pre-cartilaginous cells. In addition to the blastema in the limb bud, PNA has been used to visualize sclerotomal condensations, the vertebrae precursors, in the developing somites of both chick and human embryos.[42–44]

It has been shown that nonchondrogenic tissues can be induced to produce endochondral bone,[45] suggesting that chondroprogenitor cells reside in non-chondrogenic tissues. This hypothesis was confirmed by testing the ability of fractionated embryonic calvaria[46–50] and muscle tissue[51] to undergo chondrogenesis *in vitro*.[47,51] The PNA-binding fraction formed aggregates of round cells and produced cartilage specific collagen type II and aggrecan. These results were dependent on the initial plating density, consistent with previous observations that only high density micromass cultures, a condition that favors chondrogenesis,[52–54] permit the expression of a cartilaginous phenotype by mesenchymal cells.

Pre-cartilage condensation

The appearance of pre-cartilage condensations is one of the earliest morphological events in skeletogenesis.[1,2,4] This is a transient stage of skeletogenesis that provides the scaffold for the formation of the endochondral skeletal elements. It is at this time that the shape, size, position and number of skeletal elements are established. The importance of the cellular condensation phase for normal skeletal development was underscored by the observation of Grüneberg,[55] who named the condensation phase the 'membranous skeleton' to emphasize it as a distinct and important phase of normal skeletal development. Several mutant genes have been described that alter skeletal development and act during the condensation step, such as brachypod (bp^H), phocomelia (Pa), and congenital hydrocephalus (ch) in the mouse and $talpid^3$ in the chick (see refs 41 and 56 for discussion and references).

Cellular condensations form as a result of altered mitotic activity, failure of cells to move away from a center, or as in the limb, aggregation of cells toward a center. This active cell movement causes an increase in mesenchymal cell-packing density, i.e. an increase in cells/unit area or volume, without an increase in cell proliferation,[2,4,5,31,57–60] possibly mediated by ECM-driven locomotion. Cellular condensation is associated with an increase in cell–cell contacts through cell–cell adhesion molecules and gap junctions that facilitate intercellular communication and the transfer of small molecules between cells.[61–66] Evidence supporting the importance of cellular condensation in chondrogenesis has come from both *in vivo* and *in vitro* observations. Many classic studies have not only demonstrated the high cell density requirement for chondrogenesis to occur,[54] but have also correlated the extent of cell condensation with the level of chondrogenesis,[53,67] demonstrated the initiation of gap-junction-mediated cell–cell communication in condensing mesenchyme,[65,66] and described characteristic limb skeletal abnormalities in genetic mutants defective in mesenchymal cell condensation (reviewed in refs 41 and 56).

As stated previously, the process of mesenchymal cell condensation is crucial for chondrogenesis. This process is directed by cell–cell and cell–matrix interactions as well as secreted factors interacting with their cognate receptors. Prior to condensation, mesenchymal cells present in the limb secrete an ECM rich in hyaluronan and collagen type I that prevents intimate cell–cell interaction. As condensation begins, an increase in hyaluronidase activity is observed with a decrease in hyaluronan in the ECM. Hyaluronan is thought to facilitate cell movement and the increase in hyaluronidase and subsequent decrease in hyaluronan allows for close cell–cell interactions.[68–70] The establishment of cell–cell interactions is presumably involved in triggering one or more signal transduction pathways that initiates chondrogenic differentiation. Two cell adhesion molecules implicated in this process are neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM). Both of these molecules are expressed in condensing mesenchyme, then disappear in differentiating cartilage,[71,72] and later are detectable only in the perichondrium. Perturbing the function(s) of N-cadherin[73] or N-CAM[74] causes reduction or alterations in chondrogenesis both *in vitro* and *in vivo*, further supporting a role for these cell adhesion molecules in mediating the mesenchymal condensation step.

In addition to cell–cell interactions, cell–matrix interactions also appear to play an important role in mesenchymal cell condensation. One ECM component implicated in this process is fibronectin. Fibronectin expression is increased in areas of cellular condensation[7,15] and

decreases as cytodifferentiation proceeds. Fibronectin may facilitate a matrix-driven translocation of mesenchymal cells into cellular condensations and this process may be mediated by the amino terminal heparin binding domain.[75,76] Recent studies in our laboratory have demonstrated that fibronectin mRNA undergoes alternative splicing during chondrogenesis.[77–79] The isoform containing exon EIIIA is present during condensation but disappears once differentiation begins, suggesting that this isoform switching is important for cytodifferentiation to occur. Antibodies specific for the region encoded by exon EIIIA of the fibronectin gene inhibited chondrogenesis of limb micromass cultures *in vitro*, and when injected into chick limb buds *in vivo*, caused moderate to severe skeletal malformations.[77,78]

Limb patterning

INFLUENCES FROM THE EPITHELIUM

The development of the vertebrate limb as a functional three-dimensional structure is a complex process dependent on the interaction of various proteins, including growth factors such as fibroblast growth factors (FGFs)[80–84] and transforming growth factors (TGFs),[85–91] morphogens such as the Wnt family of secreted glycoproteins,[92–94] and transcription factors such as engrailed (En),[95] sonic hedgehog (Shh),[96,97] radical fringe (r-Frg),[98,99] and those encoded by the homeobox (Hox) gene family, such as *msx-1* and *msx-2*.[100–102] The limb develops along three axes: proximodistal (shoulder/finger) defined by the FGFs,[80–84] dorsoventral (knuckles/palm) defined by the interaction of Wnt-7A and the En-1 transcription factor domains,[94,103] and the anterioposterior (pinky/thumb) axis which is largely dependent on Shh and the Hox genes.[100–102,104–112] Although the above mentioned factors may indeed act specifically to initiate or maintain the indicated axes of the developing limbs, these axes are unlikely to exist as true independent domains, as loss of the above factors in turn will affect all limb axes. The limb can truly be considered an integrated structure, whose development consists of the cooperative integration of three axes, extensive cross-talk between numerous signal transduction pathways and reciprocal induction between ectoderm and underlying mesoderm.

LIMB INITIATION

Early investigation into growth factors responsible for limb initiation uncovered the presence of FGF-8 in lateral plate mesoderm,[80,83] and beads soaked in FGF implanted at ectopic sites in the flank of the developing chick embryo have been shown to cause the growth of an additional limb.[80,83,113] Most recently it was shown that expression of *fgf-10* presages that of *fgf-8* in intermediate mesoderm, and that ablation of *fgf-8* expression in the mesonephros at wing level still allows limb growth.[114] This recent evidence indicates that *fgf-10* may be involved in early limb induction, possibly through interaction with *fgf-8*.[84]

Though early limb growth is initiated by mesoderm,[115,116] the overlying limb ectoderm subsequently begins to play an important role in maintenance of the developing limb bud.[117] At the distal tip of the growing limb structure, the ectoderm located at the junction of the future dorsoventral boundary begins to undergo a thickening to form an area of specialized epithelium known as the AER. The AER is both induced and maintained by members of the FGF family,[80,83,113] and positioned by expression of r-Frg in the dorsal ectoderm[98,99] (Fig. 1). As the limb grows further outward, cells directly underneath the AER in a region termed the progress zone (PZ) proliferate rapidly and maintain characteristics of undifferentiated mesenchyme, while the more proximal mesenchymal cells begin to condense and differentiate into limb structures, specifically the cartilage anlage which will later be replaced by invading osteoblasts.[41]

PROXIMODISTAL AXIS

After the initial induction of the AER by the underlying mesoderm,[115,116] further limb bud development becomes dependent on signals emanating from the AER.[118] Ectopic implantation of AER to initiated limb bud results in production of a second proximodistal axis,[119] while surgical removal of the AER halts limb outgrowth and results in a truncated bud.[119–121] However, implantation of beads soaked in FGFs can substitute for the AER and maintain proximodistal growth[122,123] indicating that FGF-2, -4 and -8 secreted from the AER may be responsible for maintaining the PZ and subsequently limb outgrowth.[80,83,122,124–126] Both the AER and underlying mesoderm express members of the bone morphogenetic protein (BMP) family, *bmp-2, -4, -7*, and may function in AER or PZ maintenance, or FGF induction.[91] Until recently, the expression of *fgf-8* was considered to be the initial AER marker.[80,83] However, new work indicates that *wnt-3A* expression presages *fgf-8* expression in the AER, and inappropriate expression of the *wnt-3A* gene can in fact induce AER-specific genes such as *fgf-4, -8* and *bmp-2*.[127]

Patterning of structures comprising the proximodistal axis of the limb are possibly determined by time spent in the PZ (i.e. mesenchyme under the influence of the AER for longer will form more distal structures),[119] or the regulation of morphogens by the AER as the PZ passes areas, thereby 'fixing' particular proximodistal structures.[128] As cells leave the PZ they are left in a positional fate along all three axes.

ANTERIOPOSTERIOR AXIS

The anterioposterior axis (pinky–thumb) is the best developmentally understood of the three limb axes and is mediated by a small region of cells in the posterior mesenchyme of the limb bud known as the ZPA.[104,120,128,129] Surgical removal and reimplantation of the ZPA at more anterior sites causes digit duplication.[106–108] One candidate molecule that emerged as the putative polarizing region morphogen is retinoic acid, which when introduced into an anterior site can mimic the ZPA in anterior mesenchyme[130] and cause digit duplication.[131,132] However, it has been shown that both retinoic acid from the mesoderm[106] and FGF-4 from the AER exert their anterioposterior effects via activation of Shh in the ZPA[133] (Fig. 1). Shh in turn regulates *bmp-2, -4, -7* and possibly the expression of *gli-1, -3* (zinc-finger transcription factors) in a gradient through the limb mesoderm.[134–139]

It is thought that this regulation of *bmp*s gives rise to anterioposterior structures possibly through *hox* gene regulation.[106,140] The *hox* genes each contain a highly conserved 60 amino acid motif called the homeobox, a helix-turn-helix DNA-binding domain. *Hox* genes are organized into four clusters in an order colinear with the anterioposterior axis of the developing limb such that the 3′ most

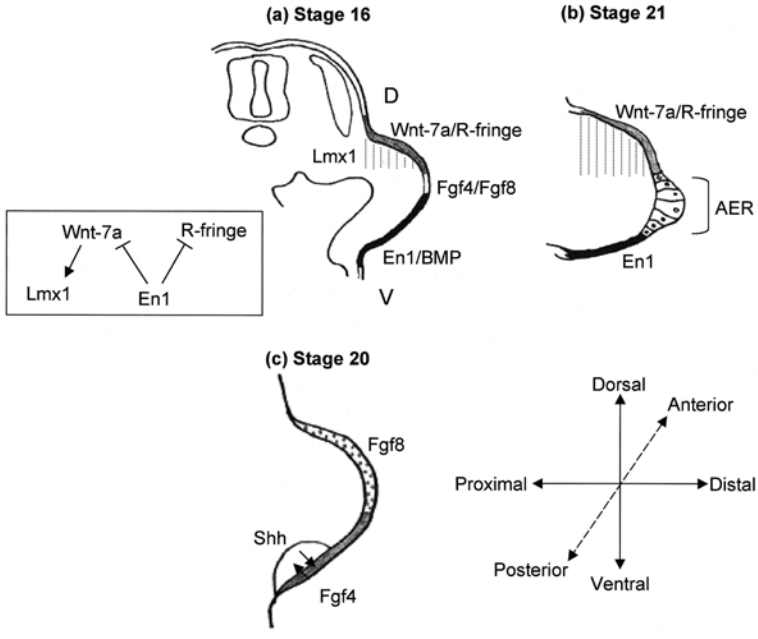

Fig. 1. Expression profiles of patterning genes during chick embryonic limb development. (a) Stage 16, pre-limb bud. *Wnt-7a* and *radical fringe* (*R-fringe*) are expressed in the dorsal ectoderm and *engrailed1* (*En1*) and *BMPs* are expressed in the ventral ectoderm of the pre-limb bud. *En1* restricts expression of *Wnt-7a* and *R-fringe* to the dorsal ectoderm. *Wnt-7a* induces the expression of *Lmx1* and therefore, *Lmx1* is restricted to the dorsal mesenchyme (see schematic in box). Members of the fibroblast growth factor (FGF) family are expressed throughout both dorsal and ventral ectoderm. (b) Stage 21, early limb bud. The apical ectodermal ridge (AER) is formed at the junction of cells that are either expressing or not expressing *R-fringe*. (c) Stage 20. *Fgf4* is expressed in the posterior ridge ectoderm and *Fgf8* is expressed throughout the entire ridge ectoderm. The arrows indicate a positive feedback loop between *Fgf4* and *sonic hedgehog* (*Shh*) in the posterior mesenchyme. Each is required for the maintenance of expression of the other. *Shh* has been demonstrated as the principal protein responsible for establishing proper anterior–posterior patterning of the limb. Figure adapted from refs 153 and 154.

genes are expressed in the most anterior areas (for a review of Hox gene structure and function see ref. 141). *Hox* genes are expressed throughout both the AER and the PZ.[101,110,142–146] and appear to respond to soluble signals emanating from the ZPA and the AER such as Shh,[97,112] and FGFs,[81,82] and Indian hedgehog (Ihh), a member of the hedgehog family and a regulator of cartilage differentiation[147] (see below). The *hox* genes are thought also to be responsible for encoding individual portions of the proximodistal axis.[141,148,149]

DORSOVENTRAL AXIS

Concerning limb axial development, the least understood and last to be examined of the three axes has been the dorsoventral axis. As with AER formation, ectodermal signals have been implicated in the dorsoventral patterning of the limb. There are four genes known to be differentially expressed along the dorsoventral axis: *en-1, r-Fng, wnt-7a, lmx-1* (a homeodomain protein expressed in response to *wnt-7a*)[94,95,133,150–152] (Fig. 1). Both *lmx-1* and *wnt-7a* loss of function mutations transform dorsal limb structures to ventral fate,[94] and conversely loss of function En-1 mutants cause dorsalization similar to *wnt-7a/lmx-1* overexpression.[103,151,152] A model has been proposed postulating that En-1 functions in the ventral ectoderm to inhibit expression of Wnt-7a and therefore Lmx-1, giving rise to ventral structures.[98,99,103,153,154]

Chondrocyte differentiation and maturation

The chondrocytes in the center of the cartilaginous templates are stimulated to proliferate and then proceed through stages of maturation and hypertrophy. In the region of hypertrophy the chondrocytes are replaced by invading osteoblasts and the tissue is replaced by bone and bone marrow. This process radiates outward with the formation of the growth plates at the distal ends of the long bones that separate the epiphysis from the diaphysis. Two signaling molecules, *Ihh* and parathyroid hormone-related peptide (PTHrP) have been identified as regulators of the process of chondrocyte maturation and hypertrophy.

Ihh is a member of the *hedgehog* family of structurally conserved, secreted proteins that are known to provide key signals in embryonic patterning in many organisms. In *Drosophila* development, the segment polarity gene *hedgehog* (*hh*) regulates embryonic segmentation and anterior–posterior patterning of the imaginal disks.[155–157] In higher vertebrates, there are at least three hedgehog genes: *Shh*, *desert hedgehog (Dhh)* and *Ihh*. The products of the *hedgehog* genes undergo autoproteolysis generating amino- and carboxy-terminal domains, with the former likely to be responsible for the short- and long-range signaling activities of the *hedgehog* proteins.[158,159] The different hedgehog proteins act through the same signal transduction pathway. In the *Drosophila* hedgehog signal transduction pathway, there are two key genes involved: *patched* (*ptc*), which encodes a transmembrane protein, and *cubitus interruptus* (*ci*), which encodes a transcription factor. The vertebrate homologues of these genes are *Patched* (*Ptc*) and *Gli*, and as in *Drosophila*, both are required for cellular response to hedgehog signaling.[160]

The expression of *Ihh* is detected in the endoderm of the developing midgut and lung and in the cartilage of the developing long bones.[147] In the developing long bones, *Ihh* is expressed in the transitional region from proliferating

to hypertrophic chondrocytes. However, both *Ptc* and *Gli* are expressed in the perichondrium, suggesting that the effect of *Ihh* is mediated by the perichondrium. Overexpression of *Ihh* in the forming cartilage of chick embryonic wings resulted in broader and shorter cartilage elements that lacked hypertrophic chondrocytes.[147] PTHrP was found to be upregulated following *Ihh* misexpression in the periarticular regions.

PTHrP is a paracrine factor that is synthesized in multiple tissues. It is structurally homologous to parathyroid hormone (PTH) and both bind the same receptor (PTH/PTHrP receptor). Much of what is known about PTHrP in chondrogenesis comes from mouse knockout studies. Mouse embryos deficient in PTHrP develop relatively normally despite the wide tissue expression of this gene. The detected morphological abnormalities are restricted to the skeletal system. The PTHrP null mice die shortly after birth and are characterized by a domed head, short snout and mandible, protruding tongue, disproportionately short extremities, and a narrow thoracic cage.[161] These abnormalities are the result of advanced differentiation and mineralization of chondrocytes resulting in premature endochondral ossification.[161,162] The result of the PTHrP knockout clearly demonstrates a role for this gene in the differentiation and maturation of chondrocytes into hypertrophic chondrocytes.

The PTH/PTHrP receptor is a member of a new G protein-coupled family[163,164] and acts through the cAMP/protein kinase A (PKA) and the phospholipase C/protein kinase C (PKC) signaling pathways when activated by its ligands.[165,166] The PTH/PTHrP receptor is expressed in many tissues including pre-hypertrophic and hypertrophic chondrocytes of endochondral bones.[162,164,167] This opens the possibility that the PTH/PTHrP receptor is the mediator of the PTHrP signal in the feedback loop that regulates chondrocyte differentiation. Homozygous null mice for the PTH/PTHrP receptor demonstrated a phenotype similar to the PTHrP knockout in that they also exhibited a domed head, short snout and mandible, protruding tongue and disproportionately short limbs.[168] Alizarin red S staining of these embryos revealed inappropriately accelerated mineralization in bones formed by endochondral ossification. In addition, the mouse embryonic cell line ATDC5, which can be induced by insulin *in vitro* to undergo endochondral ossification, exhibits a cytodifferentiation-dependent expression of the PTH/PTHrP receptor. When induced by insulin, confluent cultures of ATDC5 cells undergo cellular condensation with subsequent differentiation into cartilaginous nodules. Expression of PTH/PTHrP receptor mRNA is detectable in these cells at the early stages of chondrogenesis, prior to the induction of aggrecan gene expression, and was not seen in the undifferentiated cells. Levels of PTH/PTHrP receptor mRNA drastically increased concomitantly with that of collagen type II, a marker of differentiated chondrocytes, suggesting that the expression of the PTH/PTHrP receptor is associated with the onset of chondrogenesis.[169]

The phenotype of the *Ihh* knockout mice is directly opposite to that of the PTHrP and PTH/PTHrP receptor knockout mice,[168] suggesting that these genes are involved in a negative feedback loop that regulates the differentiation and maturation of chondrocytes. The following mechanistic model has been proposed for PTHrP and *Ihh* in cartilage development: as proliferating chondrocytes enter into hypertrophy, they express high levels of the PTH/PTHrP receptor and begin to express *Ihh* until they become fully hypertrophic. The *Ihh* signal acts on the Ptc/Gli expressing cells in the perichondrium adjacent to the pre-hypertrophic zone inducing the expression of PTHrP. PTHrP then signals back to the PTH/PTHrP receptor expressing chondrocytes and ultimately prevents undifferentiated chondrocytes from proceeding into the hypertrophic pathway.[147]

Cell adhesion in mesenchymal cell condensation

Cell adhesion is mediated by two major groups of cell–cell adhesion molecules, Ca^{2+}-independent and Ca^{2+}-dependent.[170–178] The Ca^{2+}-independent group is composed of the large immunoglobulin supergene family of membrane glycoproteins known as cell adhesion molecules (CAMs), and the Ca^{2+}-dependent group consists largely of a class of transmembrane glycoproteins called cadherins. Two adhesion molecules, N-cadherin and N-CAM, have been shown to have an important role during the pre-cartilaginous condensation phase in endochondral ossification.[73,74]

N-CADHERIN

The cadherin superfamily has many members and can be divided into six gene subfamilies based on structural homology: classical cadherins type I (e.g. E-, N-, P-, R-cadherin), classical cadherins type II (cadherin-6 to -12), cadherins found in desmosomes (desmocollins, desmogleins), cadherins with a very short cytoplasmic domain or none (LI-, T-cadherin), protocadherins, and the more distantly related gene products including the Drosophila *fat* tumor suppressor gene, the *dachsous* gene and the *ret*-proto-oncogene.[179]

The classical cadherins are a group of Ca^{2+}-dependent, single transmembrane glycoproteins which mediate cell–cell adhesion by homotypic protein–protein interactions through their extracellular domain. Classical cadherins are synthesized as precursor polypeptides and are then processed into their mature form. The extracellular domain of the mature protein consists of five tandem repeat domains termed cadherin-repeats, each of which consists of approximately 110 amino acids. The cadherin-repeats form four Ca^{2+}-binding domains and the N-terminal repeat confers the cadherin specific adhesive property of the molecule. Classical cadherins have a single transmembrane domain followed by a highly conserved cytoplasmic domain responsible for binding to the actin cytoskeleton via the catenin molecules[180] (Fig. 2).

The cadherin family of molecules exhibit spatiotemporally unique patterns of gene expression[177] and demonstrate homotypic binding through their extracellular domain, suggesting that cadherins may function as morphoregulatory molecules during development. N-cadherin, named for its initial identification in neural tissues, was one of the first identified cadherins and its functional involvement in cell–cell adhesion and development have been extensively studied.[181–188] N-cadherin plays a major role in neural development but has also been shown to be expressed in other mesodermal tissues, including developing limb mesenchyme.

N-cadherin is expressed in the developing embryonic limb bud in a manner suggestive of a role in cellular condensation.[73] Immunohistochemical localization of N-cadherin in the embryonic chick limb reveals a sparsely

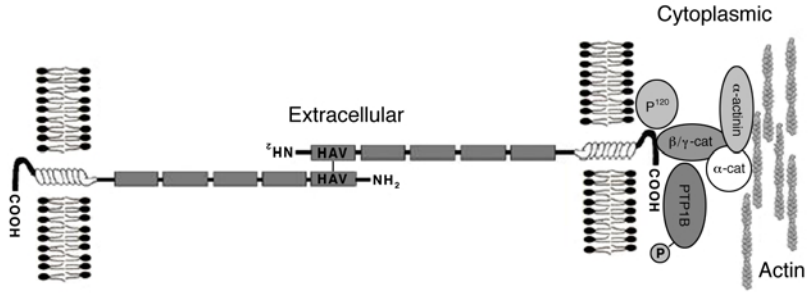

Fig. 2. Schematic of N-cadherin–catenin complex. N-cadherin is a Ca^{2+} dependent, single pass transmembrane protein which mediates cell–cell adhesion by homotypic protein-protein interactions through its extracellular domain. The extracellular domain is composed of five tandem repeats, termed cadherin repeats, which form four Ca^{2+} binding sites. The fifth cadherin repeat confers its homotypic specificity by the HAV (histidine–alanine–valine) amino acid sequence. The cytoplasmic domain binds the actin cytoskeleton via interactions with the catenin family of proteins. The cytoplasmic domain binds β/γ-catenin (β/γ-cat) directly which in turn binds α-catenin (α-cat). Subsequently, α-catenin binds the actin cytoskeleton directly or in conjunction with α-actinin. Other proteins also bind the cytoplasmic domain of N-cadherin, such as $p120^{ctn}$ and the nonreceptor protein tyrosine phosphatase 1B (PTP1B). Both of these proteins bind the cytoplasmic domain and regulate cell adhesion.[243,244,265,266]

scattered expression pattern in the central core mesenchyme during the pre-cartilage stage (Hamburger–Hamilton stages 17/18 to 22/23).[189] Expression dramatically increases in the condensing central core at stage 24/25, and by stage 25/26 the condensed central core region begins to lose N-cadherin expression while cells along the periphery of the limb bud begin to express N-cadherin. By stage 29/30, the mature cartilage is completely devoid of N-cadherin while the condensing, perichondral cells surrounding the forming cartilage still exhibit high levels of N-cadherin. As the limb bud continues to develop, the cartilaginous core region continues to grow appositionally and it is likely that the N-cadherin positive cells along the periphery contribute to this growth.[73]

In micromass cultures *in vitro*, dissociated limb mesenchymal cells aggregate to form cellular condensations which ultimately differentiate into cartilaginous nodules, separated by fibroblasts and myocytes.[54] N-cadherin protein is synthesized by the aggregating (condensing) mesenchyme by 12 h after initiation of the culture, whereas the cells outside the condensation centers display no evident N-cadherin expression. Expression of N-cadherin becomes more intense as a function of time, with maximal expression at 18 h. As the cells in the center of the condensations differentiate they lose their N-cadherin protein and the cells along the immediate periphery of the forming nodules maintain N-cadherin expression. Thus, the expression pattern of N-cadherin *in vitro* recapitulates that in the developing limb *in situ*.[73]

N-cadherin expression is localized to the prechondroblastic cells of the limb bud and maximal expression is seen during mesenchymal cell condensation, after which it is down-regulated, suggesting that cellular condensation is dependent on N-cadherin-mediated cell–cell interactions.[71,73] Evidence to support this theory comes from studies designed to perturb N-cadherin function. Oberlender and Tuan[71] were able to demonstrate a significant inhibition of cellular condensation and chondrogenesis *in vitro* and *in vivo* using a function-blocking monoclonal antibody, NCD-2, directed against N-cadherin.[182] These findings correlate well with previous findings that exogenous Ca^{2+} significantly stimulates chondrogenesis *in vitro* when added prior to condensation but has little effect when added after.[53,67]

In similar studies, addition of the transforming growth factor-β family member, BMP-2, to chick limb bud or the C3H10T1/2 murine multipotential cell line plated at high density micromass cultures stimulated chondrogenesis on the basis of Alcian blue staining, collagen type II and link protein expression, and an increase in (^{35}S) sulfate incorporation.[85,190–192] Further investigation revealed that BMP-2 treatment of C3H10T1/2 cells stimulated N-cadherin mRNA levels four-fold within 24 h and protein levels eight-fold by day 5 in culture, while an N-cadherin peptidomimic containing the His–Ala–Val sequence was able to inhibit chondrogenesis in a dose-dependent manner.[193] To specifically examine the influence of altered N-cadherin expression or activity on chondrogenesis, C3H10T1/2 cells were stably transfected with N-cadherin wild type or dominant negative N-terminal deletion constructs. Cells expressing the wild type N-cadherin at a moderate level (two-fold) increased chondrogenesis, whereas cells expressing a four-fold increase in N-cadherin or the dominant negative construct had an initial, inhibitory effect on BMP-2 stimulation of chondrogenesis.[193] These data strongly support a functional and activity-dependent role for N-cadherin in cellular condensation and chondrogenesis.

N-CAM

The glycoprotein N-CAM is a member of the immunoglobulin superfamily.[194] N-CAM is composed of five immunoglobulin-like domains, each consisting of about 100 amino acids folded into beta sheets usually linked by a disulfide bond.[195] There is only one N-CAM gene; however, different forms of N-CAM can be generated through alternative splicing of its mRNA as well as varying degrees of glycosylation (sialic acid).[196–198] The major mRNA splicing differences occur near the carboxy-terminal with some forms displaying altered cytoplasmic domains or missing the transmembrane domain. Homotypic binding of N-CAM occurs near the amino terminal,[199,200] and does not appear to be affected by alternative splicing.

N-CAM expression in the developing limb follows that of the previously described N-cadherin, however, the expression of N-cadherin mRNA occurs earlier than that of N-CAM mRNA.[72] N-CAM expression *in vivo* is observed in all limb bud cells by stage 22.[14] N-CAM expression increases and is enriched in the condensing mesenchyme at stage 27. By stage 30, the cells in the center of the condensations

differentiate and N-CAM expression is lost in mature cartilage, although strong N-CAM expression is maintained in the surrounding perichondrium.[14,74,201] The *in vitro* N-CAM expression pattern parallels that of the *in vivo* expression. In micromass cultures *in vitro*, N-CAM is expressed after 1.5 days in the aggregating, pre-cartilage condensations, with a zone of moderately N-CAM expressing cells surrounding the condensations. By 4 days in culture, the condensations have differentiated into cartilaginous nodules and lose the expression of N-CAM at their center but retain N-CAM expression at their periphery.[74]

The functional role of N-CAM in cellular condensation and chondrogenesis was determined by perturbation studies *in vitro* using aggregation assays and micromass cultures. Aggregation of dissociated stage 23 chick limb bud cells was reduced when incubated with anti-N-CAM antibodies in suspension culture and compared to cells incubated with non-immune Fab fragments.[74] In the presence of anti-N-CAM antibodies, both the number and size of aggregates is reduced by 50–60%. Micromass cultures of chick limb bud mesenchyme demonstrated a reduction in both the area occupied by condensations and the degree of cartilage differentiation when incubated with anti-N-CAM antibodies compared to the control cultures incubated with nonimmune Fab or anti-fibroblast Fab. The effect observed was dose-dependent.[14,74] Overexpression of N-CAM in micromass cultures results in enhanced aggregation of mesenchymal cells, forming large cell aggregates which differentiated into cartilaginous nodules and were collagen type II-positive.[74]

A naturally occurring genetic mutation, Talpid^2, is an autosomal recessive disorder which manifests multiple skeletal disorders including poly- and syndactyly.[202] The limb buds exhibit abnormally large pre-cartilage condensations and the mesenchymal cells were shown to have greater adhesiveness than control cells.[203] Micromass cultures of Talpid^2 limb bud cells revealed fused pre-cartilage condensations and a much greater amount of chondrogenesis compared to cultures from normal embryos. In addition, anti-N-CAM antibodies reduced the size of cellular condensations and the degree of chondrogenesis in the Talpid^2 cultures, suggesting that the increased condensations and chondrogenesis observed in the Talpid^2 chicks are at least partially mediated by an increase in N-CAM expression.[14]

Cell matrix interactions during mesenchymal condensation

During condensation, communication between neighboring cells via adhesion and ECM molecules is critical in order to establish both temporal and spatial regulation of chondrogenesis in the developing limb. The ECM not only functions in adhesive roles during condensation, but also serves as regulators of growth factors and morphogens, through presentation of molecules to their cognate receptors. As an example, presentation of TGFβ to its type II receptor via the ECM proteins betaglycan and endoglien.[204,205] Furthermore, ECM molecules function in the transmission of signals from the surrounding environment of the cell to the cytoskeleton, cytoplasm and nucleus through intricate signal transduction pathways, or by propagating inside out signals from within the cells to the pericellular environment. The ECM is thus a versatile regulator of chondrogenesis through various functions during mesenchymal condensation and differentiation.

ECM MOLECULES REGULATED DURING CHONDROGENESIS

In vivo, pre-cartilage condensations coincide with transient up-regulation of several ECM proteins including collagen type I, fibronectin and various proteoglycans.[7,15,206,207] During this time there is also controlled enzymatic hydrolysis of hyaluronan, resulting in decreased extracellular and intercellular space which allows more intimate cell–cell contact during condensation.[10,70] As mesenchymal cells begin to condense and differentiate into rounded chondrocytes, ECM production switches rapidly from collagen type I to collagen type IIa (an alternatively spliced form of collagen type II), and there is an increase in the production of sulfated proteoglycans such as tenascin and the chondroitin-sulfate rich aggrecan.[31,206,208] These molecules will constitute the majority of the environment surrounding mature chondrocytes in cartilage elements.[52]

Transition from collagen type I to collagen type IIa is also paralleled by a switch from α1 to α3 integrin expression patterns that are indicative of the onset of chondrogenesis and may prompt cellular differentiation.[209] It has been shown recently that the aggrecan-rich chondrocyte pericellular matrix is anchored to the cell surface via hyaluronan receptors (CD44) which function to direct the assembly of the chondrocyte pericellular matrix.[210] Another ECM component, fibronectin, appears to promote cell attachment and spreading over substratum,[211] an event necessary for mesenchymal condensation, but may be inhibitive for chondrocyte function itself.[212,213] In older high-density micromass cultures, fibronectin is lost from the cell surface of differentiating chondrocytes.[214,215] Interestingly, tenascin, which is up-regulated during differentiation, is thought to inhibit chondrocyte attachment to fibronectin.[11,216]

Fibronectin is a dimeric glycoprotein present in plasma and in the ECM of many tissues, and plays an important role in cell–matrix and matrix–matrix interactions, cell migration, differentiation and the maintenance of cell morphology. The fibronectin monomer consists of a linear arrangement of amino acid repeats known as type I (~45 AA), type II (~60 AA) and type III (~90 AA) repeats to yield a polypeptide chain of approximately 250 kDa. Fibronectin is usually found as a dimer joined in the C-terminal end by a pair of disulfide bonds and binds many cell types through its integrin and nonintegrin receptors. Fibronectin is encoded by a single gene but undergoes alternative splicing of its mRNA to give rise to many isoforms. Regions EIIIA or EIIIB are spliced to be either totally included or excluded; however, the V region can be spliced in several regions.[217–220]

Expression of fibronectin in the chick limb bud has been shown to be distributed throughout the intercellular space of mesenchymal cells prior to condensation, accumulates in condensations, and reaches its maximal level of expression just prior to overt chondrogenesis.[7,15,221–223] As cartilage matrix accumulates, fibronectin seems to diminish, but digestion of the limb with hyaluronidase reveals the presence of fibronectin throughout the cartilage matrix.[221,223] Therefore, fibronectin is present throughout chondrogenesis and remains in differentiated cartilage. Most interestingly, the exact structure of fibronectin (i.e. the isoform expressed) changes during the process of chondrogenesis. Mesenchymal fibronectin contains both the

EIIIA and EIIIB exons (B^+A^+), cartilage fibronectin has the EIIIA exon spliced out (B^+A^-), and plasma fibronectin has both the EIIIA and EIIIB exons spliced out (B^-A^-).[77–79,224]

Previous work in the laboratory using exon-specific antibodies has shown that antibodies directed against exon IIIA, an affinity-purified monoclonal antibody specific for the region encoded by exon IIIA of the fibronectin gene, decreases the number of chondrogenic nodules formed in micromass cultures of chick limb mesenchyme.[78] The effect seen was dose-dependent and time-dependent. Specifically, the addition of exon IIIA antibody to cultures at earlier times, after plating, decreases the area of the cultures that forms nodules. Application of the antibody after cellular condensation, i.e. 48 h after plating, has no effect on nodule formation. Injection of exon IIIA antibody into stage 23/24 chick limb primordia *in vivo* disrupted normal limb chondrogenesis resulting in moderate to severe skeletal malformations.[78] The spatial and temporal expression pattern of fibronectin and its isoform switching suggest that exon IIIA may play a role in cellular condensation, and that the cartilage isoform of fibronectin (B^+A^-) may have some role in the maintenance of mature cartilage. It has been suggested that these alternative forms of fibronectin play a functional role in the conversion of mesenchyme to chondroblasts,[225] possibly through internal signaling through the $\alpha5\beta1$ integrin fibronectin receptor and its associated integrin linked kinase (ILK) (see below).

SIGNAL TRANSDUCTION FROM THE ECM

There is extensive cross-talk between signaling pathways activated by growth factor receptors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin receptor and integrins, particularly at the level of Ras, phosphoinositol-3 kinase (PI-3K) and focal adhesion kinase (FAK) (for reviews see refs 226–228). Upon binding fibronectin, the $\beta1$ subunits of integrins have the ability to promote the assembly of focal adhesions and activate FAK through autotyrosine phosphorylation. Activated FAK may then interact with the src homology (SH2) domains of Src or Fyn,[229] activate PI-3K[230] and eventually culminate in the regulation of mitogen-activated protein kinases (MAPK), extracellular signal related kinase (ERK), and Jun N-terminal kinase (JNK).[231,232]

Though the above pathways undoubtedly exert multiple effects on condensation and chondrogenesis through ECM induced gene regulation, a more direct pathway between integrin signaling is possible through regulation of the ILK. In contrast to FAK, which is active in response to cell adhesion, ILK serine/threonine kinase appears to be inhibited in response to fibronectin attachment to the integrin receptor.[233] It has been shown that overexpression of ILK stimulates fibronectin matrix assembly in epithelial cells and reduces cellular E-cadherin levels.[234] Furthermore, in more recent work, overexpression of ILK in mammary epithelial cells resulted in down-regulation of epithelial cadherin (E-cadherin) and translocation of β-catenin, a cadherin-associated and Wnt signal transducing protein, to the nucleus. In the nucleus β-catenin forms an active transcription complex with the lymphoid enhancing factor (LEF-1), a context-dependent Wnt inducible transcription factor. This overexpression of ILK also induced upregulation of LEF-1 protein levels and caused rapid cell detachment from the ECM.[235] Finally, it has been shown that ILK can inhibit the activity of glycogen synthase kinase-3β (GSK-3β), another key mediator of the Wnt signaling pathway, through a PI–3K dependent mechanism.[236] Since Wnts are postulated to be involved in chondrogenesis in the developing limb because of their temporal and spatial expression patterns,[93,237] and their ability to inhibit chondrogenesis *in vitro*,[238] the activity of ILK via fibronectin matrix assembly is a plausible link between secreted morphogens and the ECM.

Tyrosine phosphorylation of adherens junctions has been shown to regulate adhesiveness through cadherins and their associated catenins.[239–242] Furthermore, ECM proteins have recently been identified as regulators of phosphorylation via phosphatases. Specifically, membrane-associated intracellular tyrosine phosphatase, protein tyrosine phosphatase-1B (PTP1B) like phosphatase, which is thought to regulate phosphorylation of the N-cadherin associated β-catenin,[243,244] has been shown to be regulated by interaction with the proteoglycan neurocan, a member of the aggrecan family of chondroitin-sulfate-rich proteoglycans.[245,246] Upon interaction of neurocan with its cell surface ligand, N-acetylgalactosaminylphosphotransferase (GalNAcPTase), PTP1B activity is lost, resulting in increased tyrosine phosphorylation of junctions and decreased adhesiveness,[247] an event which possibly plays a role in chondrogenesis, particularly following condensation.

GAGS AND WNT REGULATION

The cartilage matrix consists of a large amount of highly charged glycosaminoglycans (GAG) such as heparan-sulfate, keratan-sulfate, chondroitin-sulfate and dermatan-sulfate in the form of high molecular weight proteoglycans, including hyaluronan. These function to retain water, thereby maintaining distance between cells, and serving as shock absorbers. Interestingly, proteoglycans and GAGs also act as molecular tethers of soluble factors involved in both condensation and differentiation of mesenchymal chondroprogenitor cells. Recently it was determined that the heparan-sulfate cell-surface proteoglycan Dally, originally identified as a regulator of decapentaplegic (Dpp) signaling, may act as a co-receptor for Wg and potentiate its signal during development.[248,249] Dally would then be a plausible candidate for coordinating input from BMPs and Wnts during chondrogenesis. It has also been shown that disruption of the ionic environment surrounding chondrogenic mesenchymal cells with the cationic cross-linker, poly-L-lysine (PL), stimulates chondrogenesis,[53,250] as does addition of heparan-sulfate, heparin, dermatan-sulfate and dextran-sulfate to chick limb mesenchymal micromass.[53]

PL is thought to exert its stimulatory effects by altering GAG biosynthesis and distribution, cross-linking proteoglycans and cells, altering cell shape, or by affecting the activity of agents which regulate chondrogenesis.[250] Interestingly, we have recently shown that addition of PL to embryonic chick limb micromass not only stimulates chondrogenesis but also seems to do so via regulation of N-cadherin expression.[250] Hence, there is a plausible link between Wnt signaling via GAGs and N-cadherin modulation during mesenchymal chondrogenesis. Although PL does not exist as an *in vivo* molecule, the chondro-enhancing effects seen on chick mesenchymal micromass strongly suggests that there may be biological counterparts to PL, such as highly charged and localized domains of ECM molecules. In fact, the matrix of cartilage contains a large number of cationic proteins, and similarly, numerous

cationic proteins and polypeptides are able to bind to the ECM of cartilage cells.[251] A specific example is fibronectin which contains within its N-terminus repeated, lysine-rich heparin-binding domains[252] and is an attractive candidate for an endogenous PL-like molecule. In fact, it has been shown that the N-terminal domain of fibronectin acts to enhance limb mesenchymal chondensations in a matrix translocation mechanism[75] consistent with the action of poly-L-Lysine in promoting chondrogenesis.[53,250]

As mentioned earlier, the Wnt (*Drosophila* Wg) gene family is thought to be involved in chondrogenesis in the developing limb in view of its temporal and spatial expression profiles,[93,237] and the ability of some Wnts to inhibit chondrogenesis *in vitro*.[238] Recently, it has been shown that the secreted Wg protein interacts noncovalently with the cell surface and can bind heparan sulfate directly, conferring possible ECM regulation on Wg activity.[253–255] Since Wg/Wnt proteins are conserved, it is likely that proteoglycans and PL may regulate Wnt activity during chondrogenesis when sulfated proteoglycans increase dramatically in the extracellular matrix. As further proof of a relationship between heparin-like GAGs and Wnt signaling, it has been shown recently that the *Drosophila* mutants *kiwi* and *sugarless*, which have mutations in the UDP-glucose dehydrogenase gene, an enzyme required for GAG biosynthesis, mimic the Wg phenotype.[256,257]

Signaling molecules involved in mesenchymal condensation and chondrogenesis

While cell–cell adhesion plays an integral part in the condensation stage of chondrogenesis, it has been shown that a number of signaling molecules, such as growth factors (TGFβs, FGFs), and their downstream effectors (SMADs, MAPKs) are responsible for initiation and maintenance of the chondrogenic activity within the developing limb. Similarly, transcription factors such as Hox, En and LEF-1 are also important for limb patterning, whereas members of the Wnt family may be involved in limb initiation and maintenance. The current understanding of the action of these signaling molecules is summarized below.

WNTS IN CHONDROGENESIS

As stated earlier, cadherin-dependent adhesion and function appear to be regulated in part via the cytoplasmic associated proteins, α-, β-, γ-catenin, and the newly identified p120ctn.[179,242,243,245,247,258–266] Interestingly, β-catenin is known to exist in three subcellular pools: membrane bound in association with adhesion molecules,[179] a cytoplasmic pool where β-catenin binds the adenomatous polyposis coli (APC) tumor suppressor protein through an internal repeat with amino acid homology to the catenin binding region of E-cadherin,[267,268] and a nuclear pool in association with LEF and T-cell factors (LEF-1/TCF).[269–272] Due to the apparent 'promiscuity' of the β-catenin molecule, and the plausible titration between various pools in either a signaling or adhesive capacity, it is possible that mesenchymal condensation may also be dependent on the temporal and spatial availability of β-catenin, possibly through regulation via Wnt signaling.

Recently it was determined that the interaction of APC with β-catenin is regulated by GSK-3β, a key mediator of the Wnt (*Drosophila* wingless: Wg) signaling pathway.[273,274] The Wnt family consists of at least 15 cysteine-rich secreted glycoprotein members, involved in cell fate determination, induction of neural tissue, kidney tissue and muscle and mammary glands, and has been shown to effect axis determination in early embryos.[237,275,276] Wnt signaling is mediated by interaction with its membrane receptor (*Drosophila* frizzled: DFz2),[277,278] and following regulation of various intermediate effectors, functions to inactivate GSK-3β serine/threonine kinase activity.[279–281] In the absence of Wnt signal, GSK-3β phosphorylates APC, causing increased β-catenin binding to the GSK-3β-APC complex,[274] and this binding is quickly followed by N-terminal phosphorylation of β-catenin[282] via GSK-3β.[273] The subsequent phosphorylation of β-catenin serves as a tag which targets the molecule for degradation by the ubiquitin/proteasome pathway[283–285] (Fig. 3).

Both GSK-3β and the ubiquitin pathway involved in degradation of β-catenin are regulated by PKC activity,[281,284,286] and serine/threonine phosphorylation of β-catenin may be reversed by the protein phosphatase type 1 and 2 family of phosphatases.[273] In the presence of Wnt and an inactive GSK-3β, β-catenin accumulates in the cytoplasm, presumably in a signaling capacity, and eventually translocates to the nucleus via binding to nucleoporins,[287] where it interacts with LEF-1/TCFs in an active transcription complex[269–271,288,289] (Fig. 3). The pool of β-catenin available for Wnt signaling is considered to be of a very low level, and β-catenin nuclear signaling may also be regulated by adhesion molecules which sequester catenins.[290]

Interestingly, Wnts may not be the only regulators of β-catenin and LEF-1 activity. As mentioned earlier, overexpression of ILK-1 is able to increase expression levels of LEF-1 and concomitantly down-regulates E-cadherin in epithelial cells.[235] The increased levels of LEF-1 caused translocation of β-catenin to the nucleus and activation of LEF-1 responsive promoters. Although Wnts have been identified as the primary candidates in regulation of β-catenin, this recent evidence indicates that at least one, and possibly more, pathways intersect with the β-catenin-LEF-1 complex regulation. Though LEF-1/TCF-β-catenin has been shown to have a positive regulatory effect on certain promoters when present together, it has not been dismissed that β-catenin and LEF-1 in complex may also have inhibitory qualities. Recently it was determined that at times when there are low levels of β-catenin in the nucleus, the cyclic AMP response element binding protein binding protein (CBP), a factor known exclusively for its coactivation properties, binds and acetylates TCF, causing decreased affinity for β-catenin and inhibition of transcription from TCF responsive promoters.[291] LEF-1 and TCF-1 have been identified in developing mouse limb bud mesenchymal cells and in tail pre-vertebrae,[292] and are induced by BMP-4 during murine tooth and hair development.[293] Furthermore, the LEF-1-β-catenin complex has recently been shown to bind the E-cadherin promoter.[270]

Wnt-1 in PC12 cells has been shown to increase cell adhesion through β-catenin–cadherin interaction[294,295] and has been found to cause skeletal abnormalities in developing mouse limb when ectopically expressed in transgenic mice.[296] Interestingly, some Wnts have been postulated to exert antagonistic effects towards each other in regulation of embryonic responses, possibly through influencing adhesion. Specifically, Wnt-5A appears to block *Xenopus* dorsalizing response to Wnt-1, causing decreased Ca^{2+}-dependent cell adhesion, an effect mimicked by

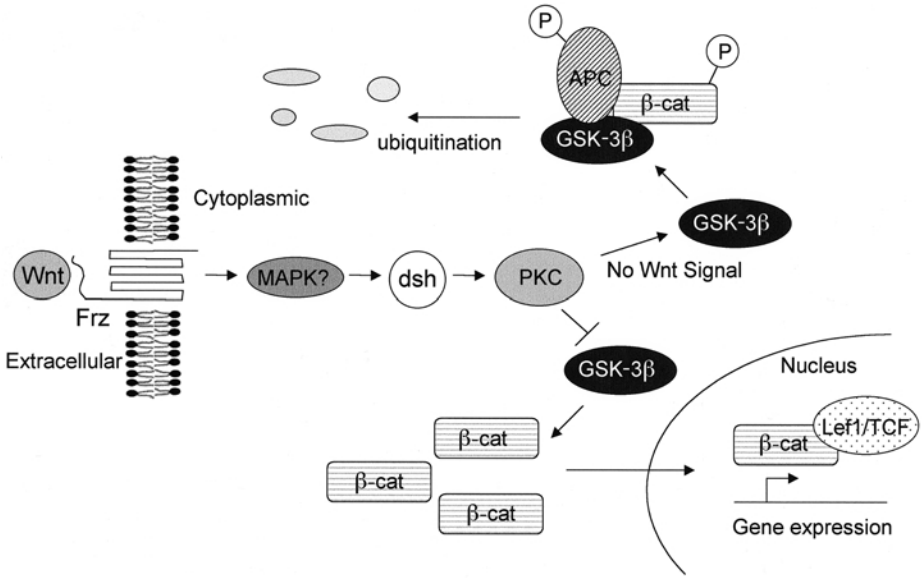

Fig. 3. Wnt signaling pathway. Wnt signaling is mediated by its interaction with its membrane receptor frizzled (Frz). Upon Wnt ligand binding, glycogen synthase kinase 3β (GSK-3β) is inactivated by a series of kinases and β-catenin (β-cat) accumulates in the cytoplasm and translocates into the nucleus where it interacts with Lef1/TCF transcription factors initiating gene transcription. In the absence of Wnt binding, GSK-3β) phosphorylates adenomatous polyposis coli tumor suppressor gene (APC) causing an increased binding of β-catenin to the APC–GSK-3β complex followed by phosphorylation of β-catenin. This complex is quickly degraded by the ubiquitin/proteosome pathway.

over-expression of a dominant negative N-cadherin.[297] However, in this report, Wnt-5A was unable to block dorsalization induced by injection of dominant negative GSK-3β or β-catenin, indicating that antagonism may occur before the Wnt pathway reaches GSK-3β.

Various Wnts are found throughout the developing limb. Wnt-3, -4, -6, and -7B are expressed uniformly throughout the limb ectoderm, Wnt-5A is expressed throughout the distal mesenchyme,[93,237] and Wnt-7A, expressed in dorsal ectoderm,[237] appears to act as dorsalizing signal[94,133,151] since mice lacking Wnt-7A develop ventralized paws.[94] Both Wnt-7A and Wnt-1 have been shown to inhibit chondrogenesis in chick limb bud micromass cultures, and the inhibition by these Wnts occurs after induction of adhesion molecules and aggregation at the late-blastema/early-chondroblast stage apparently by inhibition of chondroblast differentiation.[238] Wnt-4 has been implicated in mesenchymal condensation in kidney development,[298] and Wnt-3A, which has been isolated in the AER,[127,299] leads to induction of BMP-2.[127] Though the conserved nature of Wnt proteins implies that they act through similar pathways, Wnt-7A was recently found to exert its dorsoventral influence through a pathway other than the β-catenin/LEF-1 complex, whereas Wnt-3A in the AER appears to utilize β-catenin and LEF-1 in signaling.[127]

Our recent findings provide further support for the functional involvement of Wnt signaling in chondrogenesis. In high density micromass cultures of chick embryonic limb mesenchyme cells, expression of Wnt-3, -5A, and -7A is observed, with Wnt-7A expression showing down-regulation over the course of chondrogenesis (Woodward and Tuan, unpublished data). In cultures of the murine multipotent C3H10T1/2 mesenchymal cells, maintained as high density micromass to enhance cell–cell interaction and cellular condensation, Wnt-3 expression is up-regulated in response to the chondro-enhancer BMP-2 while Wnt-7A message is down-regulated upon addition of BMP-2 to cultures (Fischer and Tuan, unpublished data). Furthermore, lithium, a Wnt mimetic by virtue of its inhibition of GSK-3β, inhibits chondrogenesis in both embryonic chick limb mesenchyme (Woodward and Tuan, unpublished data) and in BMP-2-treated C3H10T1/2 micromass cultures (Haas and Tuan, unpublished data; Fischer and Tuan, unpublished data).

TGFβ SUPERFAMILY IN CHONDROGENESIS

A number of growth factors have long been known to regulate the initiation and maintenance of the developing limb.[300,301] Earlier in this review the FGF family was discussed in the context of limb initiation, maintenance of limb outgrowth, and reciprocal induction between the AER and the PZ of the limb bud. In addition to the FGF family, members of the TGFβ superfamily of cell–cell signaling molecules have also been implicated in chondrogenesis[302] and cell fate specification during embryogenesis.[81,303–311] The TGFβ family members contain a conserved C-terminal domain with several cysteine residues and consists of BMP-2–8, as well as TGFβs, activins/inhibins and Müllerian inhibiting substance.[312,313] Members of the TGFβ family have been shown to be multifunctional regulators of cell growth, differentiation, and apoptosis, and act on a large number of different cell types including osteoblasts and chondroblasts as well as neural and epithelial cells.[301,312]

Among the TGFβs, BMP-2 and BMP-4 are the most highly conserved. The BMP molecule exists as a 30–38 kDa dimer which undergoes cellular processing by cleavage of an inactivating N-terminal domain.[87,314] Further regulation of their biological activity occurs through presentation of the growth factors to their receptors by ECM proteins such as betaglycan and endoglien which, due to their regulatory effects, are considered to be type III receptors for TGFβs.[204,205,315] The other protein receptors for the TGFβs exist in two forms:[316–318] the growth factor

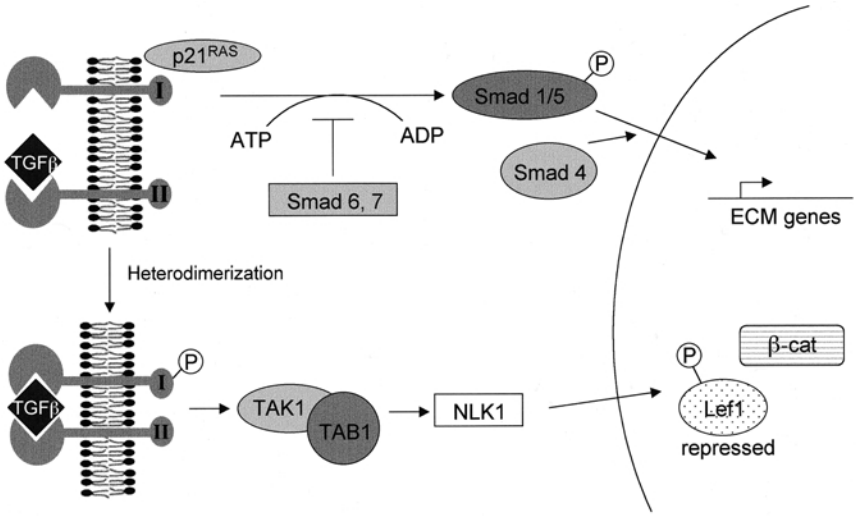

Fig. 4. TGFβ signaling pathway. TGFβ binds the type II receptor that subsequently recruits and transphosphorylates the cytoplasmic tail of the type I receptor. The MAP kinase kinase kinase TGFβ activated kinase (TAK1) and its binding partner TAK1 binding protein (TAB1), through the activity of NEMO-like kinase (NLK), negatively regulates the Wnt pathway by directly phosphorylating Lef1, thereby preventing its binding to β-catenin (β-cat). TGFβ can activate $p21^{RAS}$ by directly activating the type I receptor. SMAD proteins are phosphorylated in response to TGFβ receptor activation. These proteins form a complex (SMAD 1/5) and enter the nucleus to activate gene transcription. Other SMADs (SMAD 6,7) act to antagonize the SMAD 1/5 complex.

initially binds the upstream type II (TII) single pass membrane receptor which recruits and transphosphorylates the cytoplasmic tail of one of two type I receptors (TIa and TIb).[319,320] The activated TI receptor then, by virtue of a cytoplasmic serine/threonine kinase domain, mediates signaling through various intracellular pathways (Fig. 4).

The SMAD family of signaling molecules, homologues of *Drosophila* mothers against decapentaplegic (MAD),[321–323] are activated in response to TGFβ binding to its receptor. Although it is known that some SMADs translocate to the nucleus in singular or as hetero-dimers following interaction with TGFβ receptors,[324] it is yet unclear whether all SMAD family members have specificity for individual members of the TGFβ family, or whether the growth factors utilize various members of the SMADs to carry out various effects in different cell types.[325] Few genes are currently known to be direct targets of BMP and TGFβ signaling through SMADs,[326] although recently it was reported that MAD2, upon TGFβ stimulation, binds to the Mix.2 transcription factor promoter.[327]

The TGFβs also appear to signal through the MAPK pathway via the MAP kinase kinase kinase, TAK1 (TGFβ activated kinase),[328] and its binding partner, TAB1 (TAK1 binding protein).[329] Recent evidence indicates that TAK-1, through NEMO-like kinase (NLK), negatively regulates the Wnt pathway via direct phosphorlyation of LEF-1 and inhibition of the ability of LEF-1 to bind β-catenin (Fig. 4).[330,331] Finally, TGFβ has been shown to activate $p21^{ras}$ in epithelial cells,[332] possibly through direct interaction with the type I TGFβ receptor,[333] and cellular response to BMP-4 in *Xenopus* necessitates ras, raf and AP-1 function.[334] As mentioned earlier, BMP-2 and -4 have been shown to induce and maintain the expression of several Homeobox genes, including *Msx-1* and *-2*,[88,335,336] as well as induce LEF-1.[293] BMP-4 has been shown to mediate axis formation in *Xenopus* via Mix.1,[337] and molecules up-regulated in response to TGFβ treatment of cells include ECM molecules such as osteopontin, osteonectin, tenascin, decorin and collagen types I, III, VI, and X (reviewed in ref. 319).

Pioneer studies performed by Urist[45] first suggested that factors endogenous to demineralized bone were able to induce endochondral ossification, i.e. chondrogenesis and osteogenesis. A series of ensuing biochemical and molecular studies identified numerous growth factors within demineralized bone, including the BMPs.[300,338–341] Subsequent studies showed that injection of partially purified BMPs,[342] recombinant human BMP-2a and BMP-4,[343,344] and *Drosophila* Dpp, a homologue of BMP-2,[345] resulted in ectopic cartilage and/or bone formation. A number of *in vitro* studies have shown the osteogenic and chondrogenic effects of BMP-2 and TGFβ.[346–355] Furthermore, addition of BMP-2 to embryonic chick limb bud mesenchyme or the multipotent mouse C3H10T1/2 mesenchymal cell line plated at high density initiates and/or enhances chondrogenesis,[190,191,353,356] and increases expression of N-cadherin protein and mRNA.[190,191,356]

Examination of the developing limb identified BMP-5 within condensations near the posterior tips of buds,[357] and mouse genetic mutants deficient for BMP-5 develop defects during early skeletal formation at the time of mesenchymal condensation.[358] BMP-2 has been identified in both condensing vertebrae[86] and posterior limb bud mesenchyme where it acts as an anterior/posterior polarizing signal.[82,85,90,91] BMPs have also been implicated in inducing apoptosis in interdigital spaces while TGFβs induce chondrogenesis in developing digits.[90] Both *in vitro* and *in vivo* studies combine to strongly implicate important signaling and morphoregulatory function for members of the TGFβ superfamily in both skeletal formation and chondrogenesis. Finally, Cyr61, a growth factor-inducible heparin-binding protein, has been recently shown to have expression profiles within mesenchymal cells undergoing chondrogenesis, and has been shown to promote chondrogenic differentiation in cultures plated at subthreshold cell densities.[359]

OTHER SIGNALING PATHWAYS REGULATING CHONDROGENESIS

The above factors are likely to act in conjunction with other signaling molecules secreted from limb mesenchyme or overlying limb ectoderm. During initial condensation of *in vitro* cultured mouse and chick limb bud mesenchyme, it was determined that there is a modest increase in the intracellular cAMP levels.[360,361] Furthermore, addition of exogenous cAMP derivatives has been shown to enhance cartilage differentiation in *in vitro* studies,[54,362–366] possibly via enhanced GAG synthesis.[367] Since the cAMP signal transduction system is primarily modulated by cAMP-dependent protein kinases (PKA) (for a review see ref. 368), researchers began examining the involvement of PKA in cAMP-induced chondrogenesis, and recent reports have indeed identified PKA as a chondro-enhancer.[369] Activated PKA leads to regulation of cAMP responsive genes via cAMP response elements within promoters, cAMP response element binding protein (CREB), and CBP, a protein also known to be involved in LEF-1 regulation.[291]

Increasing evidence indicates that PKC is involved in the modulation of chondrogenesis. Stuarosporine, a PKC inhibitor, has been shown to enhance chondrogenesis,[370,371] while modulation of PKC via tetradecanoylphorbol acetate (TPA) inhibits chondrogenesis,[369,372] and leads to a decrease in the phosphorylation of CREB.[369] Interestingly, some reports suggest that certain isoforms of PKC are required for chondrogenesis.[373] It has been shown that PKC's positive contribution to chondrogenesis may be due to inhibition of Erk-1 which in turn down-regulates the expression of various adhesion proteins such as N-cadherin, fibronectin, and the $\alpha 5\beta 1$ integrin receptor,[374] therefore regulating cellular differentiation. As mentioned earlier, PKC also appears to be involved in the regulation of Wnt signaling,[281,284,286] and in this capacity may regulate chondrogenesis. Due to the large number of PKC isoforms present within any given cell (for a review see ref. 375) it is possible that certain PKCs may contribute to the chondrogenic pathway while others exert a negative effect on chondrogenesis.

Patterning of cartilage development

Sculpting of the developing limbs results from the spatial and temporal cooperative interactions between factors secreted from limb epithelium, Wnts, and *Hox* genes within the limb mesenchyme. The fact that TGFβs act in part to regulate the response of *Hox* genes, Shh, and the ECM either directly or indirectly, indicates the strong functional link between extracellular signals and an internal genomic response of the mesenchymal cells during cartilage development. The following section reviews the Hox and homeobox genes and discusses the possibility that they may represent one of the targets under ECM regulation.

HOX AND HOMEOBOX GENES

In both mouse and human, genomic analysis has identified the presence of 38 genes organized in four different chromosomal complexes (*Hox a, b, c, d*), arranged such that all the genes in each cluster are oriented in the same 5′ to 3′ direction.[101,141] Genes located at the 3′ end of the cluster are expressed prior to those at the 5′ end, and genes more 3′ generally extend more anteriorly in the developing limb. Several Hox genes are expressed during limb initiation within the limb bud. Expression of *Hoxa-10, -11* and *-13* initiates distally at the posterior margin of the bud (near the ZPA), and subsequently expands such that the final boundaries of the expression domains are perpendicular to the proximodistal axis.[149,376–378]

The *Hox d* genes have been extensively studied in mouse and chick and can be ectopically activated by the combined influence of Shh[97,112] and FGFs.[81,82] It has been determined in early wing bud that *Hoxd-9* and *10* are uniformly expressed throughout the mesenchyme, whereas *Hoxd-11, -12*, and *-13* are found more posteriorly.[379] Furthermore, *Hoxd-13* expression is located at the furthest posterior boundary, whereas that of *Hoxd-10, -11* and *-12* extend more anterior into limb mesenchyme. As limb growth progresses, the expression domains of *Hoxd-9* and *-12* extend further, though *Hoxd-13* is restricted to the most distal cells of the bud.[380] When digit differentiation begins, *Hoxd-13* is expressed perpendicular to its initial orientation.[149] These spatiotemporally unique expression profiles of the *Hox d* genes suggest that they are functionally important in early events of limb patterning.

Both *Hox-7* and *Hox-8* (*Msx-1* and *Msx-2*) are expressed in both AER and the PZ,[100,149,381–384] and are considered the earliest mesenchymal markers of the developing limb. Possible functions of these genes in the PZ include maintenance of an undifferentiated population of cells or activating apoptosis.[385,386] Furthermore, the AER is required for maintenance of the expression of both *Hox-7* and *Hox-8* within the undifferentiated mesenchyme of the PZ.[387] Candidate factors contributing to *Hox* gene maintenance include BMP-2 and -4, which have been found to be co-expressed with *Msx* genes at numerous sites, and can induce expression of *Hox-7* and *-8 in vitro*.[88,335,388] Interestingly, Ihh misexpression disrupts cartilage differentiation and results in the misexpression of *hox* genes,[147] indicating that the Hh family of transcription factors influence *Hox* gene expression along with, or as a consequence of, the action of various growth factors.

Recent *in vivo* studies have proved to be quite revealing regarding the function of *hox* genes. The construction of null alleles of various *Hoxa* and *Hoxd* genes in mice has been enlightening with regard to their role in development of the vertebrate limb. For example, *Hoxa-10, -11* and *-13* knockouts led to upper, middle limb defects and digital reductions, respectively.[148,389–391] Also, loss of *Hoxa-11* activity led to defects that suggested a relationship between proximodistal and anterioposterior patterning.[148] Complete loss of the *HoxD* locus resulted in ulnar dysplasia,[392,393] while analysis of individual *Hox d* genes showed progressive mutations of limb from upper (*Hoxd-10*) to middle (*Hoxd-11*) to digital (*Hoxd-12, -13*).[143,148,394–400] The results indicate that Hox genes largely regulate the growth rates of mesenchymal condensations within their zone of influence, and furthermore can exert influence at numerous times of development.[394,401–405] Hox gene mutations also appear to have synergistic effects, indicating coordinated gene activity as well as unique functions.[394,401–405]

Inappropriate expression of Hox genes has also been shown to result in aberrant limb formation. For example, proximal misexpression of *Hoxd-13* in mouse results in truncation of the radius–ulna and tibia–fibula,[392,393] while misexpression of *Hoxb-8* causes duplication of the ZPA and digit duplication.[109] *Hoxd-11*, normally expressed in the primordia of the posterior three digits of chick limb, was misexpressed in the primordia of the most anterior digit using retroviral infection; a morphological transformation of digit I into digit II was observed.[406] Furthermore

mis-expression of *Hoxa-13* in chick limb resulted in transformation of the cartilage primordia of the zeugopodia from elongated condensations to short condensations similar to the morphology seen only in wrist elements, and appeared to suppress cell death.[407]

SOX GENES

The Sox genes comprise a large group of developmentally regulated genes encoding transcription factors that have critical functions in many developmental processes, including sex determination, neural induction, and skeletogenesis.[408] Sox genes belong to the HMG box superfamily [Sry-type high mobility group (HMG) box] of genes that encode transcription factors. Members of the Sox subfamily have an amino acid similarity of >50% to the HMG domain of SRY, the testis determining factor, which contains a domain similar to the nonhistone chromosomal proteins HMG-1 and HMG-2. Sox proteins bind to the (A/T)(A/T)CAA(A/T)G concensus motif in the minor groove of DNA and induce DNA bending.[409] Binding to the minor groove and the ability to bend DNA has led to the hypothesis that Sox proteins may also function as architectural building blocks promoting the assembly of biologically active multiprotein complexes.[410]

One member of the Sox subfamily, Sox9, has been demonstrated to have a role in skeletogenesis. In humans, haploinsufficiency of SOX9 results in a disease known as campomelic dysplasia (CD) and XY sex reversal.[411] Patients with CD have abnormalities in most cartilage derived structures, resulting in bowing and angulation of the long bones and craniofacial defects (reviewed in ref. 412). The embryonic expression pattern of Sox9 shows an association of Sox9 mRNA expression with early events of skeletal formation.[413] During mouse development, Sox9 expression is seen in the mesenchyme of the head, the sclerotome of the somites, and the condensations of the limbs. The expression of Sox9 clearly preceded the deposition of cartilage in these sites, suggesting that Sox9 is a determinant of chondrocyte differentiation rather than a consequence.[413] Sox9 is also expressed in nonskeletal tissues such as the notochord, otic vesicle, neural tube, brain and the developing gonads, suggesting that Sox9 may have other functions (reviewed in ref. 414).

During mouse embryonic development, Sox9 and collagen type II (*Col2a1*), the major structural component and marker of cartilage demonstrate very similar expression patterns with the expression of Sox9 slightly preceding that of *Col2a1*.[413,415,416] Sox9 has been shown to bind to a consensus sequence in the *Col2a1* enhancer region responsible for chondrocyte-specific expression[417–420] and mutations in this site abolishes Sox9 regulation.[418,419] Recently, it has been shown that two other Sox proteins, L-Sox5 and Sox6, form a complex with Sox9 to cooperatively activate *Col2a1* gene expression.[421] Sox9 is also able to activate *Col2a1* reporter constructs containing chondrocyte-specific enhancer sequences when Sox9 expression constructs were co-transfected into fibroblasts and nonchondrogenic cell lines.[418] In mouse $Sox9^{-/-}$ and wild type chimeras, $Sox9^{-/-}$ cells were excluded from the condensing mesenchyme; in addition, teratomas derived from $Sox9^{-/-}$ embryonic stem cells did not form cartilage, consistent with the assumption that Sox9 is required for the cartilage phenotype.[422]

In summary, the importance of Sox9 in skeletal development is clearly demonstrated by the identification of its role in the human skeletal disorder, CD, while its embryonic expression patterns implicates action during the very early stages of chondrogenesis, most likely serving as a transcription factor to induce the expression of the collagen type II gene, perhaps by being an important architectural component of a biologically active protein complex.

OTHER PATTERNING MOLECULES

In addition to the Hox gene families, the ets gene superfamily, which contains over 30 members, encodes a class of evolutionarily conserved transcription factors that bind a purine rich sequence through an 85 amino acid ETS domain (reviewed in ref. 423). The chicken erg gene (ck-erg) has been cloned.[424] Expression patterns of ck-erg and the closely related c-ets-1 gene are almost superimposable during early development, but distinct regions of ck-erg expression appear during later stages of development. These areas are the pre-cartilage condensations and the cartilaginous blastemas preceding bone formation. The functional role of these genes during endochondral ossification has yet to be determined.

A new homeodomain-containing gene, cartilage homeoprotein-1 (Cart-1), has been cloned from a rat chondrosarcoma tumor and contains a paired-type homeodomain.[425] Cart-1 is selectively expressed in pre-cartilage mesenchymal condensations and in early chondrocytes. A more detailed expression study of Cart-1 showed that this gene is expressed in a subset of mesenchymal cells which are either precursors of chondrogenic cells or have the potential of becoming chondrogenic (e.g. mesonephros and tendon).[426] Expression of Cart-1 was often seen in mesenchymal cells prior to the initiation of cellular condensation, as well as in chondrogenic mesenchymal cell condensations. These observations suggest a role for Cart-1 in specifying the cells destined to become cartilage.[426]

Conclusion

The transformation of loosely packed mesenchymal cells into highly organized and patterned skeletal structures requires the careful orchestration of cell–cell, cell–matrix, and growth factor mediated signaling events which ultimately result in the regulation of gene transcription and function. ECM mediated mesenchymal cell condensation facilitates the requisite cell–cell interactions which presage changes in ECM composition and differentiation of mesenchymal cells into chondrocytes. Maturation and hypertrophy of chondrocytes is regulated by the two secreted proteins Indian hedgehog and parathyroid hormone-related peptide, which act through their cell surface receptors, Patched and PTH/PTHrP receptor, respectively, to initiate intracellular signal transduction pathways. In addition to its structural characteristics, the ECM is extremely dynamic and functions to transmit signals from the surrounding environment of the cell to the cytoskeleton and subsequently activate signal transduction pathways, or propagates inside-out signals from within the cells to the pericellular environment. Furthermore, recent molecular genetic advances have identified numerous genes, many of which encode specific transcription factors and bioactive growth factors, expressed within limb ectoderm and mesoderm that are responsible for the patterning of the embryonic skeletal structures. The regulated cross-talk between these genes acts spatially and temporally to guide the

patterning of each of the skeletal elements. It is becoming increasingly evident that the above signaling pathways are not linear but intersect at various points and are responsible for the strict control of the development of the embryonic skeletal structures. Thus, perturbations of the highly orchestrated series of events in cartilage development, either genetically or teratogenically, inevitably result in skeletal birth defects.

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Glossary of terms

AER	Apical ectodermal ridge
APC	Adenomatous polyposis coli
BMP	Bone morphogenetic protein
CAM	Cell adhesion molecule
CBP	CREB binding protein
CD	Campomelic dysplasia
CREB	cAMP response element binding protein
Dhh	Desert hedgehog
Dpp	Decapentaplegic
ECM	Extracellular matrix
EGF	Epidermal growth factor
En	Engrailed
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GSK-3β	Glycogen synthase kinase-3β
Hh	Hedgehog
Hox	Homeobox
Ihh	Indian hedgehog
ILK	Integrin linked kinase
JNK	Jun N-terminal kinase
LEF	Lymphocyte enhancing factor
MAD	Mothers against decapentaplegic
MAPK	Mitogen activated protein kinase
N-CAM	Neural cell adhesion molecule
NLK	Nemo like kinase
PDGF	Platelet derived growth factor
PI-3K	Phospoinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PL	Poly-L-lysine
PNA	Peanut agglutinin
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related reptide
PTP1B	Protein tyrosine phosphatase-1B
PZ	Progress zone
r-Frg	Radical fringe
Shh	Sonic hedgehog
TAB-1	TAK binding protein
TAK-1	TGF-β activated kinase
TCF	T-cell factor
TGF	Transforming growth factor
TPA	Tetradecanoylphorbol acetate
ZPA	Zone of polarizing activity