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Successive Formative Stages of Precartilaginous Mesenchymal Condensations In Vitro: Modulation of Cell Adhesion by Wnt-7A and BMP-2

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High-density chick limb bud cell culture is a useful model to study mesenchymal condensatifons and chondrogenesis. Most previous studies have focused on the effects of soluble reagents on terminal chondrogenic differentiation and have not defined the early cellular processes and signaling events. In this study, we defined five successive stages in the differentiation process: 1) dissociated cells, 2) small aggregates, 3) formation of cell clusters, 4) precartilaginous condensations, and 5) cartilage nodule. We used RCAS retrovirus-mediated Wnt-7a gene transduction to test the effect of Wnt-7a on the differentiation process. We found that Wnt-7a suppressed chondrogenic differentiation. Wnt-7a did not inhibit the initiation of condensation formation but blocked the progression of precartilaginous condensations to cartilage nodules. The Wnt-7a-transduced cultures showed characteristics of a less mature culture with persistent expression of NCAM, N-cadherin, wider distribution of integrin β1 and fibronectin, and suppression of tenascin-C. BMP-2 is known to enhance chondrogenic differentiation in these cultures by promoting cell clusters to form continuous sheet-like precartilaginous condensations. However, cultures exposed to both BMP-2 and Wnt-7a showed inhibition of chondrogenic differentiation. Different signaling molecules such as Wnt-7a and BMP-2 may have antagonistic effects on cartilage differentiation and the gradient of the two molecules may be involved in defining the boundaries of the initial precartilaginous condensation. We propose that the shape of the precartilaginous condensations may be modulated by local concentrations of signaling molecules, such as Wnt-7a and BMP-2, which act to alter cell-substrate and cell-cell adhesions. J. Cell. Physiol. 180:314–324, 1999. © 1999 Wiley-Liss, Inc.

In embryonic limb development, the limb bud first appears as a rounded protrusion from the lateral flank and consists of homogeneous mesenchymal cells capped by a specialized epithelium, the apical ectodermal ridge (AER). Beneath the AER is the progress zone, composed of mesenchymal cells that remain in a proliferative nondifferentiated state. These cells contribute to the outgrowth of the limb (reviewed in Tickle, 1995; Johnson and Tabin, 1997). As cells leave the influence of the AER, they undergo condensation and subsequent chondrogenic differentiation to form a central cartilaginous core. This central cartilaginous core is then shaped to become the cartilaginous anlage of the future bony skeleton (reviewed in Hall and Miyake, 1995). High- density chick limb bud cell or micromass cultures are ideal to analyze in vitro the processes of mesenchymal condensation and chondrogenic differentiation, which we term "chondrogenesis" (Ahrens et al., 1977; DeLuca et al., 1977; Caplan, 1991). Since micromass cultures can be easily manipulated and are isolated from the effects of overlying ectoderm, studies using this culture system can provide a different perspective to studies that manipulate the in vivo limb patterning process (Johnson and Tabin, 1997).

Many of the limb patterning molecules have now been identified (reviewed in Johnson and Tabin, 1997). Among them, Wnt-7a and engrailed are involved in dorsoventral axis determination, sonic hedgehog and Hox D in anteroposterior axis determination, and fibroblast growth factor family (FGFs) members, Msx and Hox A in proximodistal axis determination. In vivo, the shape of the formative skeleton is thus determined by the interaction between competent mesenchymal cells and their environment. We have been using micromass

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cultures to study the effect of "patterning" genes on the regulation of the initiation and differentiation of the cartilaginous anlage (Stott and Chuong, 1997) . Many growth factors expressed in the early developing limb bud have been shown to modulate chondrogenic differentiation in this system (Carrington et al., 1991; Chen et al., 1991; Jiang et al., 1993; Downie and Newman, 1994; Lee and Chuong, 1997). Adhesion molecules expressed in the precartilaginous condensation in vivo such as fibronectin, NCAM, and N-cadherin have also been shown to be involved in the formation of the precartilaginous condensation in micromass culture (Frenz et al., 1989; Widelitz et al., 1993; Oberlender and Tuan, 1994).

To expand further the usefulness of the micromass culture system, we have recently developed a new method to apply RCAS retroviral technology (Morgan and Fekete, 1996) to micromass cultures (Stott et al., 1998). We have used this technique to show that the *hedgehog* signaling pathway induces characteristics of hypertrophic cartilage in chondrogenic cell culture (Stott and Chuong, 1997) as well as inhibits cartilage differentiation in vivo indirectly via PTHrP (Vortkamp et al., 1996). This technique allows us to explore the role of many factors involved in chondrogenesis, when the proteins are either not available or are intracellular molecules, and therefore not accessible to antibody perturbation. Here we describe the use of this technique to study the effect of Wnt-7a on mesenchymal cell condensation and differentiation in micromass cultures.

Multiple members of the Wnt gene family of secreted growth factors have been shown to be expressed in the developing limb bud (Dealy et al., 1993; Parr et al., 1993). Wnt genes play important roles in growth control and fate determination in other areas of embryonic development including the developing nervous system and kidney (Moon et al., 1997a). This suggests that these genes may also play similar critical roles in the developing limb. One member of the Wnt gene family, Wnt-7a, is expressed in the dorsal ectoderm of the developing limb bud prior to the appearance of the precartilaginous condensation (Dealy et al., 1993; Akita et al., 1996). The ectoderm is known to inhibit cartilage formation in micromass culture in vitro (Zanetti and Solursh, 1986). Therefore, we chose to study the roles of Wnt-7a in chondrogenesis in micromass cultures. While our work was in progress, an independent study showed that overexpression of Wnt-1 and Wnt-7a can inhibit cartilage formation in high-density limb bud cell culture (Rudnicki and Brown, 1997). However, their work was done by adding retroviruses directly into micromass cultures.

Our-two stage protocol for retroviral infection of micromass culture includes a preliminary 48-h low-density plating period and leads to a high rate of exogenous gene expression at the time of high-density plating (Stott et al., 1998). This is crucial as it can take up to 18 h for RCAS- mediated gene expression to be detectable (Morgan and Fekete, 1996). Many key events in chondrogenic differentiation occur in the first 18 h after high-density plating so it is important to use this protocol to perturb early events in precartilaginous condensation formation.

Here we report our findings and further analyses. The responses to Wnt genes in other systems have been linked to changes in cell adhesion and cell movements (reviewed in Moon et al., 1997a). Because cell adhesion molecules play important roles in cartilage formation in vivo and in vitro (Chuong et al., 1993; Newman, 1996), we asked whether the inhibition of chondrogenic differentiation induced by Wnt-7a could be linked to alterations in cell condensation and cell adhesion molecule expression. We identified five successive formative stages and characterized the cellular events of each stage. We then used the newly developed retroviral protocol (Stott et al., 1998) to ectopically express Wnt-7a in micromass cultures. Using this technique, we were able to map the chondroinhibitory effect of Wnt-7a to the specific formative stage of precartilaginous condensation and to show the profound effect on the distribution of several cell-cell and cell-substrate adhesion molecules.

Bone morphogenetic proteins (BMPs) have been shown to be involved in skeletal development and regeneration (reviewed by Riley et al., 1996; Yamashita et al., 1996; Reddi, 1998; Wozney and Rosen, 1998). BMP-2 is thought to be one of the signals that controls recruitment of cells into the developing cartilaginous condensation (reviewed in Reddi, 1998). Overexpression of BMP-2 in the embryonic chick limb bud by either retrovirus (Duprez et al., 1996a) or implanted BMP-2-soaked beads (Macias et al., 1997) leads to increased skeletal volume and radial growth of differentiated cartilage. The total cell number is not increased, suggesting recruitment of undifferentiated mesenchymal cells into the chondrogenic differentiation pathway rather than increased proliferation of existing chondrogenic cells. We have shown that, in micromass culture, BMP-2 produces a sheet- like condensation of cartilage rather than nodules of cartilage, an effect that appears to be mediated through the protein kinase A (PKA) pathway (Lee and Chuong, 1997). Because of the ability of BMP-2 to recruit cells into a chondrogenic lineage, we tested the effect of BMP-2 on Wnt-7a-expressing micromass cultures. We found that Wnt-7a-treated cultures were resistant to the chondrogenic effect of BMP-2.

MATERIALS AND METHODS

Materials

Fertilized chicken eggs were purchased from SPAFAS (Preston, CT). Chicken embryos were staged according to Hamburger and Hamilton (1951). Replication competent avian retrovirus vectors RCAS BP (A) and RCAS BP (A) carrying mouse Wnt-7a were kind gifts from Dr. Stephen H. Hughes, Dr. Lee Niswander, and Dr. Andrew McMahon. Mouse anti-Gag antibody was provided by Dr. Bruce Morgan. In situ probes against mouse and chick Wnt-7a were kind gifts from Dr. G. Shackleford and Dr. Anthony Brown, respectively. Recombinant BMP-2 was obtained from the Genetics Institute (Cambridge, MA). Primary antibodies include rabbit anti-NCAM (Chuong and Edelman, 1985), mouse anti-tenascin-C M1B4 (Chiquet-Ehrismann et al., 1986), mouse anti- fibronectin B3D6 (Gardner and Fambrough, 1983), mouse anti-desmin D3 (Danto and Fischman, 1984), rabbit anti-collagen II (Dr. Richard Mayne), and mouse anti-N-cadherin (6B3) (from Dr. K. A. Knudsen). M1B4, B3D6, and D3 are

from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA).

Scanning electron microscopy

Micromass cultures were fixed in one-half strength Karnovsky's fixative overnight at 4°C, postfixed in thiocarbohydrazide, and followed by osmium fixation. The specimens were critically point dried and coated with gold palladium. They were viewed with a Hitachi S-570 scanning microscope.

Retrovirus transduction in micromass cultures

Retrovirus was prepared as described by Morgan and Fekete (1996) and Stott et al. (1998). Briefly, dorsal hypodermal fibroblasts from SPAFAS chicken E8 embryos were used as retrovirus-producing cells. Cells were transfected with retroviral vector DNA using lipofectamine (Life Technologies, Rockville, MD). They were subsequently subcultured into 100-mm dishes (Falcon, Beaumont, TX) and grown in complete avian medium (Ham's F-12, 12.5% fetal calf serum [FCS], 5% chicken serum). The retrovirus-containing medium was collected when cells reached 70% confluence and filtered through 0.45-μm filters and stored at -70°C. The retrovirus was titered using anti-gag immunostaining (Morgan and Fekete, 1996).

Retroviral infection of micromass cultures was performed as described in Stott et al. (1998). Dissociated mesenchymal cells were prepared from the distal one third of stage 23/24 leg buds. 3×10^6 cells were incubated with 3 ml of viral medium for 2 h at 4°C. The cells were then plated at low density (5.5×10^4 cells/cm^2) on 100-mm collagen type I-coated dishes (Falcon). They were then cultured in defined medium (60% Ham's F-12 nutrient mixture/ 40% Dulbecco's modified Eagle medium [DMEM], 5 μg/ml insulin, 5 μg/ml transferrin, 50 μg/ml L-ascorbic acid, 100 nM hydrocortisone) without serum (Paulsen et al., 1994) at 37°C/5% CO_2/95% air. At 48 h, the cells were trypsinized with 0.015% trypsin-collagenase, passed through a Cell Microsieve (20 μm; BioDesign, Carmel, NY) to ensure a single cell suspension, resuspended at a concentration of 2×10^7 cells/ml, and plated as a 10-μl drop on a spot precoated with collagen type I (UBI, NY; 50 μg/ml). After 2 h of incubation to allow attachment, the dishes were flooded with 1.5 ml of defined medium. Cultures were incubated at 37°C/5% CO_2/95% air and stopped at defined points and analyzed.

In situ hybridization

cDNA encoding 400 bp of mWnt-7a was obtained in Bluescript SK+. Sense probe was linearized with BamH1 and transcribed with T3. Antisense probe was linearized with Xho1 and transcribed with T7. The probes were labeled using a digoxigenin RNA-labeling kit (Boehringer- Mannheim, Indianapolis, IN). Cell in situ hybridization was performed as described by O'Keefe et al. (1994), with some modification. Cultures were hybridized at 55°C in 50% formamide (Sigma Chemical Co., St. Louis, MO), 5 × SSC, 0.5 mg/ml sonicated herring sperm DNA (Life Technologies), 0.25 mg yeast tRNA, 20% dextran sulfate, and 1 × Denhardt's solution, probe concentration 100 ng/ml. Posthybridization washes included a 30-min digestion with RNase A 50 μg/ml at 37°C and a 10-min wash in 50% formamide, 2 × SSC at 55°C. The cultures were preblocked with Tris-NaCl (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 10% heat inactivated FCS, incubated with a 1:500 dilution of sheep anti-digoxigenin Fab fragments, and alkaline phosphatase labeled (Boehringer-Mannheim) for 3 h. After washing with Tris-NaCl, the cultures were incubated with BM purple (Boehringer-Mannheim). Color development at room temperature was stopped by the addition of a solution containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

Immunocytochemistry

Immunostaining was performed as described in Lee and Chuong (1997). Micromass cultures were fixed with Bouin's fixative or 2.5% paraformaldehyde for 20 min followed by washing with 70% ethanol. Specimens were incubated with primary antibodies overnight, followed by secondary biotinylated horse anti-mouse antibodies and avidin-alkaline phosphatase conjugate (Vector, Burlingame, CA) or alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega, Madison, WI). NBT/BCIP were used as a substrate for alkaline phosphatase.

Quantitative measurement of micromass cultures

DNA assay. A fluorimetric method (Labarca and Paigen, 1980) was used to quantify DNA per culture. Cells were scraped off the dish into 500 μl of 0.05 M Tris, pH 7.5, then briefly sonicated. A 100-μl aliquot was mixed with 1.9 ml of assay buffer containing 2 M NaCl, 0.05 M Na_2HPO_4, pH 7.4, and 10 ng/ml Hoechst dye. The emission at 458 nm was read using a Hitachi (Tokyo, Japan) 2000 fluorescent spectrophotometer and the reading compared to a standard curve constructed using calf thymus DNA.

Quantitation of chondrogenic differentiation. Micromass cultures were fixed with 2.5% paraformaldehyde in phosphate-buffered saline (PBS) and stained with 1% Alcian blue 8GX in 0.1 N HCl, pH 1 (Lev and Spicer, 1964; Hassell and Horrigan, 1982) for 3 h, which stains cartilage-specific sulfated proteoglycans (Leonard et al., 1991). Stained cultures were destained with 70% ethanol. The bound Alcian blue dye was extracted with 0.5 ml 4 M guanidine HCl (pH 5.8) and quantified by measuring absorbance at OD 600 nm.

Alkaline phosphatase assay. Alkaline phosphatase activity was quantitated as described by Pacifici et al. (1991) with some modification. Briefly, a 100-μl aliquot of sonicated cells was incubated with 0.05 M Tris, pH 7.4, with 0.02% Triton X for 30 min on ice and then mixed with 750 μl of 1.5 M Tris, pH 9.5, 1 μl 1 M $MgCl_2$ and 49 μl of H_2O to a final volume of 1 ml. Spectrophotometric readings at 410 nm were made at fixed time points and converted to nanomoles of p-nitrophenol released per minute per milligram of DNA using a standard curve.

RESULTS

In micromass cultures, the formation of cartilage nodules from dissociated mesenchymal cells is characterized by five successive stages

When stage 23/24 chick limb bud cells are plated as high-density cultures, they first form small aggregates

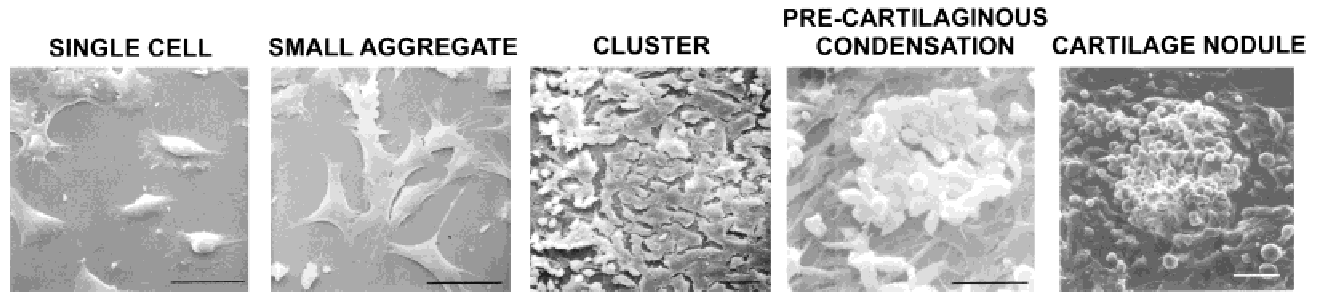

Fig. 1. Successive stages of cartilage nodule formation in micromass cultures. Scanning electron microscopic pictures from chicken limb bud micromass cultures. Five stages of precartilaginous condensation from micromass cultures are shown. The two left panels are from 4-h cultures. The right three panels are from 18, 48, and 72-h cultures, respectively. Note the randomly orientated spindle-shaped cells make contact to form small cell aggregates. Some aggregates progress to become cell clusters as cells become polygonally shaped. Cells then round up and rearrangement occurs during the formation of precartilaginous condensations. Finally, the condensations differentiate into cartilage nodules. Scale bar, 30 μm for the left four panels; scale bar, 40 μm for the far right panel.

that then condense to form precartilaginous condensations, and finally cartilage nodules. Extracellular matrix, rich in sulfated proteoglycans, is then secreted to form mature chondrogenic nodules (Ahrens et al., 1977; Chen et al., 1995). To more closely assess the effect of Wnt-7a on chondrogenic differentiation, we first analyzed the successive stages of cartilage nodule formation. The cells were plated at a density of 6.6×10^6 cells/ml, a density that is one third of the density normally used for regular micromass cultures. This density allowed a clearer view of cell distribution but did not prevent cartilage formation. We were able to identify five stages in the evolution of cartilage nodules (Fig. 1). These stages were defined to facilitate analysis, but we should remember that the formation of cartilage nodules is a continuous process. With many new signaling molecules identified recently (Tickle, 1995; Johnson and Tabin, 1997), this staging will be helpful for the identification of the signaling molecules involved in the progression or inhibition of each stage. We have also noted that when limb bud cells are plated as a single drop, a cell density gradient forms with decreasing density from the center to the periphery. Therefore, maturation is more advanced in the center, providing a spectrum of several intermediate stages that can be observed in one single culture. The following are summarized from observations of 4, 18, 48, and 72-h cultures.

Dissociated mesenchymal cells. Immediately after initial plating, there are many individual cells. At the periphery, some are completely isolated and some have contact with others through the cellular process.

Small cellular aggregates. Within the first few hours, small cellular aggregates appear. Aggregates are defined as at least three cells. Many cells are spindle shaped and some are radially orientated toward the center of the aggregates, suggesting high cell motility.

Cell clusters. The aggregates stabilize and grow in size to form cell clusters that are about 20 cells in size. The cells in the center of the aggregate become polygonal in shape and closely opposed to each other. The contour of clusters gradually become round, but the outside boundary is still uneven.

Precartilaginous condensations. Cell clusters progress to form precartilaginous condensation. This stage is characterized by the beginning of specification of cell fate. Cells in the central region of the condensation remain polygonal and intercellular boundaries become unclear. Cell layers at the outer edge become elongated and apposed to the perimeter of the condensation, thus forming the putative perichondrium. This leads to the smooth and more organized appearance of the precartilaginous condensations.

Cartilage nodules. Cells begin to differentiate and form more compact cartilage nodules. Maturation of the nodule starts in the center and spreads radially to the border.

Many signaling molecules are involved in the progression of these stages. In this study, we studied the effect of Wnt-7a and BMP-2.

Ectopic expression of Wnt-7a disrupts the formation of precartilaginous condensations

To express Wnt-7a in high-density micromass cultures, we infected the limb bud cells with the replication competent avian sarcoma retroviral vector RCAS BP (A) carrying the mWnt-7a construct (RCAS Wnt-7a) (Yang and Niswander, 1995), using a protocol recently developed in our lab (Stott et al., 1998).

As expected, limb bud cells infected with the RCAS vector showed the normal pattern of differentiation with well-defined cartilaginous nodules (Fig. 2A,B). In contrast, the limb bud cells infected with the RCAS Wnt-7a virus showed a change in cellular morphology by 72 h of high-density plating (Fig. 2A',B'). Over the first 48 h, these cultures formed large flattened irregular clusters of cells connected by longitudinally aligned cells. These cell clusters remained irregularly shaped and did not progress to form the smooth-contoured precartilaginous condensations or cartilage nodules as seen in normal micromass condensations by 72 h. These irregular clusters did not express Alcian blue positive glycosaminoglycan nor type II collagen at 72 h. Quantitative measurement can be seen in part of Figure 5. Continued culture past 96 h showed that the condensations remained arrested at that point with no further progression to form cartilage nodules (not shown).

To confirm the presence of mWnt-7a, we carried out in situ hybridization using a 400-bp probe that hybridizes to the 3′ end of the mWnt-7a gene. This probe does not recognize the endogenous chicken Wnt-7a gene. In

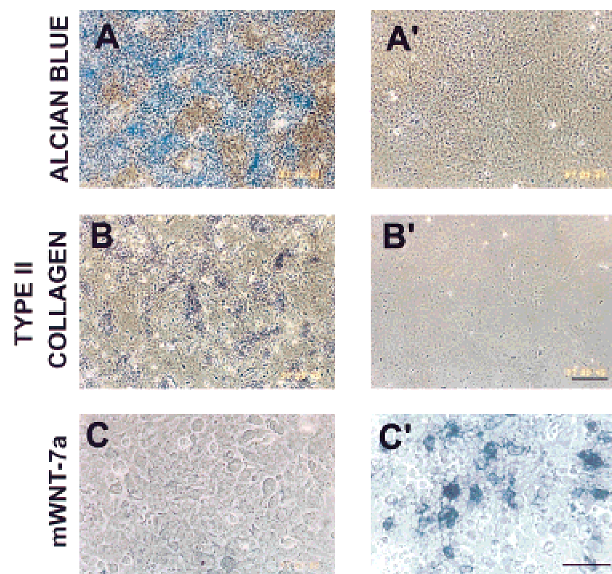

Fig. 2. Effect of Wnt-7a gene expression on chondrogenic differentiation. High-density cultures were prepared from primary limb bud cells that had been infected with either RCAS virus or RCAS Wnt-7a virus. **A, A':** Higher power view of cultures in A,B. Alcian blue stained nodules are clearly seen in the RCAS-infected cultures (A). RCAS Wnt-7a cultures show a flattened appearance with large irregularly shaped condensations that are negative for Alcian blue staining (A'). **B, B':** Micromass cultures immunostained for type II collagen after 3 days of high-density culture. RCAS-infected cultures express type II collagen strongly in the cartilaginous nodule (B) while RCAS Wnt-7a-infected cultures are negative for type II collagen (B'). Note: Cells were plated at a density of 6.6×10^6 cells/ml. Scale bar for B, B' is 200 μm. **C, C':** Retroviral-induced ectopic mWnt-7a expression. In situ hybridization shows many cells exposed to RCAS Wnt 7a do express mWnt-7a mRNA (C'). Cells infected with RCAS vector are negative for mWnt-7a mRNA (C). Scale bar for C, C' is 150 μm.

situ hybridization 5 h after high-density plating showed a random distribution of mWnt-7a positive cells, representing the source of exogenous Wnt-7a (Fig. 2C, C'). RCAS-infected cultures were negative when probed with the antisense construct. (Sense probes were also negative. Data not shown.) However, hybridization with chick Wnt-7a at 5 and 20 h after secondary high-density plating did not show any endogenous Wnt-7a expression in RCAS or RCAS mWnt-7a-infected cultures.

Ectopic expression of Wnt-7a alters the distribution of cell-cell and cell-substrate adhesion molecules

The responses to Wnt genes in other systems have been linked to changes in cell adhesion and cell movements (reviewed in Moon et al., 1997a). We asked, therefore, if the response to Wnt-7a in micromass culture could also be transduced through alterations in cell adhesion molecule expression. Infection with the RCAS viral vector does not alter the temporospatial expression pattern of several cell adhesion molecules. The difference between RCAS control and RCAS Wnt-7a-infected cultures is not obvious during the first 48 h of culture (not shown). At 72 h, the difference became striking (Fig. 3). This suggests that Wnt-7a does not inhibit the initiation of the precartilaginous condensation, but perturbs the subsequent differentiation into a cartilage nodule. This is the stage that we focused on in our immunostaining experiments.

NCAM. In both nonvirus-transduced and RCAS-infected cultures, NCAM expression was initially enriched in the areas of condensation. By 3 days, NCAM staining had diminished from the cartilage nodule. In the RCAS Wnt-7a-infected culture, the cultures continued to express NCAM even after 3 days of high-density culture.

N-cadherin. By 3 days, N-cadherin staining in the precartilaginous condensation had reduced. In the RCAS Wnt-7a culture, the cultures continued to express N-cadherin at 72 h.

β1 integrin. In control, β1 integrin was expressed widely in clusters, then it became restricted to the precartilaginous condensations, finally disappearing from the nodules. In the RCAS Wnt-7a-infected cultures, β1 integrin remained widely distributed.

Fibronectin. Fibronectin staining was enriched in the center of the cartilage nodules in the RCAS-infected cultures by 72 h. In the RCAS Wnt-7a-infected cultures, fibronectin was expressed at high levels in the irregular condensations and in a much more diffuse pattern.

Tenascin-C. Tenascin-C was highly expressed in the RCAS-infected culture in the central cartilaginous nodule at 3 days but was also expressed in cells connecting the cartilage nodules. In contrast, immunostaining for tenascin-C remained very low in the RCAS Wnt-7a cultures.

Desmin. Immunostaining for desmin, a myogenic marker, was also performed as a control. Both the control and the mWnt-7a-transduced cultures contained a small number of desmin positive cells, suggesting that muscle lineage is unaffected.

The above results imply that overexpression of Wnt-7a has a profound effect on the distribution of several major cell-cell and cell-substrate adhesion molecules that have been shown to be involved in the formation of precartilaginous condensation.

Wnt-7a alters the responsiveness of limb bud cells to BMP-2

Members of the BMP family of growth factors act to modulate chondrogenic differentiation in vitro and in vivo (Carrington et al., 1991; Chen et al., 1991). BMP-2 promotes chondrogenesis in chick limb bud mesenchymal cells (Lee and Chuong, 1997) and appears to have a maximal effect at the time of cellular condensation (Roark and Greer, 1994) . We asked whether exogenous BMP-2 added to the media could overcome the inhibition of chondrogenic differentiation induced in RCAS Wnt-7a-infected cultures. Cultures were plated at a density of 2×10^7 cells/ml and recombinant human BMP-2, concentration 100 ng/ml, was added to the media at time of plating as a single dose. This concentration of BMP-2 was chosen as it leads to maximal effect in our system (Lee, 1996). The RCAS-infected control cultures showed a marked increase in chondrogenic differentiation after staining with the Alcian blue dye (Fig. 4A–D). In contrast, the RCAS Wnt-7a-infected cultures, with or without BMP-2, did not show any staining with the Alcian blue dye (Fig. 4E–H).

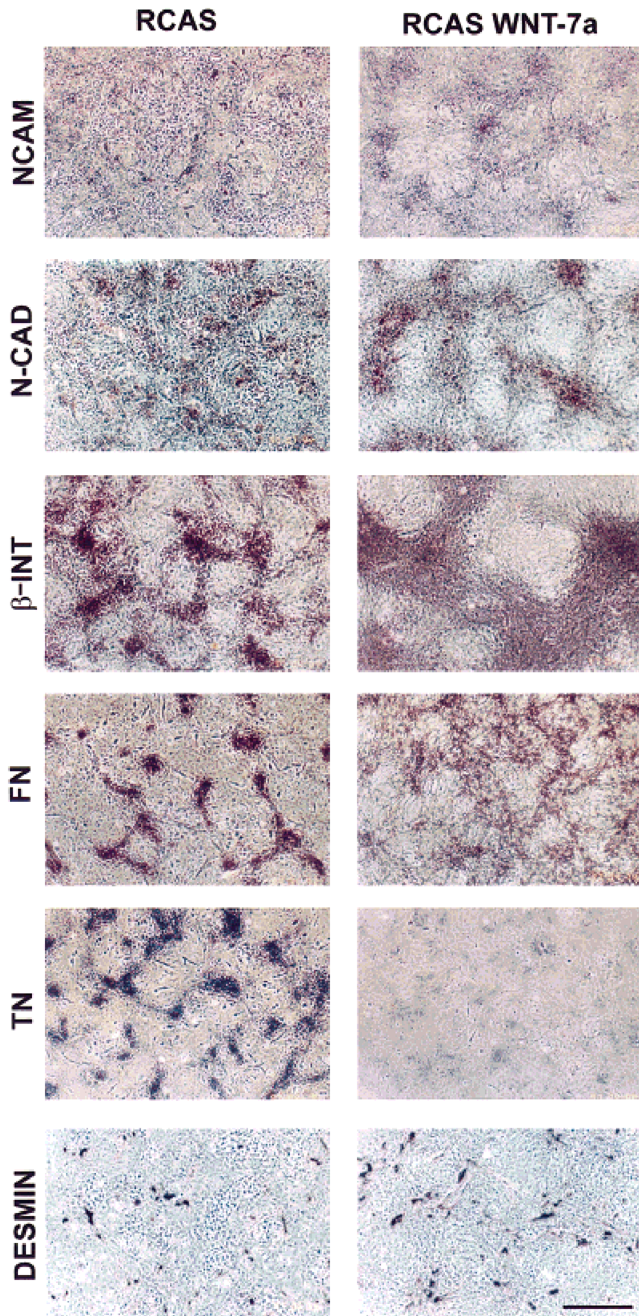

Fig. 3. Effect of Wnt-7a overexpression on expression of NCAM, N-cadherin, fibronectin, and tenascin-C. Immunostaining of RCAS (**left column**) and RCAS Wnt-7a (**right column**)-infected high-density cultures after 72 h of high-density culture. NCAM: NCAM expression is transient and at this stage it has disappeared from the normal cartilaginous nodules, but continues to be present in the cell clusters in the RCAS Wnt-7a-infected cultures. N-CAD: In RCAS-infected cultures, N-cadherin expression in the cartilaginous nodules is much diminished. In RCAS Wnt-7a-infected cultures, the expression levels of N-cadherin remain high. β-INT: In control cultures, integrin β1 is enriched in the nodules and in cells that connect nodules. In Wnt-7a-infected cultures, integrin β1 remains diffusely distributed. FN: In the RCAS-infected control culture, fibronectin continues to be expressed, but is limited to the cartilaginous nodule. In RCAS Wnt-7a-infected culture, fibronectin is diffusely distributed. TN: In control, tenascin-C is strongly positive in the normal cartilaginous nodules but is expressed at very low levels in the RCAS Wnt-7a-infected cultures. Desmin: Desmin is a myogenic marker. Single cells positive for desmin are seen in both RCAS and RCAS Wnt-7a-infected cultures. Scale bar, 350 μm.

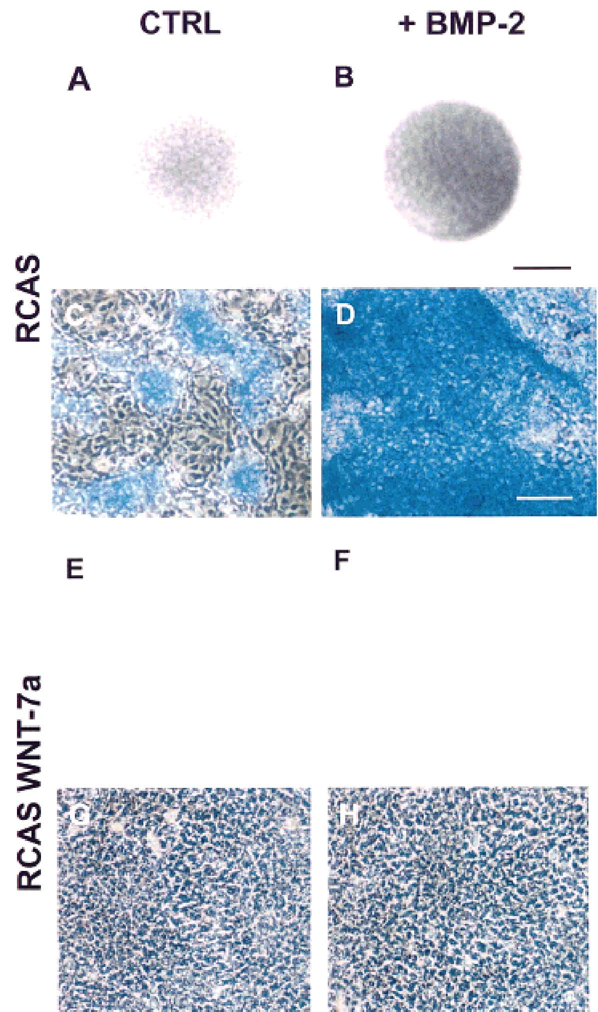

Fig. 4. Effect of BMP-2 on chondrogenic differentiation in Wnt-7a-infected cultures. A–H: Whole mount micromass cultures stained with Alcian blue after 4 days of culture. Cultures were infected with RCAS virus or RCAS Wnt-7a virus for 2 h and then plated at low density for 48 h. A single dose of BMP-2, 100 ng/ml, was added to the defined media at the time of high-density plating. RCAS-infected cultures show increased Alcian blue staining in response to BMP-2, both at low power (**A,B**) and high power (**C,D**). RCAS Wnt-7a-infected cultures (**E–H**) show no Alcian blue staining, with or without BMP-2. The results show that Wnt-7a has altered the responsiveness of the limb bud cells to BMP-2. Scale bar: A,B, 2 mm; C–H, 150 μm.

BMP-2 did not significantly change DNA content in either the RCAS-infected cultures or the RCAS Wnt-7a-infected cultures (Fig. 5A). However, extraction of the Alcian blue dye and normalization to DNA content showed that BMP-2 induced a threefold increase in Alcian blue positive staining in the RCAS-infected controls (average of three independent experiments ± SD) (Fig. 5B). A twofold increase in alkaline phosphatase activity was also seen in the RCAS cultures exposed to BMP-2 100 ng/ml (Fig. 5C). In contrast, the RCAS Wnt-7a-infected cultures did not show any increase in Alcian blue dye uptake or in alkaline phosphatase activity in response to BMP-2.

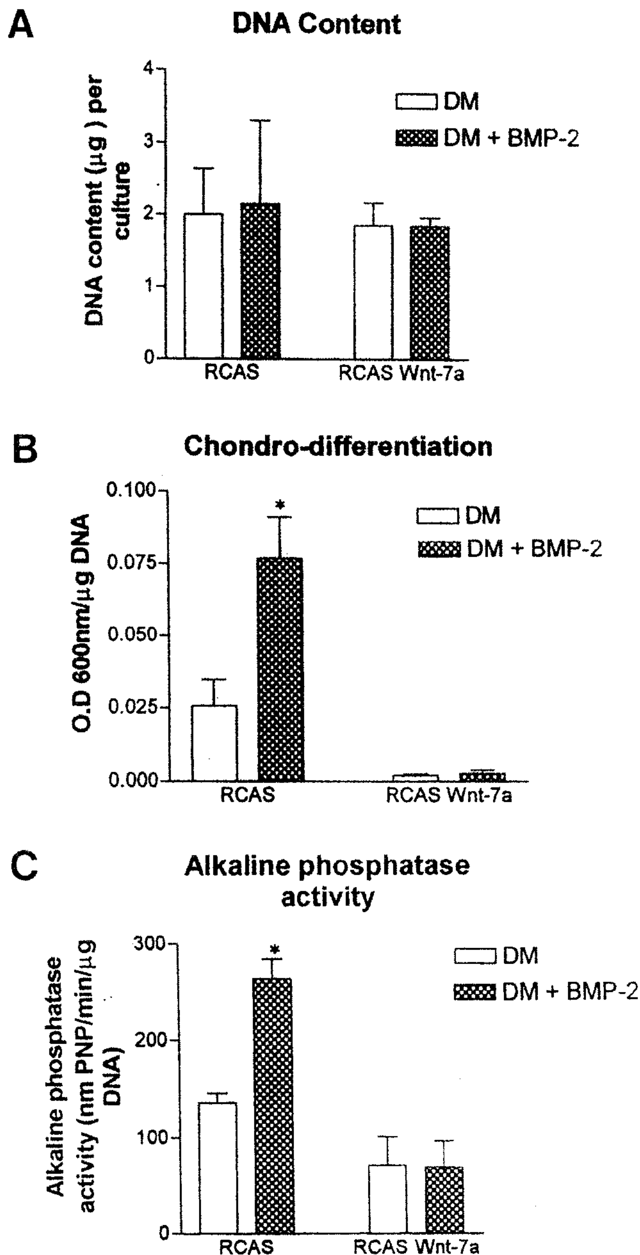

Fig. 5. Effect of BMP-2 on alkaline phosphatase activity and DNA content in RCAS Wnt-7a-infected cultures. **A:** Bar histogram of DNA content per culture after 4 days of high-density culture. DNA content was not increased by BMP-2 in either the RCAS-infected cultures or the RCAS Wnt-7a-infected cultures. Each bar represents the mean ± SD of three independent experiments. DM, defined media. **B:** Bar histogram of extracted Alcian blue dye normalized to DNA content. OD readings for BMP-2-treated cultures were normalized to the nongrowth factor-treated cultures. Note that readings for RCAS Wnt-7a-infected cultures were at background level in both BMP-2-treated and nontreated cultures. Each bar represents the mean ± SD of three independent experiments, $P < 0.05$ for Student's t-test. **C:** Bar histogram of alkaline phosphatase activity normalized to DNA after 4 days of secondary high-density culture shows increased alkaline phosphatase activity in the BMP-2-treated RCAS-infected cultures. The mWnt-7a-infected cultures do not show any change in alkaline phosphatase activity when treated with BMP-2. Each bar represents the mean ± SD of three independent experiments, $P < 0.05$ Student's t-test.

DISCUSSION

Cartilage nodules in micromass cultures are formed by successive morphogenetic stages

We and others have shown that progression from individual mesenchymal cells to cartilage nodules in in vitro micromass cultures is associated with a defined series of changes in adhesion molecule expression (Chuong et al., 1993; Chen et al., 1995; Hall and Miyake, 1995). These changes modulate mesenchymal cell-cell and cell-substrate interactions and help to recruit cells into the formation of initial cell aggregates. NCAM, N-cadherin, tenascin, and fibronectin have been previously shown to have highly specific temporospatial expression patterns in micromass culture (reviewed in Lee et al., in press; Hall and Miyake, 1995; Chuong et al., 1993). To further study the process of chondrogenesis, we first demonstrated the stepwise progression from dissociated mesenchymal cells to cartilage nodules in the limb bud micromass cultures (Fig. 6, Table 1). While the dissection of the process aids in the analysis, we should remember that it is actually a continuous process. The first stage is used to show the beginning point, the cells as dissociated cells. In the second stage, cells start to explore the environment. Since they are adhesive, they start to form small aggregates. These aggregates then enlarge into clusters of approximately 100 µm in diameter by a process of cell division and cell recruitment into the aggregate. This is a unique property since other chicken embryonic fibroblasts such as those from developing skin will not form dermal condensations without the presence of epithelium (Chuong et al., 1996). This is also different from fibroblastic cell lines that will form monolayers or whirls in the culture dish. The formation of multiple condensations in these cultures offers a unique opportunity to study periodic pattern formation, similar to work we have reported for dermal condensations (Jung et al., 1998). Since here Wnt-7a appears to act in later stages (see below and Fig. 6), we have focused on the stage when the already formed aggregates/clusters become precartilaginous condensation.

The process of condensation formation is cell density dependent (Ahrens et al., 1977). The differentiation process is more advanced in the center of the culture, the condensations forming faster in areas where the cell density is higher. To improve the visualization and analysis of the progression of the condensation process, we modified the high-density culture to a slightly lower cell density similar to the lower cell density region at the periphery of the regular culture (Fig. 1). These regions also form condensations and nodules, but events are more clear allowing better visualization of interactive events between the cells.

To test the modulatory effect of limb patterning molecules on the formation of precartilaginous condensations, we have explored the roles of Wnt-7a and BMP-2 and how they may act together. We showed that these limb patterning signaling molecules can modulate the formation of precartilaginous condensations by altering the expression of adhesion molecules. The approach here also acts as a paradigm for further analyses of the relationships between signaling molecules and adhesion molecules during limb patterning.

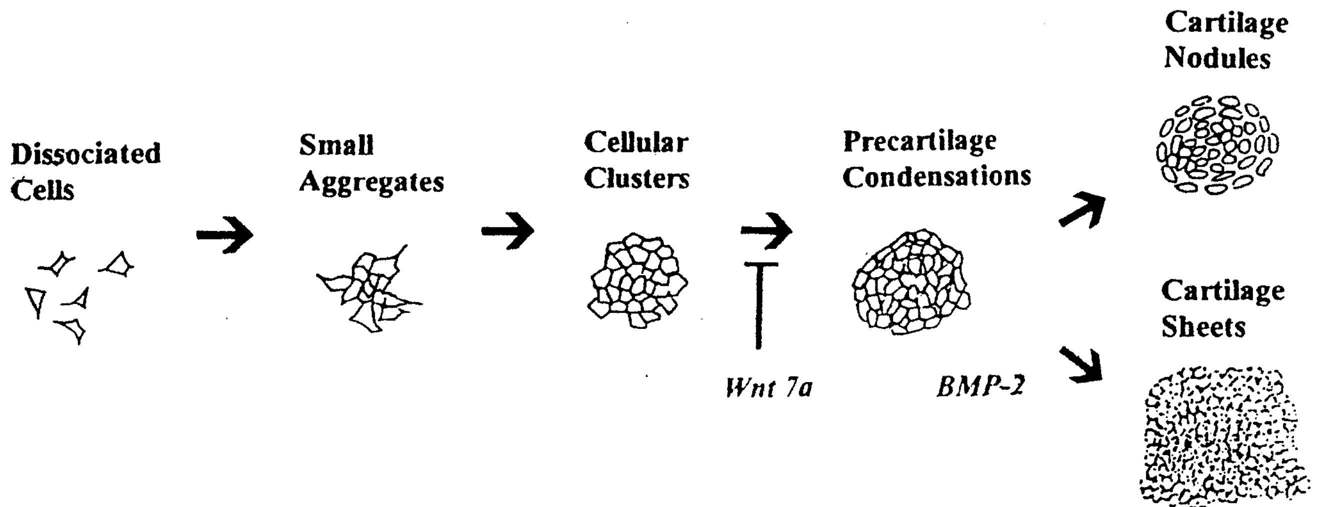

Fig. 6. Schematic summary of the successive stages of cartilage nodule formation in vitro and the modulatory effect of Wnt-7a and BMP-2. Different growth factors may either modulate chondrogenic differentiation or act to mold different shapes of skeleton. Formation of a sheet of cartilage induced by BMP-2 may branch off at the cell cluster stage or the precartilaginous condensation stage. Wnt-7a blocks progression through the precartilaginous condensation stage.

TABLE 1. Successive stages of precartilage condensation formation

	Dissociated cells	Small aggregates	Cellular clusters	Precartilage condensations	Cartilage nodules
Cell number	1	3–20	Around 20	Up to hundreds	Up to hundreds
Cell arrangement	Randomly distributed	Randomly or radially oriented	Rearranged for maximal neighboring	Closely arranged/ circumferentially arranged	Tightly packed
Cell shapes	Spindle	Spindle	Polygonal	Round inside, elongated outside	Round inside, elongated outside
Contour of cell groups	NA	Highly irregular	Round, but uneven	Smoothed by perichondrium-like cells	Wrapped in putative perichondrium
Reversibility	NA	+	+	−	−
Differentiation	—	−	−	Beginning from the center	+

Wnt-7a arrests the formation of cartilage nodules but not the initiation of precartilaginous condensation formation

Ectopic expression of Wnt-7a in high-density limb bud cell culture causes a dramatic change in morphology with production of flattened spread out cells that form large irregular condensations but do not undergo chondrogenic differentiation. The RCAS Wnt-7a-infected cultures do not produce extracellular matrix containing sulfated proteoglycans and do not express type II collagen. In situ hybridization demonstrated a random distribution of exogenous Wnt-7a gene expression just after secondary high-density plating and at 20 h after high-density plating in the Wnt-7a-infected cultures. The altered phenotype was, however, seen in all cells. This finding is consistent with studies of other Wnt genes which have shown non cell autonomous signaling and suggests that Wnt genes, like Wg, act as long range secreted morphogens (reviewed in Siegfried and Perrimon, 1994). A limitation of the RCAS retroviral delivery system is that it is not practical to do a Wnt-7a dose response curve to test the effect of lower doses of Wnt-7a on chondrogenic differentiation. As well, we cannot generate the gradient of Wnt-7a levels in this model that would be seen in vivo. Certainly, one future direction will be to test the response to Wnt-7a protein when this becomes available. Micromass cultures are heterogeneous, however the Wnt-7a transcript was not expressed during the early stages of aggregate formation, suggesting that other factors act to specify aggregate initiation and boundaries in micromass culture.

Cell adhesion molecules are thought to influence development by effects on cell interaction, migration, and sorting. Prior to the onset of condensation, the limb bud cells produce a matrix rich in fibronectin (Dessau et al., 1980; Frenz et al., 1989), hyaluronic acid (Knudson and Toole, 1985), tenascin (Mackie et al., 1987), and type I collagen (Dessau et al., 1980). These extracellular matrix molecules are thought to drive the translocation of cells, promoting the formation of the initial cellular aggregate via alterations in cell-substrate adhesion. The cell-cell adhesion molecules, NCAM and N-cadherin, are both expressed transiently in early condensations but disappear from the mature cartilage nodule (Widelitz et al., 1993; Oberlander and Tuan, 1994). Perturbation of NCAM or N-cadherin-mediated cell adhesion inhibits limb bud mesenchymal cell aggregation

and subsequent chondrogenesis (Wideiltz et al., 1993; Oberlender and Tuan, 1994). Both fibronectin and tenascin are strongly expressed in the maturing cartilage nodule. Different regions and isoforms of fibronectin may play different roles in the condensation process (Frenz et al., 1989; Gehris et al., 1997). The increased expression of tenascin is thought to facilitate cell rounding to form precartilaginous condensation (Mackie et al., 1987). Finally, chondrogenic differentiation is accompanied by the disappearance of many of these adhesion molecules such as NCAM and N-cadherin.

The alteration in morphology in the RCAS Wnt-7a-infected cells was first detected by light microscopy after 48 h. Both RCAS and RCAS Wnt-7a-infected cultures formed condensations over the first 48 h. However, the condensations in the Wnt-7a-infected cultures were more irregular in shape than those seen in control cultures and did not progress to the smoother contoured, more compact precartilaginous condensations seen in control cultures. Rudnicki and Brown (1997) noted that expression of proliferating cell nuclear antigen (PNA) and NCAM was normal at 48 h in Wnt-7a-infected cultures. We also found that the expression of cell adhesion molecule expression was similar to controls over the first 48 h. However, by 72 h, the Wnt-7a-infected cultures showed characteristics of a less mature culture, suggesting the inhibition step is not the initiation of the condensation, but the differentiation into the precartilaginous condensation stage. If this is correct, there should be persistent expression of adhesion molecules similar to their expression pattern in early stages. There is indeed high expression of NCAM and N-cadherin persisting beyond their normal expression period. This implies that the adhesive properties between cells are altered and are consistent with the failure for the cells to rearrange and progress into precartilaginous condensations.

There is also a wider distribution of fibronectin and integrin β1, suggesting an effect of Wnt-7a on cell-substrate interactions. Fibronectin has been shown to be involved in the formation of precartilaginous condensation (Frenz et al., 1989) and can modulate the size and shape of precartilaginous condensations (Downie and Newman, 1994). Tenascin expression, which is highly expressed in the center of precartilaginous condensations, remains at a low level in the Wnt-7a-treated cultures. Expression of tenascin-C has been suggested to be necessary for maintenance of the chondrogenic phenotype (Pacifici, 1995). Tenascin-C has been shown to have an antiadhesive effect and to induce cell rounding, which is thought to be required for adoption of the chondrogenic cell fate. Fibronectin and tenascin have opposite effects on cell rounding (reviewed in Chiquet-Ehrismann et al., 1995). The high levels of fibronectin expression coupled with the low expression of tenascin-C in the RCAS Wnt-7a-infected cultures suggest that cell rounding may be retarded in these cultures by an excess of fibronectin-induced cell-substrate adhesion. Whatever the molecular mechanism may be, the results are consistent with the notion that Wnt-7a, directly and/or indirectly, alters cell-cell and cell-substrate interactions and arrests the progression from cluster to precartilaginous condensation stage (Fig. 6).

Wnt-7a may restrict regions of chondrogenic differentiation by modulating the competence of limb bud cells to respond to other signaling molecules such as BMP-2

Undifferentiated mesenchymal cells in the embryonic limb bud are exposed to signals from many regions, some short range and some long range. These signals control both the early differentiation pathway adopted by a single mesenchymal cell and the subsequent patterning of the developing skeletal elements. Activin and other members of the transforming growth factor beta (TGF β) superfamily including the BMPs are all expressed in the developing limb and act to promote chondrogenesis in vitro (Kulyk et al., 1989; Leonard et al., 1991; Jiang et al., 1993; Roark and Greer, 1994). In this study, we examined how mesenchymal cells would interpret BMP-2 signaling in the presence of exogenous Wnt-7a. We found that exogenous BMP-2 added to the RCAS Wnt-7a-infected high-density cultures could not overcome the inhibition of chondrogenic differentiation. As well, the Wnt-7a-infected cultures did not show the expected increase in alkaline phosphatase activity in response to BMP-2. The data suggested that Wnt-7a had a dominant effect over BMP-2 even when the amount of BMP-2 that gave the maximal effect in our system was used.

BMPs are thought to act to enhance chondrogenesis both through regulation of apoptosis and recruitment of undifferentiated precursors into the developing cartilage nodule (Duprez et al., 1996b). BMP receptors are part of a larger family of serine/threonine kinases and exist as two types, type I and type II. Two type I BMP receptors, BMPR-1A and BMPR-1B, are expressed in the chick embryonic limb. BMPR-1B is involved in precartilage condensation formation both in vivo and in vitro, while BMPR-1A regulates chondrocyte differentiation from prehypertrophic to hypertrophic cartilage (Zou et al., 1997). In the micromass cultures, RCAS-mediated transduction of dominant negative BMPR-1B, but not dominant negative BMPR-1A, suppresses cartilage nodule formation, suggesting that the receptors mediating chondrogenetic effect are 1B. One explanation for the lack of response by the RCAS Wnt-7a-infected culture to BMP-2 is that Wnt-7a has altered the competence of these limb bud mesenchymal cells to respond to BMP-2. This may be achieved by changing the level of BMPR-1B or other molecules that act on the BMP signaling pathway such as noggin or Smads.

Another member of the Wnt gene family, Wnt-1, has been reported to induce skeletal malformation in mice (Zakany and Duboule, 1993) and localized failure of cartilage formation in chickens (Rudnicki and Brown, 1997) when expressed ectopically in the mesenchyme. Very recently, a mutation in Wnt-7a has been shown to be responsible for a classical mouse mutation, postaxial hemimelia (Parr et al., 1998). The frizzled gene family of serpentine receptors has been identified as receptors for several of the Wnt genes (reviewed in Moon et al., 1997b). A frizzled homolog, FRZB, has been shown to be present in the cartilaginous core of developing embryonic long bones in humans, suggesting that Wnt genes may act on skeletal morphogenesis through members of the frizzled gene family (Hoang et al.,

1996). Exploring the temporospatial expression and function of frizzled in the micromass culture system and the interactions with different Wnt genes will be another future direction.

In conclusion, the shaping of the skeleton is a complex and still poorly understood process. A key element in the shaping of the skeleton is the early determination of the locations and boundaries of the condensing cartilage. Different signaling molecules such as Wnt-7a and BMP-2 may have antagonistic effects on cartilage differentiation. The gradient of the two molecules may be involved in defining the boundaries of the initial precartilaginous condensation, through modulation of cell-substrate and cell-cell adhesion molecule expression. Finally, the methodology used in this study will be useful to analyze the roles of other limb patterning molecules in in vitro mesenchymal condensation and chondrogenic differentiation models (Stott et al., 1998). As more genes are identified, the use of such organotypic models will become increasingly useful in clarifying the roles of genes in chondrogenesis.

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