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EARLY EVENTS DURING PRECARTILAGE CONDENSATION IN LIMB BUD MICROMASS CULTURES

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Abstract

This paper reviews the molecular and cellular events during precartilage condensation and describes the application of a new retroviral gene transduction procedure. In micromass cultures, dissociated mesenchymal cells interact to form cartilage nodules. Cells first form small aggregates, then bigger clusters. Next, through rearrangement, they consolidate to form precartilage condensations which then differentiate into cartilage. We mapped molecular expression in these early stages and showed that the sequence of molecular expression was tenascin-C, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), and phos-phorylated CREB (P-CREB) $\rightarrow$ neural cell adhesion molecules $\rightarrow$ integrin β1 and fibronectin $\rightarrow$ collagen II and Alcian blue. We then used a newly developed replication competent avian sarcoma (RCAS) mediated gene transduction procedure to alter the gene expression in micromass cultures. We showed that RCAS-(antisense NCAM) can suppress cartilage formation by forming smaller and fewer cartilage nodules. Sonic hedgehog directs mesenchymal cells to form novel nodules with characteristics of hypertrophic cartilage. The use of retroviral transduction, together with the addition of growth factors and Fab' antibodies to the media, constitute powerful approaches to the analysis of the molecular cascade of chondrogenesis in micromass cultures. It also provides a means to engineer the fate of mesenchymal cells through modulation of adhesion molecules and transgene expression of signaling molecules.

Key Words: Micromass culture, retrovirus, adhesion molecules, neural cell adhesion molecules (NCAM), tenascin-C, fibronectin, integrin β1, sonic hedgehog, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), collagen.

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Introduction

The formation of skeleton depends first on the formation of precartilage condensation anlage. The process of precartilage condensation involves a series of cell interactions (reviewed by: Hall and Miyake, 1995), including initiation, propagation, and termination of propagation (setting up the boundary). Individual limb bud cells have the ability to form small cellular aggregates. The size of the aggregates continues to increase through a process of cell rearrangement. The expansion of cell aggregates ceases when the condition of boundary is reached and precartilage condensations take shape. Chondro-differentiation is triggered in precartilage condensations and ultimately cartilage forms. This developmental process involves cooperative cell activities including cell migration, cell adhesion, intracellular signaling, and cell proliferation, which result in specific arrangements of precartilage condensations and subsequent differentiation.

Previous studies have shown that adhesion molecules including the cell-cell adhesion molecules, neural cell adhesion molecules (NCAM; Widelitz *et al*., 1993) and extracellular matrix molecules (ECM), fibronectin (Fn; Tomasek *et al*., 1982) and tenascin-C (Tn-C; Mackie *et al*., 1987) are enriched in precartilage condensations. In micromass cultures, antibodies to NCAM and exon IIIA region of Fn suppress the formation of precartilage condensations and chondrogenesis (Chuong *et al*., 1992; Gehris *et al*., 1997). In contrast, over-expression of NCAM causes the formation of large precartilage condensations (Widelitz *et al*., 1993). Hox genes can regulate cell adhesion by altering the expression of cell adhesion molecules such as NCAM and Tn-C (Jones *et al*., 1993; Yokouchi *et al*., 1995; reviewed by: Newman, 1996). Integrin receptors are expressed in pre-chondrogenic mesoderm (Mushler and Horwitz, 1991). Fn and Tn-C have opposite effects on chondrogenesis. Fn promotes cell attachment and spreading and inhibits chondrogenesis (Swalla and Solursh, 1984). Tn-C causes limb bud cells to round-up and promotes chondrogenesis (Mackie *et al*., 1987). Extracellular matrix macromolecules such as hyaluronan also can modulate the condensation process. It is shown that hyaluronan

hexasaccharides interfere with hyaluronan-cell interactions, resulting in a delay in the formation of condensations and chondrogenic differentiation in micromass cultures (Maleski and Knudson, 1996).

Intracellular signaling molecules and transcription factors plan integrating roles in regulating the progression from mesenchymal cells to chondroblast. For example, homeobox gene Msx-2 is expressed in the progress zone and is involved in the regulatory network that delineates the boundaries of the progress zone (Ferrari *et al*., 1998). Intracellular signaling molecules, such as NFκB, are expressed in prechondrogenic mesenchyme. Over-expression of IkB with retroviral vectors leads to the reduction of NFκB activity and the suppression of limb development (Bushdid *et al*., 1998; Kanegae *et al*., 1998). Activation of the cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA) pathway leads to stimulation of chondrogenesis (Solursh *et al*., 1981; Rodgers *et al*., 1989; Lee and Chuong, 1997). Recently, a specific kind of protein, kinase A, was found to be associated with chondro-differentiation (Zhang *et al*., 1996). These results suggest the importance of intracellular signaling molecules in mediating cell interactions during precartilage condensation formation.

Many of the limb patterning molecules have recently been identified (Tickle, 1995). The apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) direct limb outgrowth and skeletal patterning through epithelium-mesenchyme interactions (Saunders, 1948; Summerbell, 1974). The ZPA is in the posterior limb bud and contains sonic hedgehog (SHH) activity (Riddle *et al*., 1993) that is involved in the anterior-posterior axis of the limb bud.

fibroblast growth factor (FGF) pathway mediates AER activity and plays a key role in proximo-distal axis determination. Wnt-7a and engrailed are considered to be involved in dorso-ventral axis determination. Many other extracellular signaling molecules are expressed in the early limb bud and are shown to modulate chondrogenic differentiation. These include members of the transforming growth factor-β (TGFβ; Jiang *et al*., 1992; Downie and Newman, 1994; Lee and Chuong, 1997); bone morphogenetic protein (BMP) and BMP receptors (Duprez *et al*., 1996; Yamashita *et al*., 1996; Zhou *et al*., 1997); Wnt (Rudnicki and Brown, 1997); etc. Some of them have been shown to have antagonistic effects that may be used to set up the borders of precartilage condensations (e.g., FGFs and BMPs; Buckland *et al*., 1998) and some work in succession (e.g., TGFβ 1.2 and BMP-2; Roark and Greer, 1994). Other examples are being worked out (Hoang *et al*., 1996). The shaping of precartilage condensations *in vivo* is driven by the force of cell adhesion and determined by the interaction between the competence of a mesenchymal cell and the extracellular environment. The environment is a complex landscape made of extracellular matrixes and multi-directions of gradients of signaling molecules. To ascertain how the *in vivo* "patterning" is translated into specific skeletal morphology, we have to resort to a more simplified and analyzable *in vitro* model.

Micromass cultures, originally developed by Solursh (Ahrens *et al*., 1977; Paulsen *et al*., 1988; Daniels *et al*., 1996) have been an excellent model for analyzing the mechanisms of chondrogenesis. The methods to perturb micromass cultures involve the addition of drugs, peptides, growth factors (Frenz *et al*., 1989; Leonard *et al*., 1991; Chen *et al*., 1993; Downie and Newman, 1994; Paulsen *et al*., 1994) and antibodies (Chuong *et al*., 1992; Widelitz *et al*., 1993; Oberlander and Tuan, 1994). Here, we first summarize the early molecular expression events occurring in the early stages of chondrogenic nodule formation in micromass cultures. We then report the application of the newly developed replication competent avian sarcoma (RCAS) technology (Morgan and Fekete, 1996) to micromass cultures (Stott *et al*., 1998). We used two sets of experiments to demonstrate the usefulness of this approach. One is the role of NCAM. Earlier, we reported that over-expression of NCAM enhances chondrogenesis (Widelitz *et al*., 1993). Here, we show that suppression of NCAM activity by RCAS transduced anti-sense NCAM can suppress the formation of precartilage condensation, further demonstrating that NCAM is physiologically involved in precartilage condensation formation (although other redundant adhesion molecules are also involved). SHH is involved in patterning, but also has effect on osteo-chondro differentiation (Stott and Chuong, 1997). Here, we review the unexpected novel hypertrophic cartilage nodules induced by ectopic expression of SHH in micromass cultures. Since later in development, Indian hedgehog (IHH) is expressed in the pre-hypertrophic zone of long bone to regulate the rate of chondrocyte differentiation (Vortkamp *et al*., 1996); this work provides further information on the role of hedgehog pathway in skeletal morphogenesis. We then discuss the roles of various molecules in precartilage condensation formation: adhesion molecules are mediators, extracellular signaling factors modulate adhesion and differentiation events; and intracellular signaling factors determine the competence and types of responses of the mesenchymal cells. There are also feedback loops among these three categories molecules, which work as a team to achieve the formation of precartilage condensations.

Methods and Materials

Materials

Chicken eggs were obtained from SPAFAS (Preston, CT). Embryos were staged according to Hamburger and Hamilton (1951). The following reagents were used: rabbit

polyclonal antibodies against chicken NCAM (Chuong and Edelman, 1985), CREB (cyclic (MP response element binding protein) and P-CREB [Upstate Biotechnology (UBI), Lake Placid, NY], and Tn-C (Jiang and Chuong, 1992); Monoclonal antibodies against Fn (Frenz *et al*., 1989), integrin β1 (Muschler and Horwitz; 1991), chicken collagen type II (a kind gift from Dr. Richard Mayne, Hybridoma Core Facility of the Multipurpose Arthritis Center, University of Alabama, Birmingham); biotinylated peanut agglutinin (PNA; Vector, Burlingame, CA); retroviral glycosaminoglycans (GAG; a kind gift of Dr. Morgan, Harvard University, Cambridge, MA); chicken NCAM cDNA (pEC1402; Hemperly *et al*., 1986); RCAS and Cla 12 adapter plasmid (kind gifts of Dr. Hughes; Givol *et al*., 1992; Hughes *et al*., 1987); RCAS-SHH and *in situ* hybridization probes to SHH (kind gifts from Drs. Tabin and Riddle; Riddle *et al*., 1993).

Limb bud cell cultures

Limb bud cell cultures were performed based on Ahrens *et al*. (1977) and Lee and Chuong (1997). The distal third of the chicken wing buds at stage 23-24 were dissected. Epithelium was removed after soaking in 2x calcium and magnesium free medium (CMF) on ice. Pooled limb buds were digested with 0.1% trypsin and 0.1% collagenase (Worthington, Lakewood, NJ) in 1x CMF at 37°C for 10 minutes. After stopping the digestion with fetal calf serum (FCS), tissues were gently triturated, washed by centrifugation, resuspended in Dulbecco's modified Eagle's medium (DMEM), and passed through Cell Microsieve netting (20 μm; BioDesign, Carmel, NY) to ensure a single cell suspension. Fifteen microliters of 7 x 10^6 cells/ml or 2 x 10^7 cells/ml cell suspension as a drop were plated on 35 mm tissue culture dishes (Corning, Oneota, NY). Cells were allowed to attach to the culture dish for 1 hour, then were cultured in 1.5 ml defined medium (60% Ham's F12 nutrient mixture/40% DMEM, 5 mg/ml insulin, 5 mg/ml transferrin, 50 mg/ml ascorbic acid, 100 nM hydrocortisone without serum) supplemented with 10 mg/l of gentamicin and 0.5% FCS in a humidified 37°C incubator with a 5% CO_2/95% air atmosphere (Paulsen *et al*., 1988).

Immunohistochemistry

Immunostaining was performed as described in Lee and Chuong (1992). Cultures were fixed with 2.5% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes, or with Bouin's fixative for 20 minutes followed by washing with 70% alcohol. Specimens were incubated with primary antibodies overnight, followed by secondary fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit antibodies (Vector) or alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega, Madison, WI). NBT (4-nitroblue tetryzolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) were used as substrates of alkaline phosphatase. To block endogenous alkaline phosphatase activity, levamisole (0.1 mM) was added in the substrate solution. The control experiment without primary antibodies was performed in parallel.

To consider the possibility of marked reduction owing to interference with the antibodies' access to their cognate antigens by glycosaminoglycans, immunostaining results were subject to comparison with cultures predigested with chondroitinase ABC (Boehringer Mannheim, Indianapolis, IN; 0.1 unit in PBS, pH 8.0) at 37°C for 1 hour.

Retrovirus production

For RCAS-(antisense NCAM), a Bam H1/Hind III fragment of NCAM cDNA (nucleotides 28 to 1280) was excised from pEC1402 (Hemperly *et al*., 1986). This DNA fragment was then subcloned into the adaptor plasmid, Cla 12 (Hughes *et al*., 1987). This Cla I fragment encompassing the 5' NCAM portion was then subcloned into the unique Cla I cloning site of the RCAS retroviral vector (subgroup A; Hughes *et al*., 1987). Expression of the transgene was driven by the retroviral long terminal repeat (LTR) promoter. The orientation of NCAM in the RCAS vector was determined by restriction enzyme mapping using both Sal I and Sac I.

For production of virus, dermal fibroblasts from SPAFAS chicken embryos were used and prepared as follows. E7 embryonic skins were dissected and the epithelia were removed in 2x CMF. Dermal mesenchyme were dissociated in Hank's balanced salt solution (HBSS; without Ca^{2+} and Mg^{2+}) containing 0.1% trypsin/0.1% collagenase. After adding an equal volume of FCS and washing, cells were cultured at 5 x 10^4 cells/cm^2 on 60 mm dishes in F10 medium supplemented with 12.5% FCS, 5% chicken serum, 1.2% vitamin solution (Irvine Scientific, Santa Ana, CA), 1.2% folic acid, 0.5% dimethylsulfoxide (DMSO), 1 mM L-glutamine, and 10 mg/ml gentamicin. When grown to 70% confluence, cells were transfected with 5 mg of retroviral vector DNA using the calcium phosphate method (Sambrook *et al*., 1989). Culture medium was changed on the next day. After 3 days in culture, cells were subcultured onto 100 mm dishes containing 8 ml culture medium. At 70% confluence, the medium was changed and then collected after 3 days in culture. This retrovirus-containing medium was filtered through 0.45 μm filters and stored at -70°C. Assays for reverse transcriptase activity (Rosenberg and Baltimore, 1978) and anti-GAG immunostaining were performed to determine the titer and infectious efficiency of retrovirus (Morgan and Fekete, 1996).

Retroviral transduction of micromass cultures

Micromass cultures without retroviral transduction are grown in defined medium supplemented with 0.5% FCS. For micromass cultures subjected to retroviral transduction, dissected limb buds were subject to mild dissociation conditions in HBSS (without Ca^{2+} and Mg^{2+}) containing

0.003% of trypsin/collagenase. Dissociated limb bud cells were incubated with retrovirus-containing medium (10^6 cells/ml of retroviral medium) for 2 hour at 8°C with gentle shaking. After centrifugation (150 x *g*), cells were plated at low density (5.5 x 10^4 cells/cm^2) and cultured in the defined medium consisting of 60% Ham's F12 nutrient mixture/40% DMEM, 5 mg/ml insulin, 5 mg/ml transferrin, 50 mg/ml L-ascorbic acid, 100 nM hydrocortisone, and 10 mg/l gentamicin in 100 mm dishes. At the second day, cells were re-incubated with retroviral medium (3 ml/100 mm dish) for 15 minutes to increase transduction efficiency. At the third day, the cells were trypsinized with 0.01% of trypsin/collagenase, passed through a Cell Microsieve (20 mm; BioDesign), and plated as a 15 ml drop (2 X 10^7 cells/ml) on a spot precoated with collagen type I (UBI; 50 mg/ml) at 37°C for one hour. After a 2-hour incubation, cultures were flooded with defined medium supplemented with 0.5% FCS (Stott *et al*., 1998).

Reverse transcribed-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 20 micromass cultures using Trizol reagent (Life Technologies, Gaithersburg, MD) and following the instruction recommended by the manufacturer. Reverse transcription (RT) was performed in a total volume of 50 µl containing of 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 25 mM deoxynucleotide (dNTP; Boehringer Mannheim), 50 U RNase Inhibitor (Boehringer Mannheim), 20 mM oligo $(dT)_{12-18}$ (Pharmacia Biotech, Uppsala, Sweden) 10 U avian myelobiastosis virus (AMV) reverse transcriptase (Boehringer Mannheim), 1 µg total RNA. For PCR, sense primer (5'-GGTATTTGCTTATCCCAGTGC-3') and antisense primer (5'-AGTTTCCGTCTT CTCCCATCT-3') were used, which encompass 1342-1907 bp of the chicken NCAM cDNA. Reaction was conducted in a total volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dNTP, 20 µM of each primer, 2.5 U Taq DNA polymerase (Boehringer Mannheim), 2 µl RT reaction mixture. Amplification was performed at 94°C for 50 seconds, 60°C for 50 seconds for the first cycle followed by 94°C for 50 seconds, 60°C for 50 seconds, and 74°C for 50 seconds for 35 cycles. PCR products were resolved on a 1% agarose gel and showed a single expected 565 bp DNA fragment.

Quantitation of chondrogenesis in micromass cultures

Micromass cultures were fixed with 2.5% paraformaldehyde in PBS and stained with 1% Alcian blue 8GX (pH 1; Lev and Spicer, 1964; Hassell and Horigan, 1982) for 3 hour, which stains cartilage specific sulfated proteoglycans (Leonard *et al*., 1991). Stained cultures were de-stained with 70% ethanol. Bound Alcian blue dye was extracted with 0.5 ml of 4 M guanidine HCl (pH 5.8) followed by measuring absorbance at optical density (OD) 600 nm. Parallel cultures were scraped off the dish and sonicated in 0.5 ml DNA extraction buffer {4 M NaCl, 0.1 M Na_2HPO_4, 4 mM ethylenediaminetetraacetic acid (EDTA); pH 7.4} for 20 seconds, and then subjected to fluorometric determination in the DNA buffer containing Hoechst 33258 (1 mg/ml; emission wavelength, 365 nm; excitation wavelength, 460 nm). Calf thymus DNA was used as a DNA standard (Brunk *et al*., 1979). Owing to the fact that Hoechst 33258 shows enhanced fluorescence upon association with A-T pairs, the DNA standard curve derived from different species may show significant variation resulted from species-specific difference in A-T:G-C ratio.

Results

Limb bud cell cultures were used as a model for studying precartilage condensation. In this culture system, dissociated cells are plated as a spot on a substratum. After the cells attach, they display an increasing cell density gradient from the periphery to the central region. Initially, cells form small aggregates due to intimate cell-cell interactions. Through a process of cell rearrangement, the size of aggregates expand and ultimately precartilage condensations with high cell compactness form (Fig. 1).

Spatial and temporal distribution of adhesion molecules and signaling molecules during precartilage condensation formation *in vitro*

To dissect the adhesion and signaling events involved in precartilage condensation, the spatial and temporal distribution of adhesion molecules and signaling molecules were studied using an immunochemical approach with specific antibodies. In addition, molecular markers, such as PNA binding molecules for precartilage condensation (Aulthouse and Solursh, 1987), and collagen type II for chondrogenesis were used for comparison. In this study, limb bud cell cultures (7 x 10^6 cells/ml) were examined at 4 hours, day 1, day 3, and day 4 time points. Results are summarized here as schematic drawings (Fig. 2).

The expression of NCAM was initially weak in individual cells and became evident in cell contacts of aggregates. When precartilage condensations formed, NCAM was expressed over the whole condensation, and was manifested as a gradient with high expression in the center and low expression at the periphery. Ultimately, NCAM faded away from the center where chondrogenesis occurred and cartilage nodules formed, which were positive for collagen II and Alcian blue staining. Tn-C was expressed sporadically among individual cells at the cell margin. When cells formed aggregates, the expression of Tn-C became evident at the cell contacts of aggregated cells. The extent and level of Tn-C expression continued to increase until the precartilage condensation formed. Thereafter, Tn-C disappeared in regions where chondrogenesis and

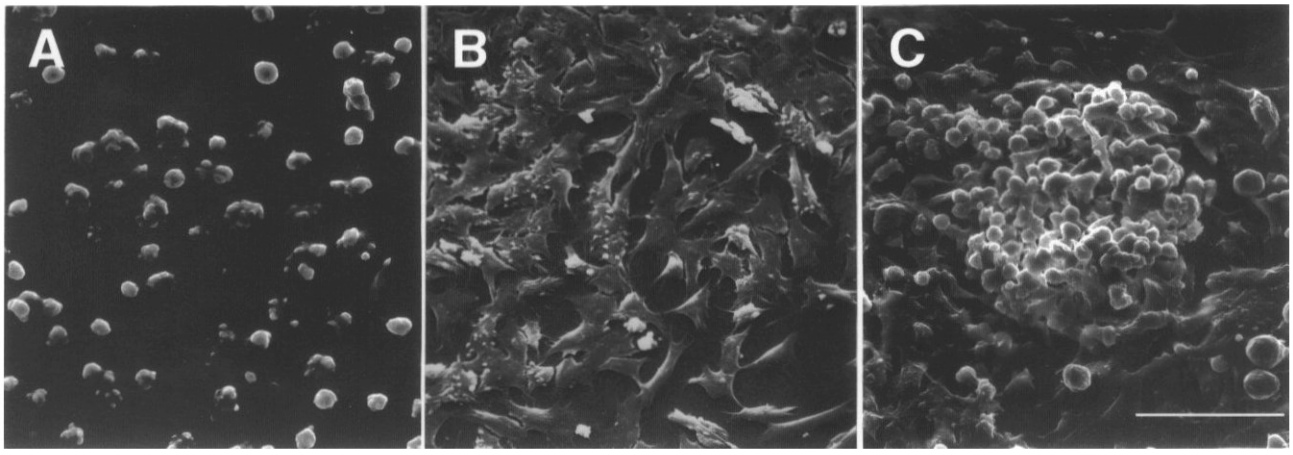

Figure 1. Morphology of micromass cultures. Scanning electron micrographs of limb bud micromass cultures show dissociated cells in the beginning cultures (**A**), small aggregates (**B**), and the mature cartilage nodules (**C**). Bar = 60 μm (A, B, and C are at identical magnification).

mineralization occurred. However, Tn-C remains in articular cartilage and may contribute to their special property (Pacifici *et al*., 1993). Fn was initially expressed at a low level in individual cells and became evident at cell contacts within aggregates containing more than about 8-12 cells. The extent and level of Fn expression increased in proportion to the size of the cell aggregates, which reached a maximum in well formed precartilage condensations. When chondrogenesis occurred, the expression of Fn decreased and finally disappeared in the chondrocytes. Integrin β1 showed a similar spatial and temporal distribution as Fn.

Undoubtedly, some signaling molecules are involved in this condensation process. We have attempted to study a transcription factor, CREB, involved in the protein kinase A pathway (Fig. 2). CREB was expressed in the cytoplasm and nucleus of all cells in the condensation phase (Lee and Chuong, 1997). The level of CREB decreased during chondrogenesis and disappeared from chondrocytes. P-CREB was sporadically expressed among cells without a specific pattern in the condensation phase. Afterwards, the level of P-CREB decreased in the condensation phase and disappeared from chondrocytes. It needs to mentioned that the efficacy of immunostaining results described above were verified without interference with endogenous alkaline phosphatase activity and the antibodies' access to their cognate antigens by various glycosaminoglycans in the cartilage matrix.

Down-regulation of NCAM by RCAS-(antisense NCAM) suppresses chondrogenesis

RCAS-(antisense NCAM) was used to suppress NCAM expression in micromass cultures. To evaluate the effect of antisense NCAM on NCAM expression, double staining using anti-retroviral GAG and anti-NCAM was performed. Under the same experimental conditions, the level of GAG expression is similar in RCAS and RCAS-(antisense NCAM) transduced micromass cultures (Figs. 3B and 3B'). However, RCAS-(antisense NCAM) cultures showed a significant decrease, but not disappearance in the expression of NCAM protein when compared to the RCAS control cultures (Figs. 3A and 3A'). This is particularly clear when comparing the similar GAG expression levels in both cultures. In addition, RT-PCR was performed to semi-quantitatively analyze mRNA level of NCAM. Results showed no changes in the mRNA level in RCAS-(antisense NCAM) transduced cultures. It is reported that antisense RNA treatment may not always result in degradation of the target mRNA. Several cases of RNA:RNA duplexes have been documented (Bass and Weintraub, 1988; Bunch and Goldstein, 1989).

The chondrogenesis of the antisense cultures was monitored. Results showed that RCAS infected cultures had a similar degree of chondrogenesis to non-infected control cultures (data not shown). However, RCAS-(anti-sense NCAM) cultures had smaller cartilage nodules. Chondrogenesis was remarkably suppressed to 35% of control levels (Figs. 3C and 3C'). Cell proliferation was marginally increased in antisense NCAM treated cultures by 120% of the control. After normalizing for the amount of DNA (cell number), there was about a 70% reduction in chondrogenesis. Since the size of NCAM cDNA (2.6 kb) is over 2 kb capacity of RCAS vector, over-expression of NCAM in this system becomes technically unavailable. Nonetheless, this is comparable to the previous studies showing reduction of precartilage condensation formation and chondrogenesis when Fab' antibody to NCAM were

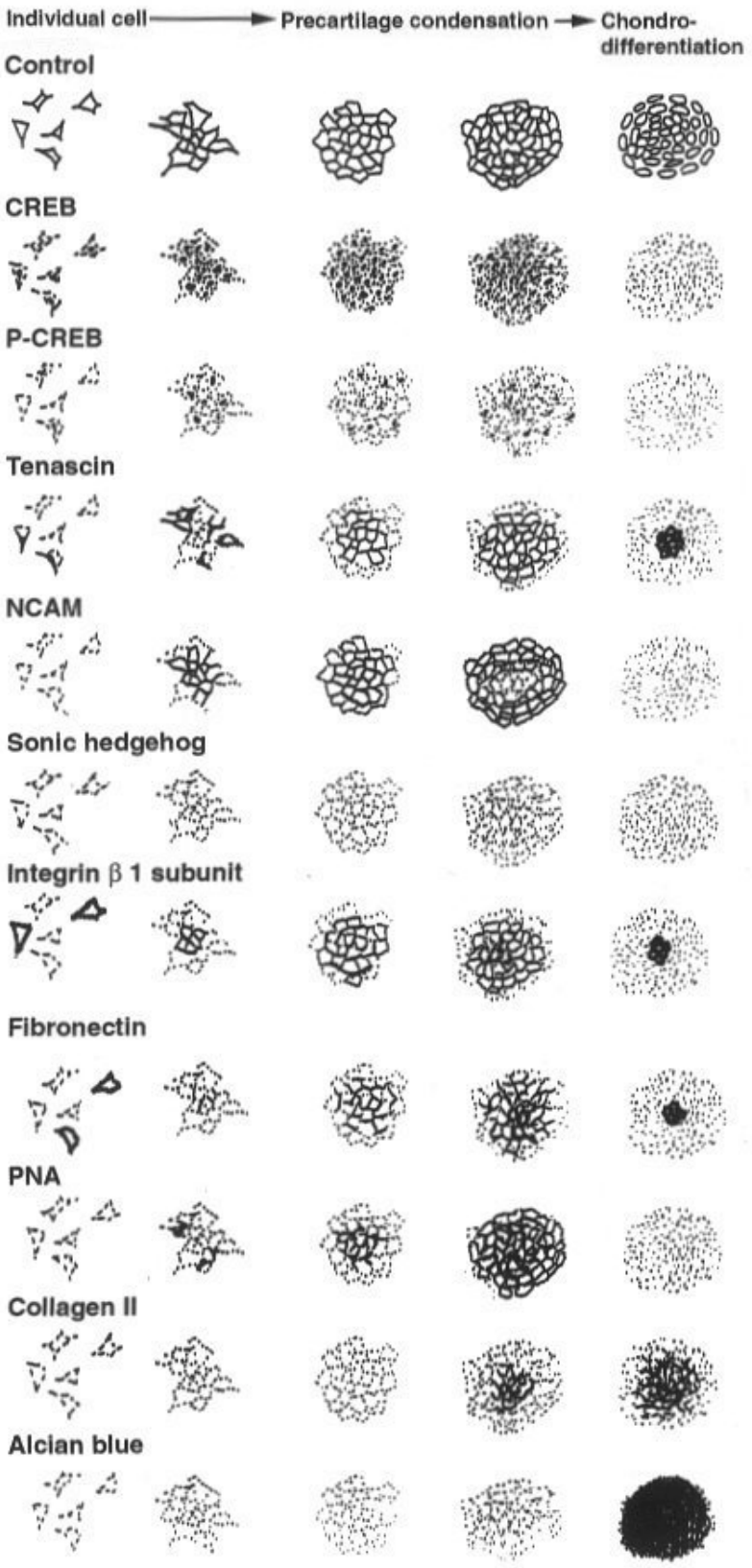

Figure 2. Schematic drawings showing the distribution of adhesion molecules in successive stages of cartilage nodule formation. Immunocytochemistry was used to follow the expression sequence of adhesion molecules and intracellular signaling molecules. The results are summarized here. Broken lines indicate cell boundaries. Solid lines indicate expression.

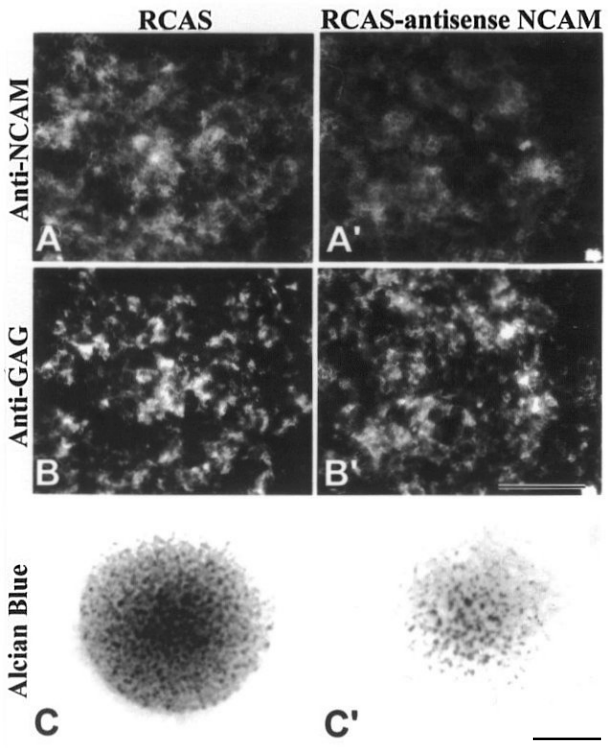

Figure 3. Formation of cartilage nodules is suppressed in micromass cultures infected with RCAS-(antisense NCAM). Micromass cultures were transduced with RCAS control vectors (A-C) or RCAS-(antisense NCAM; A'-C'). **A, A'**: Antibodies to NCAM showing the suppression, but not absence, of NCAM protein in RCAS-(antisense NCAM) transduced cultures. **B, B'**: Antibodies to GAG showing the expression of viral proteins are about equal in both cultures. **C, C'**: Reduction of Alcian blue positive nodules, in both number and size, in RCAS-(antisense NCAM) transduced cultures. Bars = 100 μm (A, A', B, and B' are at the same magnification); and 2 mm (C and C' are at the same magnification).

added to the cultures (Widelitz *et al*., 1993). These results again demonstrate the involvement of NCAM in chondrogenesis and the use of RCAS mediated antisense transduction in micromass cultures.

Ectopic expression of SHH using RCAS-SHH leads to the formation of novel nodules

Members of the vertebrate hedgehog gene family (HH) are involved in patterning and modulating differentiation. Recently ectopic expression of HH gene family members *in vivo* was shown to block chondrocyte maturation through activation of a parathyroid hormone related protein (PTHrP) dependent negative regulatory loop in the perichondrium (Vortkamp *et al*., 1996). However, the direct effect of HH on chondrocyte maturation has not been tested. Here, we tested the effect of retroviral over-expression of the chicken sonic hedgehog gene on the growth and maturation of limb bud cells in micromass cultures (reported in Stott and Chuong, 1997).

First, we used *in situ* hybridization to detect the expression of SHH in micromass cultures. Although SHH positive cells are included in the preparation, expression was lost in dissociated cultures and was not re-expressed until day 4 in culture. Dissociated cells were transduced with RCAS-SHH and replated at high density leading to the production of novel tightly whorled nodules in addition to the normal appearing cartilage nodules (Fig. 4A; compare with Fig. 1). Normal nodules (solid arrow) are strongly Alcian Blue positive while SHH nodules (empty arrow) are weakly Alcian Blue positive (Fig. 4B). We further characterized these new nodules and showed that they are strongly positive for alkaline phosphatase (Fig. 4C) and enriched in type X collagen (Fig. 4D).

SHH over-expression also increased cell proliferation, but this can not account for the formation of the new nodules. The results demonstrate that activation of the signaling pathway can modulate the normal progression of precartilage condensation formation and alter the differentiation fate of mesenchymal cells. These signals are likely to be used in physiological conditions to regulate growth and to shape the forming cartilage. It also demonstrates the use of RCAS to mediate ectopic expression of signaling molecules in micromass cultures for the study of chondrogenesis.

Discussion

The importance of precartilage condensation is reflected by its involvement in patterning the skeleton in the limb. Precartilage condensation involves cell migration, cell adhesion, and intracellular signaling, which are revealed by the spatiotemporal configuration of gene expression. The progression of precartilage condensation is directed by local differences in molecular expression and the competence of the cells to respond to interacting signals. In limb bud cell cultures, the formation of precartilage condensations is a density dependent event. When limb bud cells are plated as a drop, an increasing gradient of cell density forms from the periphery to the center. In this system, the progression from the initiation to the propagation stage is evident from the periphery to the center of the culture. This model is useful for studying the molecular expression involved in the initiation and propagation of precartilage condensation.

Here, we have showed a panel of adhesion molecules expressed in different stages of precartilage condensation formation. The involvement of cell adhesion molecules, such as, NCAM (Widelitz *et al*., 1993) and cadherin (Oberlender and Tuan, 1994), and substrate ad-

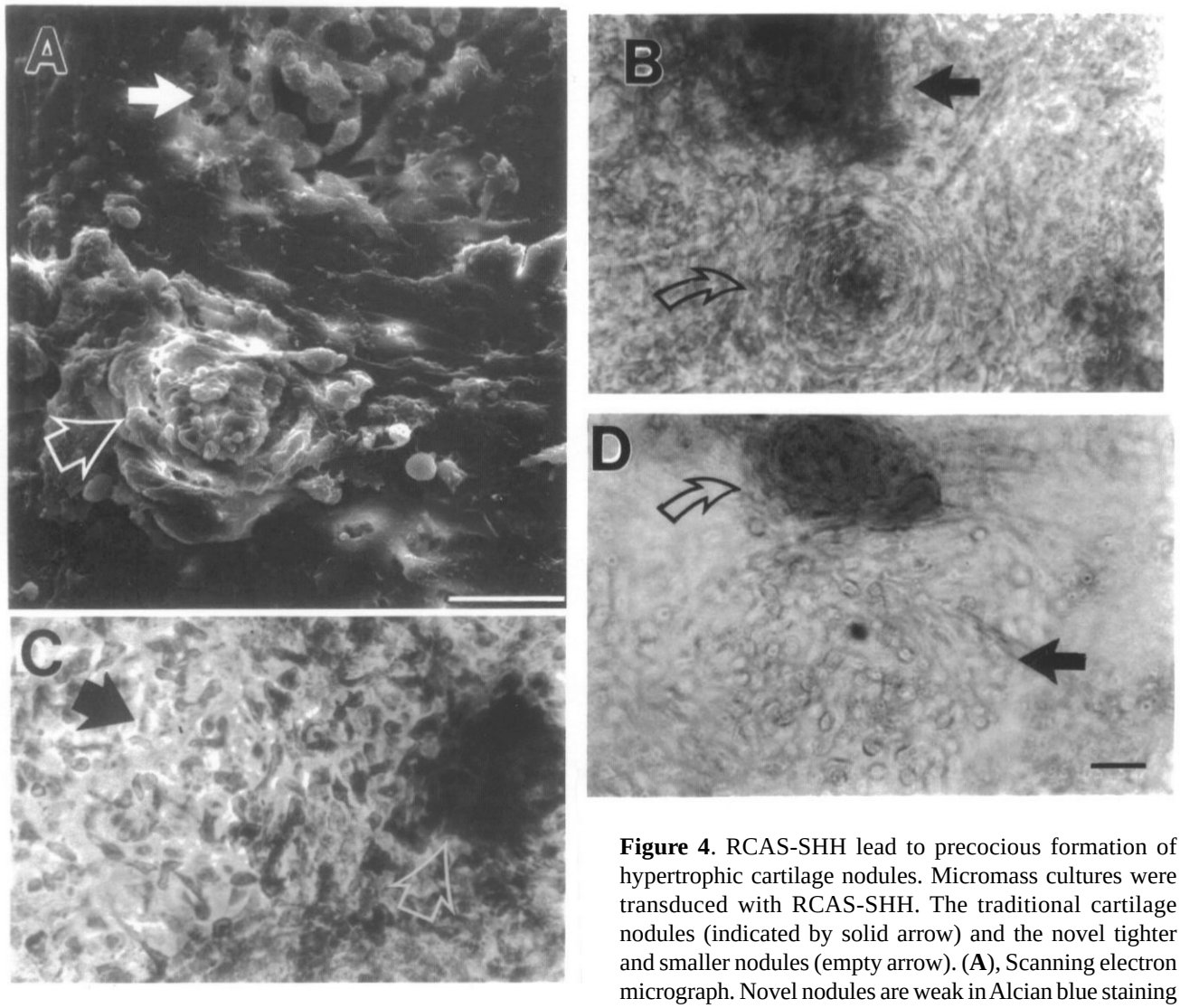

Figure 4. RCAS-SHH lead to precocious formation of hypertrophic cartilage nodules. Micromass cultures were transduced with RCAS-SHH. The traditional cartilage nodules (indicated by solid arrow) and the novel tighter and smaller nodules (empty arrow). (**A**), Scanning electron micrograph. Novel nodules are weak in Alcian blue staining (**B**) but strong in alkaline phosphatase (**C**) and collagen X (**D**) expression. Bars = 60 μm (A) and 100 μm (B, C, and D are at the same magnification).

hesion molecules, such as, tenascin (Mackie *et al*., 1987) and fibronectin (Frenz *et al*., 1989) in precartilage condensation formation have been reported. They probably act by modulating cell-cell and cell-substrate interactions (Chuong *et al*., 1992). Although these studies showed the involvement of adhesion molecules, exactly how adhesion molecules act to build and shape the cartilage nodules has not been addressed. To approach this aspect, we established the expression sequence of these adhesion molecules and potential signaling molecules in each stage of precartilage condensation formation. We hope this molecular expression sequence will help future studies to discern the molecular basis for pattern formation and chondrogenesis.

The initiation step involves cell aggregation

How does the aggregation start? Dissociated limb bud mesenchymal cells start to form aggregates in 4 hours, in contrast to the skin mesenchymal cells that will not form condensations in the absence of epithelium (Chuong *et al*., 1996). NCAM and tenascin were expressed earliest in micromass cultures. We propose that NCAM and Tn-C are involved in the initiation of precartilage condensation formation. Initially, NCAM is homogeneously expressed at a basal level among individual cells. The expression of NCAM became evident in cell contacts in small cell aggregates containing 2-3 cells. This suggests that NCAM may function in establishing the initial cellular contact. Tn-C is unevenly distributed on the surface of individual cells and also at the cell contact region in small 2-3 cell aggregates. Tn-C has been suggested to play a role in

mediating cell migration (Bonner-Fraser, 1988). Since the initiation of cell aggregation involves cell migration toward or away from an attractant followed by local cell arrangements, Tn-C may facilitate cell motility and migration during the initiation process. In this stage, the expression of integrin β1 remains low. It seems that cell adhesion favors condensation initiation.

The propagation step involves a positive feedback mechanism reinforcing cell adhesion and changes of cell shape

Cell adhesion involvement in precartilage condensation is dynamic and regulated. The expression of NCAM, Fn, Tn-C, and integrin β1 is initially low and diffusive among individual cells and then is augmented at cell contacts of expanding cell aggregates. This suggests that the expression of NCAM, Fn, Tn-C, and integrin β1 may involve a positive feedback mechanism through strengthening cell adhesion in the condensation.

The sequential change of cell shape from irregular to polygonal and finally to round is the morphological hallmark of chondrogenesis. This change of cell shape is associated with a reorganization of the actin cytoskeleton (Mitchison and Cramer, 1996). Experimental conditions that cause cells to round-up by disrupting actin, such as cytochalasin D treatment or growing cells on an agarose substratum, enhance chondrogenesis (Zanetti and Solursh, 1984). Here, integrin β1 and Fn are expressed at contacts of apposed polygonal cells in cell aggregates. Integrin is reported to modulate the shape and motility of cells through the actin cytoskeleton after ECM-integrin binding. This suggests a potential signaling network, which comprises Fn and integrin in controlling the cell shape and concomitant gene expression during precartilage condensation.

Disruption of NCAM mediated cell interactions by antibody (Widelitz *et al*., 1993) or by antisense (this paper) is consistent with this scheme. However, antisense NCAM is not able to abolish chondrogenesis completely. There are likely to be other redundant adhesion molecule pathways involved. A separate important cell adhesion molecule is N-cadherin which is highly expressed in precartilage condensations and antibodies against it can suppress chondrogenesis (Oberlander and Tuan, 1994). Finally, the novel methodology used here should allow us to further test more candidate genes and examine how other molecules are altered.

Different signaling molecules can alter cell adhesion and guide the condensations and differentiation in various ways

In vivo, it is proposed that IHH regulates the rate of cartilage differentiation by induction of PTHrP (Vortkamp *et al*., 1996). PTHrP then binds to the PTH/ PTHrP receptor on prehypertrophic chondrocytes, blocking hypertrophic differentiation (Henderson *et al*., 1996) and inhibiting the expression of IHH. PTHrP protein is detected by immunostaining on the prehypertrophic chondrocytes in the growth plate cartilage, while PTHrP mRNA is expressed predominantly in the periarticular perichondrium of developing murine limb buds (Senior *et al*., 1991; Lee *et al*., 1995). In IHH infected mouse limb explant cultures, induction of PTHrP mRNA is also restricted to the periarticular perichondrium in contrast to the more widespread induction of *Ptc* and *Gli*, two other downstream molecules in the HH signaling pathway (Vortkamp *et al*., 1996). This suggests that the effect of IHH on PTHrP transcription is mediated through relatively specialized cells in the periarticular perichondrium and/or may require other signals.

In our *in vitro* condition, dissociated limb bud cells develop into cartilage nodules after 72-96 hours in micromass cultures (Ahrens *et al*., 1977). In the early stages (first two days) of these cultures, most limb bud cells consist of primitive mesenchyme (Chuong *et al*., 1992; Widelitz *et al*., 1993). There are no perichondrium nor PTHrP positive cells (Stott and Chuong, 1997). Therefore the IHH/PTHrP/PTHrP receptor pathway observed *in vivo* is unlikely to be active in our model. This provides a unique opportunity to observe the direct action of SHH in the absence of the negative feedback loop. Our study shows that SHH acts to enhance the formation of hypertrophic cartilage nodules (Stott and Chuong, 1997). This current study shows that HH has dual complementary functions: a direct positive effect on chondrocyte hypertrophy in the absence of the PTHrP pathway, and an indirect negative feedback loop through PTHrP to prevent other less differentiated chondrocytes from becoming hypertrophic. These two complementary actions of HH coordinate the progression of cartilage maturation.

Using similar approaches, RCAS-Wnt-7a has recently been shown to suppress chondrogenesis (Rudnicki and Brown, 1997). Wnt-7a is interesting because it is expressed in the dorsal limb bud ectoderm and can specify the fate of dorsal mesenchyme. Our data suggest that Wnt-7a increases cell adhesion among mesenchymal cells in a different way and hence leads the condensed mesenchymal cells along a different pathway (Stott, Jiang and Chuong, submitted). Thus, different signaling molecules, HH, PTHrP, Wnt-7a as well as FGF and BMP (Niswander and Martin, 1993) act on limb bud mesenchymal cells in different ways to modulate skeletogenesis.

Summary

The formation of precartilage condensations requires the exquisite coordination between cell-cell and cell-ECM adhesion. The diverse combination of cell adhesion at specific times and sites coupled with intracellular

signal transduction is crucial for the shape and differentiation of skeletal elements. Perturbation can be done by antibodies, exogenous added peptides, growth factors, matrix molecules, or retrovirus mediated gene expression and suppression in micromass cultures. Results of perturbation may be a suppression of chondrogenesis or of the size of cartilage nodules such as those caused by RCAS-(antisense NCAM), or may be an alteration of cellular fate such as those caused by the powerful morphogen SHH.

During development, through physiological modulation of the precartilage condensation process, the shape and fate of the mesenchymal condensations and, in turn, the geometry of skeletal elements are laid down. For tissue engineering, it is also possible for scientists to move in the direction of modulating these adhesive processes with signaling molecules or gene therapy to achieve desired skeletal elements.

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References

Ahrens PB, Solursh M, Reiter RS (1977) Stage related capacity for limb chondrogenesis in cell culture. Dev Biol **60**: 69-82.

Aulthouse AL, Solursh M (1987) The detection of a precartilage, blastema-specific marker. Dev Biol **120**: 377-384.

Bass BL, Weintraub H (1988) An unwinding activity that covalently modifies its double-stranded RNA substrate. Cell **55**: 1089-1098.

Bonner-Fraser M (1988) Distribution and function of tenascin during cranial neural crest development in the chicken. J Neurosci Res **21**: 135-147.

Brunk CF, Jones KC, James TW (1979) Assay for nanogram quantities of DNA in cellular homogenates. Anal Biochem **92**: 497-500.

Buckland RA, Collinson JM, Graham E, Davidson DR, Hill RE (1998) Antagonistic effects of FGF4 on BMP induction of apoptosis and chondrogenesis in the chick limb bud. Mech Dev **71**: 143-150.

Bunch TA, Goldstein LS (1989) The conditional inhibition of gene expression in cultured Drosophila cells by antisense RNA. Nucleic Acids Res **17**: 9761-9782.

Bushdid PB, Brantley DM, Yull FE, Blaeuer GL, Hoffman LH, Niswander L, Kerr LD (1998) Inhibition of NFκB activity results in disruption of the apical ectodermal ridge and aberrant limb morphogenesis. Nature **392**: 615-618.

Chen P, Yu YM, Reddi AH (1993) Chondrogenesis in chick limb bud mesodermal cells: Reciprocal modulation by activin and inhibin. Exp Cell Res **206**: 119-127.

Chuong CM, Edelman GM (1985) Expression of cell adhesion molecules in embryonic induction. II. Morphogenesis of nestling feathers. J Cell Biol **101**: 1027-1043.

Chuong CM, Widelitz RB, Jiang TX, Abbott U, Lee YS, Chen HM (1992) Roles of adhesion molecules NCAM and tenascin in limb chondrogenesis: Analysis with antibody perturbation, exogenous gene expression, talpid mutants and activin stimulation. In: Limb Development and Regeneration. Fallon JF, Goetinck PF, Kelley RO, Stocum DL (eds.). Wiley-Liss, New York. pp. 465-474.

Chuong CM, Widelitz RW, Ting-Berreth SA, Jiang TX (1996) Early events during the regeneration of skin appendages: Dependence of epithelial-mesenchymal interaction and order of molecular reappearance. J Inv Derm **107**: 639-646.

Daniels K, Reiter R, Solursh M (1996) Micromass cultures of limb and other mesenchyme. Methods Cell Biol **51**: 237-47.

Downie SA, Newman SA (1994) Morphogenetic differences between fore and hind limb precartilage mesenchyme: Relation to mechanisms of skeletal pattern formation. Dev Biol **162**: 195-208.

Duprez D, Bell EJ, Richardson MK, Archer CW, Wolpert L, Brickell PM, Francis-West PH (1996) Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. Mech Dev **57**: 145-147.

Ferrari D, Lichtler AC, Pan Z, Dealy CN, Upholt WB, Kosher RA (1998) Ectopic expression of Msx-2 in posterior limb bud mesoderm impairs limb morphogenesis while inducing BMP-4 expression, inhibiting cell proliferation, and promoting apoptosis. Dev Biol **197**:12-24.

Frenz DA, Jaikaria NS, Newman SA (1989) The mechanism of pre-cartilage mesenchymal condensation: A major role of interaction of the cell surface with the amino-terminal heparin-binding domain of fibronectin. Dev Biol **136**: 97-103.

Gehris AL, Stringa E, Spina J, Desmond ME, Tuan RS, Bennett VD (1997) The region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. Dev Biol **190**: 191-205.

Givol I, Greenhouse JJ, Hughes SH, Ewert DL (1992) Retroviruses that express different *ras* mutant cause different types of tumors in chickens. Oncogene **7**: 141-146.

Hall BK, Miyake T (1995) Divide, accumulate, differentiate: Cell condensation in skeletal development

revisited. Int J Dev Biol **39**: 881-893.

Hamburger V, Hamilton H (1951) A series of normal stages in the development of the chick embryo. J Exp Morphol **88**: 49-92.

Hassell JR, Horigan EA (1982) Chondrogenesis: A model developmental system for measuring teratogenic potential of compounds. Teratog Carcinogen Mutagen **2**: 325-331.

Hemperly JJ, Murray BA, Edelman GM, Cunningham BA (1986) Sequence of a cDNA clone encoding the polysialic acid-rich and cytoplasmic domains of the neural cell adhesion molecule NCAM. Proc Natl Acad Sci USA **83**: 3041-3073.

Henderson JE, He B, Goltzman D, Karaplis AC (1996) Constitutive expression of parathyroid hormone-related peptide (PTHrP) stimulates growth and inhibits differentiation of CFK2 chondrocytes. J Cell Physiol **169**: 33-41.

Hoang B, Moos M Jr, Vukicevic S, Luyten FP (1996) Primary structure and tissue distribution of FRZB, a novel protein related to Drosophila frizzled, suggest a role in skeletal morphogenesis. J Biol Chem **271**: 26131-26137.

Hughes SH, Greenhouse JJ, Petropoulos CJ, Sutrave P (1987) Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J Virol **61**: 3004-3012.

Jiang TX, Chuong CM (1992) Mechanism of skin morphogenesis. I. Analyses with antibodies to adhesion molecules tenascin, NCAM, and integrin. Dev Biol **150**: 82-98.

Jiang TX, Yi JR, Ying SY, Chuong CM (1992) Activin enhances chondrogenesis of limb bud cells: Stimulation of precartilaginous mesenchymal condensations and expression of NCAM. Dev Biol **155**: 545-557.

Jones FS, Holst BD, Minowa O, De Robertis EM, Edelman GM (1993) Binding and transcriptional activation of the promoter for the neural cell adhesion molecule by HoxC6 (Hox 3.3). Proc Natl Acad Sci. USA **90**: 6557-6561.

Kanegae Y, Tavares AT, Izpisua Belmonte JC, Verma IM (1998) Role of Rel/NFκB transcription factors during the outgrowth of the vertebrate limb. Nature **392**: 611-614.

Lee YL, Chuong CM (1992) Adhesion molecules in skeletogenesis: I. Transient expression of N-CAM in osteoblasts during endochondral and intramembranous ossification. J Bone Min Res **7**: 1435-1446.

Lee YS, Chuong CM (1997) Activation of protein kinase A is a pivotal step involved in both BMP-2- and cyclic AMP-induced chondrogenesis. J Cell Physiol **170**: 153-165.

Lee K, Deeds JD, Segre GV (1995) Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. Endocrinology **136**: 453-461.

Leonard C, Fuld HM, Frenz DA, Downie SA, Massague J, Newman SA (1991) Role of transforming growth factor-beta in chondrogenic pattern formation in the embryonic limb: Stimulation of mesenchymal condensation and fibronectin gene expression by exogenous TGFβ and evidence for endogenous TGFβ like activity. Dev Biol **145**: 99-109.

Lev R, Spicer SS (1964) Specific staining of sulfate groups with Alcian blue at low pH. J Histochem Cytochem **12**: 38-41.

Mackie EJ, Thesleff I, Chiquet-Ehrismann R (1987) Tenascin is associated with chondrogenic and osteogenic differentiation *in vivo* and promotes chondrogenesis *in vitro*. J Cell Biol **105**: 2569-2579.

Maleski MP, Knudson CB (1996) Hyaluronan-mediated aggregation of limb bud mesenchyme and mesenchymal condensation during chondrogenesis. Exp Cell Res **225**: 55-66.

Mitchison TJ, Cramer LP (1996) Actin-based cell motility and cell locomotion. Cell **84**: 371-379.

Morgan BA, Fekete DA (1996) Manipulating gene expression with replication-competent retroviruses. In: Methods in Cell Biology. Bonner-Fraser M (ed.). Academic Press, New York. pp. 186-217.

Muschler JL, Horwitz AF (1991) Down-regulation of the chicken alpha 5 beta 1 integrin fibronectin receptor during development. Development **113**: 327-337.

Newman SA (1996) Sticky fingers: Hox genes and cell adhesion in vertebrate limb development. Bioessays **18**: 171-174.

Niswander L, Martin GR (1993) FGF-4 and BMP-2 have opposite effects on limb growth. Nature **361**: 68-71.

Oberlender SA, Tuan RS (1994) Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. Development **120**: 177-187.

Pacifici M, Iwamoto M, Golden EB, Leatherman JL, Lee Y-S, Chuong C-M (1993) Tenascin is associated with articular cartilage development. Dev Dyn **198**: 123-134.

Paulsen DF, Langille RM, Dress V, Solursh M (1988) Selective stimulation of *in vitro* limb bud chondrogenesis by retinoic acid. Differentiation **39**: 123-130.

Paulsen DF, Chen WD, Okello D, Johnson, B (1994) Stage- and region-dependent responses of chick wing-bud mesenchymal cells to retinoic acid in serum-free microcultures. Dev Dyn **201**: 310-323.

Riddle RD, Johnson RL, Laufer E, Tabin CJ (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. Cell **75**: 1401-1416.

Roark EF, Greer K (1994) Transforming growth factor-beta and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation *in vitro*. Dev Dyn **200**: 103-116.

Rodgers BJ, Kulyk WM, Kosher RA (1989) Stimulation of limb cartilage differentiation by cyclic AMP is

dependent on cell density. Cell Differ Dev **28**: 179-187.

Rosenberg N, Baltimore D (1978) The effect of helper virus on Abelson virus-induced transformation of lymphoid cells. J Exp Med **147**: 1126-1141.

Rudnicki JA, Brown AMC (1997) Inhibition of chondrogenesis by Wnt gene expression *in vivo* and *in vitro*. Dev Biol **185**: 104-118.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd Edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 16.33-16.37.

Saunders JW, Jr (1948) The proximo-distal sequence of origin of limb parts of the chick wing and the role of the ectoderm. J Exp Zool **108**: 363-404.

Senior PV, Heath DA, Beck F (1991) Expression of the parathyroid hormone-related protein mRNA in the rat before birth: Demonstration by hybridization histochemistry. J Mol Endocrinol **6**: 281-290.

Solursh M, Reiter RS, Ahrens PB, Vertel BM (1981) Stage- and position-related changes in chondrogenic response of chick embryonic wing mesenchyme to treatment with dibutyryl cyclic AMP. Dev Biol **83**: 9-19.

Stott S, Chuong CM (1997) Dual action of sonic hedgehog on chondrocyte hypertrophy: Retrovirus mediated ectopic sonic hedgehog expression in limb bud micromass culture induces novel cartilage nodules that are positive for alkaline phosphatase and type x collagen. J Cell Sci **110**: 2691-2701.

Stott S, Lee YS, Chuong CM (1998) Retroviral gene transfer in micromass cultures: Potential for genetic manipulations in organotypic cultures. Biotechniques **24**: 661-666.

Swalla BJ, Solursh M (1984) Inhibition of limb chondrogenesis by fibronectin. Differentiation **26**: 42-48.

Summerbell D (1974) A quantitative analysis of the effect of excision of the AER from the chick limb bud. J Exp Morph **32**: 651-660.

Tickle C (1995) Vertebrate limb development. Curr Biol **5**: 478-484.

Tomasek JJ, Mazurkiewicz JE, Newman SA (1982) Non-uniform distribution of fibronectin during avian limb development. Dev Biol **90**: 118-126.

Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ (1996) Regulation of rate of cartilage differentiation by Indian Hedgehog and PTH-related protein. Science **273**: 613-622.

Widelitz RB, Jiang TX, Murray B, Chuong CM (1993) Neural cell adhesion molecules (NCAM) mediate precartilaginous condensations and enhances chondrogenesis during chicken limb development. J Cell Physiol **146**: 399-411.

Yamashita H, Ten Dijke P, Heldin CH, Miyazono K (1996) Bone morphogenetic protein receptors. Bone **19**: 569-574.

Yokouchi Y, Nakazato S, Yamamoto M, Goto Y, Kameda T, Ida H, Kuroiwa A (1995) Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. Genes Dev **9**: 2509-2522.

Zanetti NC, Solursh M (1984) Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. J Cell Biol **99**: 115-123.

Zhang Q, Carr DW, Lerea KM, Scoot JD, Newman SA (1996) Nuclear localization of type II cAMP-dependent protein kinase during limb cartilage differentiation is associated with a novel developmentally regulated A-kinase anchoring protein. Dev Biol **176**: 51-61.

Zhou H, Weiser R, Massague J, Niswander L (1997) Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. Genes Dev **11**: 2191-2203.

Discussion with Reviewers

D.F. Paulsen: Was there any particular observation that led you to supplement the defined medium with 0.5% FCS for this study? Did you observe any effect of this modified medium composition on the expression patterns of any of the various antigens studied?

Authors: We supplemented the defined media with 0.5% FCS because it made molecular expression more distinct. This modification of medium composition did not cause changes of expression patterns of molecules.

D.F. Paulsen: Have you considered the possibility that the marked reduction of staining of certain cell-surface or extracellular antigens coinciding with the onset of Alcian-blue-positive matrix secretion reflects not a stage-dependent reduction in expression, but rather interference with the antibodies' access to their cognate antigens by the viscous glycosaminoglycans in the cartilage matrix? Do you obtain similar results with cultures pre-digested with, for example, chondroitinase ABC?

Authors: We have treated cultures with chondroitinases and neuraminidases and the results do not change.

D.F. Paulsen: What is the relative spatiotemporal course of expression of cell adhesion molecules during chondrogenesis? Do such relationships remain coordinately regulated when chondrogenesis is stimulated or inhibited?

Authors: The expression sequence of adhesion molecules is in sequential order. It appears that the molecular and cellular events are well coordinated; this is best illustrated in Figure 2. When chondrogenesis is enhanced by factors such as BMP, cAMP, etc., the expression of adhesion molecules is amplified but the relative relationship remains

the same. On the other hand, when the "quality" of cartilage nodules is modified such as by SHH (Fig. 4), the mesenchymal cells are shunt to a different pathway, and the expression pattern becomes totally different.

D.F. Paulsen: *In vivo*, which endogenous signals do you expect will have the greatest stimulatory effect on initiating the precartilage condensations? How do you expect that such signals act so selectively on the mesenchyme at the core of the limb?
Authors: FGF family can be a good candidate as the greatest endogenous signal initiating condensation in the developing limb. This expectation is based on the observation that FGF-2, 4, and 8 are expressed in AER at the early condensation stage and are able to induce an additional limb with cylinder cartilage core in the prospective limb region. As far as the confinement of precartilage condensation to the core of the limb bud, it may be a combination of the simple facts that cell density is higher in the core of the limb bud and that the ectoderm exerts an inhibitory effect on chondrogenesis. This can also be appreciated from micromass cultures where cell density is higher in the center regions and contain more cartilage nodules.

D.F. Paulsen: Have you considered the possibility that, rather than being induced, the condensation is a manifestation of "self-assembly" in a permissive environment? That is to suggest the possibility that signals exist in the limb periphery that inhibit mesenchymal condensation there, as a significant component of the mechanism limiting chondrogenesis to the core of developing limb? Considering what is known about factors that inhibit the expression of the adhesion molecules you have analyzed, can you suggest any candidate "anti-condensation signals" that might be operating in the limb periphery, perhaps emanating from the ectoderm as suggested by Solursh (1984)?
Authors: Indeed, we do consider that condensation process is a self-organization process in a permissive environment (appropriate cell density and cellular competence). This process is then modulated by extracellular matrix and signaling molecules. Some extracellular molecules enhance chondrogenesis while others suppress it. Recently, Wnt 7a was shown to have inhibitory effect on chondrogenesis and is secreted by the dorsal limb bud epithelium (Rudnicki and Brown, 1997). With many soluble modifiers for BMP (e.g., follistatin, noggin), FGF (e.g., heparin) and Wnt (frizzbe) identified recently, we can expect a multiple array of signaling molecules and their modulators that work together to regulate the formation of condensation boundaries.

D.F. Paulsen: In the description of the DNA assay (Brunk *et al*., 1979), the authors indicated the use of "calf thymus DNA" for the standard curve. While acceptable, this is less than ideal. Owing to significant species-specific differences in A-T:G-C ratios in genomic DNA, and to the fact that Hoechst 33258 shows quantitative fluorescence enhancement upon association with A-T pairs, it is considered much more desirable to establish standard curves for such assays using DNA from the same species being used as the tissue donor (i.e., chicken DNA in this case). Again, considering the likelihood that others may wish to adapt the authors' method to their own system (which might be primary cultures or cell lines derived from mouse, rat, human, or other species), some mention of this issue should be made.
Authors: In this work, we used fluorometric determination for DNA with Hoechst 33258 (1 mg/ml; emission wavelength, 365 nm; excitation wavelength, 460 nm). Calf thymus DNA was used as a DNA standard (Brunk *et al*., 1979). Owing to the fact that Hoechst 33258 shows enhanced fluorescence upon association with A-T pairs, the DNA standard curve derived from different species may show variation resulting from species-specific difference in A-T:G-C ratio. For comparison between different experimental conditions, this is sufficient for our purpose. To obtain the absolute amount of DNA, one should follow your advice.

R.S. Tuan: As a review paper, the manuscript should review more of the current thinking concerning the function of many of the patterning genes (e.g., Hox), extracellular molecules (e.g., hyaluronate, fibronectin), as well as the mechanistic basis of mutations that affect limb mesenchymal condensation.
Authors: The shaping of skeleton *in vivo* is driven by the force of cell adhesion and determined by the interaction between the competence of a mesenchymal cell and the extra-cellular environment. The environment is a complex landscape made of extracellular matrixes and multi-directions of gradients of signaling molecules. To ascertain how the *in vivo* "patterning" is translated into specific skeletal morphology, we have to resort to a more simplified and analyzable *in vitro* model. The purpose of this paper is to demonstrate the usefulness of micromass culture and the new addition of retroviral transduction. Here, we first summarize the early molecular expression events occurring in the early stages of chondrogenic nodule formation in micromass cultures. We then report the application of the newly developed RCAS technology (Morgan and Fekete, 1996) to micromass cultures (Stott *et al*., 1998). We use NCAM and SHH to demonstrate the usefulness of the approach. We then review the roles of various molecules in precartilage condensation formation: adhesion molecules are mediators, extracellular signaling factors modulate

adhesion and differentiation events; and intracellular signaling factors determine the competence and types of responses of the mesenchymal cells. The molecules you mentioned have been covered and we now expand their coverage as well as update with new references. The **Discussion** represents current knowledge for precartilage condensation formation.

R.S. Tuan: As a research paper, more information is needed concerning the efficacy and time course of the viral transduction protocol (at both mRNA and protein levels), the coincidence in terms of antisense N-CAM expression and reduced chondrogenic differentiation, as well as the result of over-expression of N-CAM.

Authors: Suppression of condensation formation by antibodies to NCAM and induction of large condensations by over-expression of NCAM have been shown in Widelitz *et al*. (1993). Results from RCAS-antisense NCAM experiment further demonstrate that NCAM is involved in the endogenous process. It also needs to be pointed out that since the size of NCAM cDNA (2.6 kb) is over 2 kb capacity of RCAS vector, over-expression of NCAM becomes technically unavailable to the RCAS approach.

Semi-quantitative measurement of NCAM expression in the mRNA and protein levels have been performed using RT-PCR and double labeled immunostaining with expression of viral GAG as the internal control. Results showed reduced expression of NCAM proteins but no changes in the mRNA level. Antisense RNA treatment may not always result in degradation of the target mRNA. Several cases of RNA:RNA duplexes have been documented (Bass and Weintraub, 1988; Bunch and Goldstein, 1989).

Additional Reference

Solursh M (1984) Ectoderm as a determinant of early tissue pattern in the limb bud. Cell Differentiation **15**: 17-24.