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Regulation of Early Steps of Chondrogenesis in the Developing Limb

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Abstract: In the developing limb, chondrogenesis is an important prerequisite for the formation of cartilage whose template is required for bone formation. Chondrogenesis is a tightly regulated multi-step process, including mesenchymal cell recruitment/migration, prechondrogenic condensation of the mesenchymal cells, commitment to the chondrogenic lineage, and differentiation into chondrocytes. This process is controlled exquisitely by cellular interactions with the surrounding matrix and regulating factors that initiate or suppress cellular signaling pathways and transcription of specific genes in a temporal-spatial manner. Understanding the cellular and molecular mechanisms of chondrogenesis is important not only in the context of establishing basic principle of developmental biology but also in providing research direction toward preventive and/or regenerative medicine. Here, I will overview the current understanding of cellular and molecular mechanisms contributing to prechondrogenic condensation processes, the crucial steps for chondrogenesis, focusing on cell-cell and cell-matrix interactions.

Key words: chondrogenesis, condensation, extracellular matrix, integrin, gap junction, matrix metalloproteinase, signal transduction

The body plan is established in the early embryo by precise coordination of cell migration, proliferation and differentiation. The early embryonic limb bud possesses two signaling centers, the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), which produce signals responsible for directing the proximal-distal outgrowth and anterior-posterior patterning of the limb skeletal elements, respectively (DeLise et al., 2000; Olsen et al., 2000). When the mesenchymal cells in the central core of the limb bud become located outside of the range of the AER signaling, they launch chondrogenesis into highly complicated processes of endochondral ossification (Tuan, 2004; Goldring et al., 2006; Pacifici et al., 2006). Cartilage formation is a tightly regulated multi-step process, including mesenchymal cell recruitment/migration, precartilage condensation of the mesenchymal cells, commitment to the chondrogenic lineage, and differentiation into chondrocytes (Johnson and Tabin, 1997; Sander and Adler, 1999; DeLise et al., 2000; Knudson and Knudson, 2001). The differentiated chondrocytes can then proliferate and undergo the complex process of hypertrophic maturation, followed by programmed cell death and replacement by bone (Fig. 1).

Chondrogenesis is a prerequisite event for cartilage formation, an earliest overt morphogenetic event of endochondral ossification, which begins with a process of prechondrogenic condensation (Olsen et al., 2000; Tickle and Munsterberg, 2001). Previous studies have demonstrated that high level of cell density in the chondrogenic regions is correlated with the extent of cell condensation (Ede and Shamslahidjani, 1983; Newman et al., 1985). Therefore, prechondrogenic condensation could be dependent on mitotic activity and the migration of cells toward a center at the sites where the cartilaginous templates of the endochondral bones will develop directly from the cells within the condensations (Mariani and Martin, 2003; Shum et al., 2003). During condensation, intimate cell-cell and cell-matrix interactions occur to trigger chondrogenesis. However, the exact mechanism has yet to be elucidated. This article will summarize the current understanding of cellular and molecular events contributing to condensation processes focusing on cell-cell and cell-matrix interactions.

CELL-CELL INTERACTIONS

Prechondrogenic condensation is initiated by active cell movement that causes an increase in chondroprogenitor mesenchymal cell packing density in the core of the limb bud (Iwasaki et al., 1993; Seghatoleslami and Tuan, 2002).

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This process is associated with an increase in cell-cell contacts and interaction through cell-cell adhesion molecules and gap junctions accompanied by one or more signal transduction pathways.

Cell adhesion molecules

Adhesion of cells to each other provides important information and determines cellular responses (Leckband and Prakasam, 2006). The cell adhesion molecules known to facilitate mesenchymal cell-cell contacts include neural cadherin (N-cadherin), a Ca^{2+}-dependent large transmembrane glycoprotein, and neural cell adhesion molecule (N-CAM), a Ca^{2+}-independent membrane glycoprotein composed of the large immunoglobulin supergene family. Both molecules have been demonstrated, *in vivo* and *in vitro*, to be associated with initiation and maintenance of condensation, respectively, and perturbation of these molecules results in alterations in chondrogenesis (Widelitz et al., 1993; Oberlender and Tuan 1994; Fang and Hall, 1995; DeLise and Tuan, 2002; Tuan, 2003). In fact, experiments involving plasmid and retroviral vector-mediated misexpression of wild-type N-cadherin or the dominant negative N-terminal deletion constructs in micromass cultures of primary chick limb mesenchymal cells have confirmed that transient misexpression of wild-type N-cadherin during the early time of limb mesenchyme micromass culture, corresponding to the time of endogenous N-cadherin expression, enhanced cellular condensation (Oberlender and Tuan 1994; DeLise and Tuan, 2002). On the other hand, expression of the deletion mutant form of N-cadherin, which lacks either the extracellular homotypic interaction domains or the intracellular β-catenin binding site, resulted in decreased cellular condensation and subsequently reduction of chondrogenesis (Tuan, 2003). Widelitz et al. (1993) have reported that anti-N-CAM antibody-treated chick mesenchymal cell cultures show decreased cell aggregation resulting in reduction of cell condensation and chondrogenesis. On the other hand, overexpression of N-CAM in micromass cultures results in enhanced cell aggregation followed by differentiation into cartilage nodules. These findings indicate that N-cadherin and N-CAM are required in a time- and quantity-specific manner for normal cellular condensation and chondrogenesis to occur and that those temporally inappropriate cell-cell interaction activities are inhibitory to chondrogenic differentiation, possibly because of inappropriate timing of signaling events.

Gap junctions

Gap junctions, which are generally believed to facilitate cell-to-cell diffusion of hydrophilic molecules having a molecular mass of less than 1 kD, such as cAMP, Ca^{2+}, IP_3, and ATP (Kumar and Gilula 1996), are present in many developing tissues and play roles in many processes, including signal transmission, cell proliferation, differentiation, apoptosis, and tissue homeostasis (Simon and Goodenough, 1998; Xu et al., 2001). Gap junctions are formed by members of a family of sequentially and structurally related proteins known as connexins. Approximately 20 connexins have been identified and cloned from various tissues and cells (Eiberger et al., 2001; Willecke et al., 2002; Saez et al., 2003). Six monomers of connexins consisting of four conserved membrane spanning domains and two extracellular loop domains are joined head-to-head across extracellular "gap" between two adjacent cells to form intercellular channels (Sosinsky and Nicholson, 2005; Yeager and Harris, 2007).

In the limb buds of the mouse and chick, gap junctions are present predominantly in the AER (Fallon and Kelley, 1977; Makarenkova et al., 1997; Meyer et al., 1997). One of the major gap junction proteins in chick and mouse embryonic limb is α-1 connexin or connexin43. During the limb development, connexin43 transcript is present at high levels in the central condensation of mesenchymal cells, but as differentiation proceeds, connexin43 transcript becomes restricted to the edge of the developing cartilaginous core, the site of the future perichondrium (Dealy et al., 1994; Green et al., 1994). A recent report has demonstrated that gap junction-mediated intercellular communication prevents spontaneous apoptosis (Yasui et al., 2000). This protective effect has also been described in chick leg bud mesenchymal cells, in which the blockade of gap junction-mediated intercellular communication by knockdown of connexin43 inhibited condensation of chondrogenic progenitor cells that results from increased apoptotic cell death through down-regulation of integrin β4 (Jin et al., 2008). Contrary to the roles for gap junction in cell survival signaling cascades, several researchers have suggested that these communicative structures might be implicated in cell death as well. In several experimental models, transfection with connexin genes results in induction of apoptotic cell death (Huang et al., 2001; Muramatsu et al., 2002; Kalvelyte et al., 2003). Moreover, communication via gap junctions frequently underlies the propagation of cell death between a dying cell and its healthy neighbor in ischemia-related cell injury, such as in the case of cerebral infarction (Lin et al., 1998; Contreras et al., 2004; Nakase et al., 2004). The spread of cell death through gap junctions and the "Good Samaritan" effect could reflect the two sides of a coin. The role of gap junctional communication in spreading/preventing cell death has been recently reviewed (Krysko et al., 2005).

CELL-MATRIX INTERACTIONS

Cells within the chondrogenic condensations express high levels of extracellular matrix (ECM) components such as fibronectin, tenascin, syndecan and cartilage oligomeric

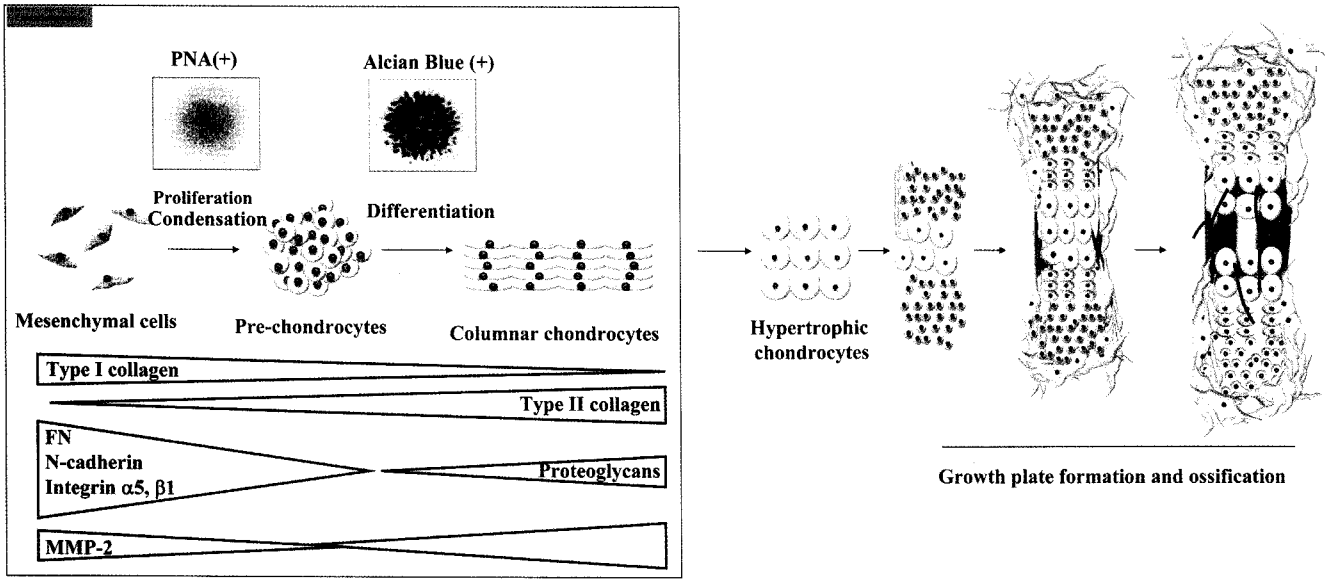

Fig. 1. The multistep process of endochondral ossification in the developing limb bud. The different stages are presented schematically, showing the temporal patterns of regulatory factors involved in each stage. Chondrogenesis in the micromass cultures was detected by PNA staining of condensing cells and Alcian blue staining of cartilage nodules.

proteins as well as cell surface adhesion molecules (Aulthouse and Solursh, 1987; Gould et al., 1995; Maleski and Knudson, 1996; Tavella et al., 1997). The temporal and cooperative functions of these molecules mediate the requisite cell-matrix interactions that initiate the condensation event. This necessary step is mimicked *in vitro* by chondrogenesis of mesenchymal cells, where high cell density results in the formation of three-dimensional spheroid structures that are chondrocytes in phenotype and are associated with synthesis and deposition of ECM components (Fig. 1). Cell-matrix interactions are mediated via transmembrane receptors including integrins and hyaluronal receptor, CD44 (Yu et al., 1991; Knudson, 2003; Takahashi et al., 2003; Onodera et al., 2005). Members of each receptor class, through interactions with their principal ligands, provide changes in the ECM environment and these changes may elicit matrix remodeling or cytoskeletal reorganization (Filipenko et al., 2005; Wood et al., 2007; Lock et al., 2008). The molecular mechanisms of these interactions are not fully understood, but it is clear that communication between neighboring cells through adhesion and ECM molecules during condensation step is critical in establishing both temporal and spatial regulation of chondrogenic differentiation in the developing limb (DeLise et al., 2000; Djouad et al., 2007; Wu et al., 2007).

Extracellula matrix (ECM)

Fibronectin plays a critical role in aggregation of mesenchymal cells to be recruited into condensations. Both *in vitro* and *in ovo* experiments of the chick limb have shown that a functional splice variant of fibronectin (FN) is highly expressed just prior to condensation, and inhibition of the FN function results in down-regulation of condensation (Frenz et al., 1989; Downie and Newman 1995; Gehris et al., 1997; White et al., 2003). It has been reported that inhibition of integrin β1 binding to FN impairs activation of focal adhesion kinase (FAK) and prechondrogenic aggregation, and subsequently blocks chondrogenesis (Bang et al., 2000). Fibronectin also acts to activate the expression of N-CAM during cellular condensation, which is down-regulated by binding of syndecan, thereby setting the condensation boundaries (Gould et al 1995; Chimal-Monroy and Diaz de L, 1999). Mesenchymal condensation also requires interactions with tenascin and cartilage oligomeric proteins for chondrogenic differentiation via signaling through FAK and paxillins (Gehris et al., 1997; DeLise et al., 2000; Hall and Miyake, 2000).

Changes in cell shape from fibroblast-like to round or polygonal morphologies and production of ECMs follows prechondrogenic condensation of mesenchymal cells (von der Mark and von der Mark, 1977). These changes involve a loss of fibrillar-actin-based cytoskeleton, accumulation of cortical actin and changes in the adhesive properties (Idowu et al., 2000). Integrins stimulate the formation of signaling complexes mediating cell attachment to ECMs and play an important role in morphogenesis (Schwartz et al., 1995). Included within these signaling complexes are scaffolding proteins such as talin, paxillin, α-actinin and kinases including FAK and integrin-linked kinase (Lo, 2006). Several data suggest that actin dynamics control chondrogenesis (Daniels and Solursh, 1991, Kim et al., 2003, Hwang et al., 2006). Chondrocytes plated in a monolayer culture tend to

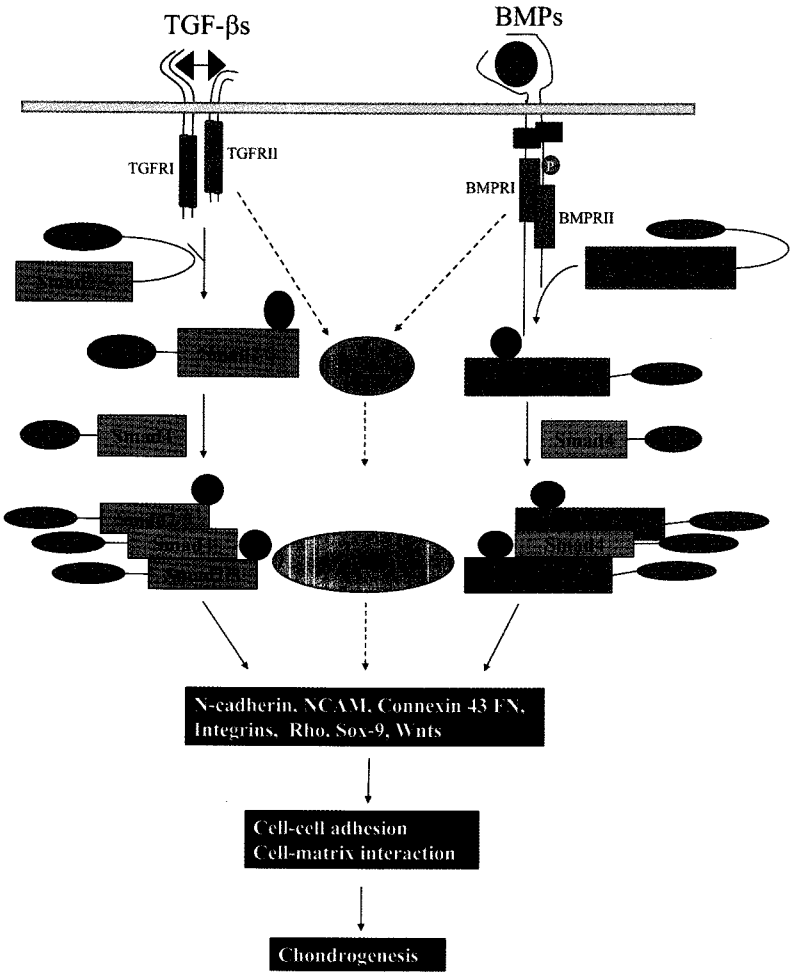

Fig. 2. Schematic diagram showing the signaling pathways of TGF-β and BMP in the regulation of cell-cell adhesion and cell-matrix interaction. (See the text).

change morphology to flattened cells and to cease production of collagen II, a typical marker of chondrogenic differentiation (von der Mark et al., 1977; Grundmann et al., 1980). These dedifferentiated chondrocytes return to round morphology and exhibit increased production of collagen II with the addition of dihydrochalasin B or cytochalasin D, inhibitors of actin polymerization (Zanetti and Solursh, 1984; Brown and Benya, 1988; Loty et al., 1995; Lee at al., 2007).

FACTORS REGULATING CELL-CELL AND CELL-MATRIX INTERACTIONS

Chondrogenesis has been shown to be under the regulation of a number of growth and differentiation factors that initiate or suppress cellular signaling pathways and transcription of specific genes (Shimizu et al, 2007). The transforming growth factor β (TGF-β) superfamily such as TGF-β proteins and bone morphogeneric proteins (BMPs) contains a conserved C-terminal domain with several cysteine residues (Massague et al., 1992; Kingsley et al., 1994) which acts through activation of respective receptors followed by down-signaling pathways (Shi and Massague, 2003). TGF-β and BMP signals are mediated by Smad transcription factors that bind to type I receptor and are phosphorylated following ligand binding to type II receptor (Fig. 2). Both the BMP receptor-associated Smads (1, 5, and 8) and the TGF-β receptor-associated Smads (2 and 3) are released into the cytoplasm upon phosphorylation, complex with Smad4 followed by translocation to the nucleus, where they regulate gene expression (Massague et al., 1997; Shi and Massague, 2003). Because TGF-β receptor- and BMP receptor-associated Smads compete for Smad4 and other downstream signaling molecules, these pathways antagonize one another depending upon cell types (Candia et al., 1997). They also have opposing effects in chondrogenesis in which TGF-β inhibits (Ballock et al., 1993; Jin et al., 2008) and BMP promotes (Leboy et al., 1997; Grimsrud et al., 1999; Jin et al., 2006a) chondrogenic differentiation. On the other hand, both TGF-β and BMP have been shown to enhance chondrogenesis in murine mesenchymal cells with the most robust effect in response to BMP signaling (Tuan

2003; Zhang et al., 2004). BMP signaling is required for both the formation of precartilaginous condensations and the differentiation of precursors into chondrocytes (Yoon et al., 2005).

TGF-β and BMP signals act in combination with other signaling pathways. In ATDC5 cells (Watanabe et al., 2001) and chick limb bud mesenchymal cells (Jin et al., 2006b), stimulation of chondrogenesis by TGF-β or BMP-2 is mediated by the p38 and Erk1/2 MAP kinase pathways as well as activation of the Smad pathways. Furthermore, the Wnt/β-catenin signaling *in vivo* appears to inhibit chondrogenesis in human mesenchymal stem cells (Day et al. 2005) and chick limb bud mesenchymal cells (Jin et al., 2006a). Wnt-5a expressed in limb mesenchyme and Wnt-7a expressed in the dorsal ectoderm of the limb bud have been proposed to be involved in the early events of chondrogenesis, particularly with respect to N-cadherin-related activities (Tuan, 2003; Tuli et al., 2003; Daumer et al., 2004; Modarresi et al., 2005). TGF-β and BMP signals act synergistically with Wnt/β-catenin signaling and modulate chondrogenesis by regulating N-cadherin- and Sox-9-related activity (Fisher et al., 2002; Zhou et al., 2004; Jin et al., 2006a), respectively. Overall, it is clear that BMP- and TGF-β-signalings are critical for regulation of chondrogenic differentiation (Fig. 2). However, these pathways are only a part of multiple signaling events that contribute to the regulation of chondrogenic commitment.

Sox9 is a transcription factor which belongs to the SRY (sex-determining region on the Y chromosome) family and contains the HMG (high mobility group) box DNA domain (Wright et al., 1995). Sox 9 is expressed in all chondro-progenitors and chondrocytes and is absolutely required for prechondrogenic condensation (Zhao et al., 1997). It has been shown that *Sox9*$^{-/-}$ cells in chimaeric mouse are excluded from the aggregating mesenchyme of the prechondrogenic cells autonomously (Bi et al., 1999). Moreover, removal of Sox9 from mouse limb mesenchymal cells prior to the onset of condensation results in complete absence of prechondrogenic condensation, leading to formation of extremely short limbs without any skeletal components (Akiyama et al., 2002). The expression of Sox9 proteins is dependent upon BMP signaling via BMP-receptors, which are functionally redundant and active in chondrocyte condensations but not in the perichondrium (Yoon et al., 2005). These studies indicate that Sox9 is indispensable for mesenchymal condensation.

Modulation of cell-matrix interactions occurs through the action of unique proteolytic systems responsible for hydrolysis of a variety of ECM components. Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell-matrix composition through the turnover of ECMs and that function as key regulators of cell-ECM interactions during development and differentiation (Vum and Werb, 2000; Somerville et al., 2003; Mott and Werb, 2004; Mannello et al., 2005). Several MMP are expressed during endochondral ossification including MMP-2, -3, -9, -10, -13, and -14 (MT1-MMP) (Bord et al., 1997; Johansson et al., 1997; Bord et al., 1998; Zhou et al., 2000). In the avian species, MMP-2, -3, -9, -13, and -16 (MT3-MMP) have been cloned (Yang et al., 1996; D'Angelo et al., 2000; Tong et al., 2003), but MMP-10 and -14 have not yet been identified. The involvement of MMPs in skeletal growth indicates the importance of proper ECM remodeling as a major limiting factor for vital parts of the long bone developmental process, including apoptosis, angiogenesis and osteoblast recruitment (Si-Tayeb et al., 2006; Amãlinei et al., 2007; Chetty et al., 2007; Shi et al., 2007). Mice lacking both MMP-9 and MMP-13 show a severe endochondral bone phenotype with drastically shortened long bones with a diminished ECM remodeling, prolonged chondrocyte survival, delayed vascular recruitment and defective trabecular bone formation (Tuckermann et al., 2000; Stickens et al., 2004; Ortega et al., 2005). MT1-MMP (MMP-14) knockout mice have severe skeletal development defects such as craniofacial dysmorphism and dwarfism due to a decreased proliferation by proliferative chondrocytes at the growth plate (Holmbeck et al., 1999). High levels of MMP-2 is detected in osteoarthritic cartilage and synovial fluid (Imai et al., 1997; Volk et al., 2003) and testican-1, an inhibitor of pro-MMP-2 activation, is expressed in joint and growth plate cartilage (Hausser et al., 2004) suggesting the involvement of MMP-2 in the remodeling of cartilage ECM. MMP-2 was also found to function as a negative regulator of the integrin β1 mediated cell-matrix interaction through FAK, thereby leading to inhibit precartilage condensation of chick leg bud mesenchymal cells (Jin et al., 2007). Together, these findings suggest that MMPs participate in the regulation of matrix turnover in cartilage and chondrogenesis. However, the detailed regulating mechanisms remain to be elucidated.

CONCLUDING REMARKS

The transformation of loosely packed mesenchymal cells into highly organized and patterned skeletal structures requires complicated cell-cell and cell-matrix interaction mediated by various regulatory molecules and cellular signaling pathways. Alteration and modification of extracellular composition is crucial for cell-cell and cell-matrix interaction during differentiation of mesenchymal cells into chondrocytes. Elucidation of mechanism integrating signals involved in cell-cell and cell-matrix interaction will contribute to a better understanding of the cellular and molecular basis of cartilage formation during normal development, and to therapeutic approaches for cartilage and bone repair.

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