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Original Article

Role of *5' HoxD* genes in chondrogenesis *in vitro*

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ABSTRACT In the present study, we have examined by *in situ* hybridization, expression of five 5' *HoxD* cluster genes *(D9, D10, D11, D12* and *D13)* during chondrogenesis of chick limb bud mesenchymal cells *in vitro*. After one day in culture, *D9* and *D13* gene expression was restricted to patches of mesenchymal cells, while expression of *D10, D11,* and *D12* gene was prominent in all mesenchymal cells. In 3-day cultures, *D9* and *D13* genes were expressed only in cartilage nodules, while *D10, D11,* and *D12* genes were expressed in both cartilage nodules and in all mesenchymal cells. These observations indicate two different patterns of expression; one for *D9* and *D13,* and a different one for *D10, D11,* and *D12*. These patterns of expression seem to correlate with patterns of cell proliferation and differentiation to chondrocytes. The role of these *HoxD* genes was further investigated by employing antisense S-oligomers. We found that oligodeoxynucleotides complementary to *HoxD (D10-D13)* mRNAs were capable of inhibiting chondrogenesis. These data suggest that expression of *HoxD* genes is required for mesenchymal condensation, and differentiation to chondrocytes. This in turn implies that these *HoxD* genes aside from their role in the patterning of the developing skeletal elements might regulate down-stream factors necessary for cartilage differentiation as well.

KEY WORDS: *limb bud, mesenchymal cells, chondrogenesis, homeobox*

Introduction

The culturing of mesenchymal cells from the developing chick limb bud provides a useful system for studying cellular and molecular mechanisms during cartilage differentiation. The onset of limb cartilage differentiation *in vivo* and *in vitro* is characterized by a transient cellular condensation or an aggregation process by which prechondrogenic mesenchymal cells become closely juxtaposed to one another prior to deposing the cartilage matrix (Ahrens *et al.*, 1977). A variety of studies have indicated that this condensation involves cell-extracellular matrix and cell-to-cell interactions and communication. Chondrogenesis can be affected by many molecules, such as, growth factors (TGF-β, bFGF, activin, inhibin and Bone Morphogenetic Protein), extracellular matrix molecules (fibronectin, collagen, proteoglycans, link protein, cartilage matrix protein), and adhesion molecules (NCAM, N-Cadherin). All these molecules show specific temporal and spatial expression during the process of chondrogenesis (Kravis and Upholt, 1985; Biddulph, *et al.*, 1988; Frenz *et al.*, 1989; Kulyk *et al.*, 1989a,b; Stirpe and Goetinck, 1989; Schofield and Wolpert, 1990; Solursh, 1990; Carrington, *et al.*, 1991; Widelitz *et al.*, 1993; Tsonis *et al.*, 1994). It has also been shown that ligands for receptors belonging to the steroid family, such as vitamin D or retinoic acid, can affect expression of the above-mentioned molecules and stimulate chondrogenesis of chick limb bud mesenchymal cells (Ide and Aono, 1988; Tsonis, 1991; Del Rio-Tsonis and Tsonis, 1994; Paulsen, *et al.*, 1994; Tsonis *et al.*, 1996). It is conceivable that such interactions require a sequence of yet uncharacterized transcriptional regulation.

In the past few years, homeobox genes *(HOX)* have been implicated in signaling pathways in limb bud morphogenesis (Dolle and Duboule, 1989; Davidson *et al.*, 1991; Izpisua-Belmonte *et al.*, 1991; Nohno *et al.*, 1991; Rogina *et al.*, 1992; Burke *et al.*, 1995; Nelson *et al.*, 1996). The homeobox genes encode transcriptional factors with a conserved DNA-binding domain, the homeo domain. In vertebrates, there are four clusters of homeobox-containing genes *(HoxA, HoxB, HoxC* and *HoxD)* (Duboule, 1994). Especially, the 5' members of the *HoxA* and *HoxD* complex are expressed in a specific spatial-temporal fashion along the anteroposterior and proximodistal axes in the developing limb.

The earliest *HoxD* gene expression observed during limb bud outgrowth, demonstrated by whole-mount *in situ* hybridization, is the uniform activation of *HoxD9* and *HoxD10* along the entire anterior/posterior extent of the early limb bud. Subsequently, *HoxD11, HoxD12* and *HoxD13* are activated sequentially at the posterior border of the limb bud (Laufer *et al.*, 1994; Nelson *et al.*, 1996). The expression of the *HoxA* genes correlates with segmentation and cartilage bifurcation along the proximo-distal axis.

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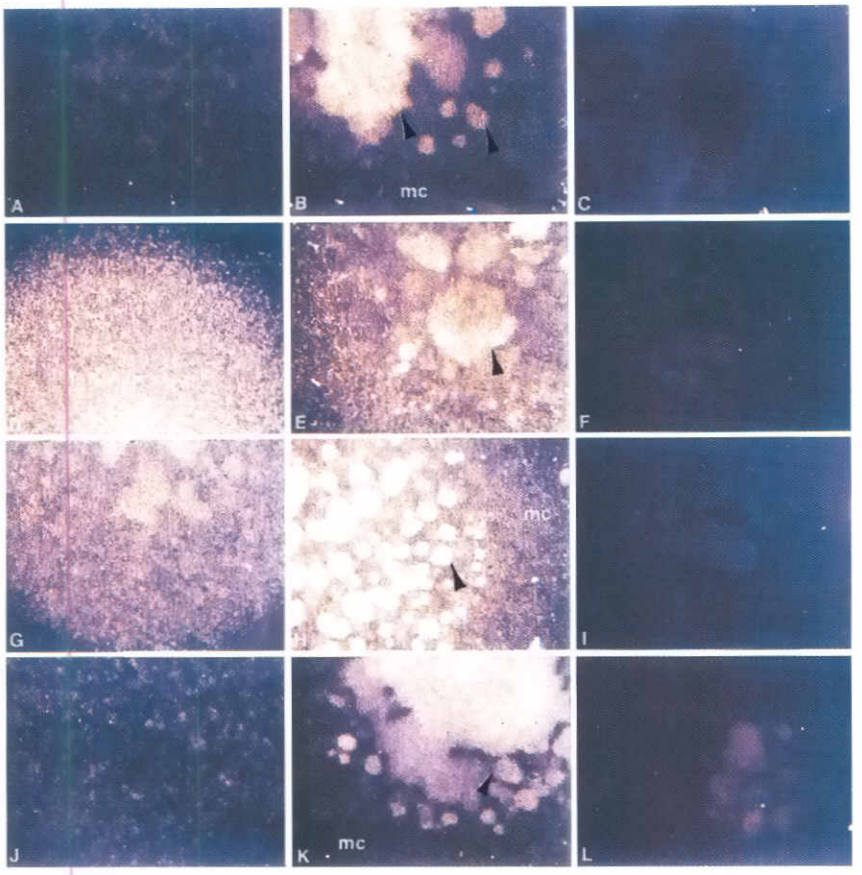

Fig. 1. Expression of *HoxD* genes during *in vitro* chondrogenesis of the chick limb bud mesenchymal cells. *These are micromass cultures. The whole culture is a circle. Usually the cartilage nodules appear first in the center while at the periphery we have the undifferentiated mesenchymal cells. These pictures have been taken from the periphery to the center so that all pertinent information can be included.* **(A,D,G,J)** *Expression of HoxD9, HoxD10, HoxD11 and HoxD13 respectively in cultures maintained for one day. At this point chondrogenesis has not started, but some patches of cells have started to organize in condensations. Note that HoxD9 (A) and HoxD13 (J) expression is not uniform in all cells, but appears patchy. Expression of HoxD10 (D) and HoxD11 (G) can be seen in all cells.* **(B,E,H,K)** *Expression of the same genes but after three days in culture. Note that transcripts of HoxD9 (B) and HoxD13 (K) are present only in the cartilage nodules (arrowheads), while transcripts of HoxD10 (E) and HoxD11 (H) are present in chondrocytes and all undifferentiated mesenchymal cells (mc).* **(C,F,I,L)** *Hybridization with the sense probes to show the background in three day cultures. These figures have been taken with wide lens and differences in the size of the nodules might affect the light.*

Although *HoxA* and *HoxD* genes were originally thought to have separate functions in the limb, paralogs share considerable overlap in expression domains, and overlap in function (Graham, 1994; Yokouchi *et al.*, 1995). This has become obvious from knockout experiments in mice where it has been shown that *HoxA* and *HoxD* genes are cooperatively determining pattern formation of the different segments of the limb. For example, *HoxA11* and *HoxD11* are required synergistically to specify the development of ulna and radius (Davis *et al.*, 1995), while *HoxA13* and *HoxD13* are required for chondrogenic patterns and development of the autopod (Fromental-Ramain *et al.*, 1996).

In vivo, misexpression studies have suggested that *Hox* genes affect both the condensation of skeletal precursors in the limb bud and the subsequent growth and elongation of these elements (Morgan and Tabin, 1994; Yokouchi *et al.*, 1995). *HoxD13* misexpression results in a decrease of the bone length due to change in cell proliferation, while *HoxD11* misexpression affects chondrogenic condensations (Goff and Tabin, 1997). As *Hox* genes are expressed in the limb mesenchyme during precartilaginous condensation it has been proposed that *Hox* genes might control the cell adhesiveness and growth/differentiation of the cartilage. This seems to be the case for *HoxA13* (Yokouchi *et al.*, 1991,1995).

Despite the considerable progress that has been made during the past decade in elucidating various mechanisms of *Hox* gene regulation during limb and patterning *in vivo*, little is known about their role during chondrogenesis *in vitro*. Given the lack of studies at the transcriptional level during *in vitro* chondrogenesis of the limb bud mesenchymal cells, we have decided to undertake a detailed study on the expression and function of some *Hox* genes in this system. Hence, we have selected five *5'HoxD* genes *(D9, D10, D11, D12* and *D13)* as key candidates for regulating chondrogenesis of the chick limb bud mesenchymal cells *in vitro*. Our studies indicate that these genes are expressed during condensation and chondrogenesis and that they also regulate this differentiation process.

Results

Expression of HoxD genes during chondrogenesis of mesenchymal cells

When the undifferentiated mesenchymal cells composing the distal subridge region of stage 24 limb buds are subjected to high density micromass culture, in serum containing media, they uniformly undergo chondrogenesis and form within three days a sheet of cartilage with little or no nonchondrogenic tissue present. The nodules are most prominent in the center, while the periphery contains undifferentiated mesenchymal cells (Ahrens *et al.*, 1977; Newman, 1977). This formation is characterized by the deposition of Alcian blue-positive cartilage matrix with an enhanced accumulation of sulfated glycosaminoglycans (GAGs) into matrix proteoglycans. After one day in culture we could observe widespread prechondrogenic condensations of cells. In the second day, appearance of nodules ensues and by the end of the third day the culture is filled with large nodules resulting from growth and differentiation. We, therefore, terminated such experiments at the end of the third day.

In day-1 cultures, *D9* and *D13* genes were expressed in restricted patches of mesenchymal cells (Figs. 1A,J; 2A,G), while *D10, D11,* and *D12* genes were strongly expressed ubiquitously in all mesenchymal cells (Figs. 1D,G; 2C,E). In day-3 cultures, *D9* and *D13* transcripts were only present in cartilage nodules (Figs. 1B,K; 2B,H), but *D10, D11,* and *D12* were highly expressed in both cartilage nodules and all undifferentiated mesenchymal cells

(Figs. 1E,H; 2D,F). Results of *HoxD12* not shown. We presume that the expression patterns for *HoxD9* and *HoxD13* seen at one day represent mesenchymal cells forming condensations that will lead to cartilage nodule differentiation. These two different patterns in the levels of *HoxD* expression can be seen in Figures 1 and 2. Figure 1 presents an overall picture of a micromass culture and has been taken using special wide lens. In Figure 2, however, we present close-ups of the same stages for further clarification of the results.

Prompted by these results as well as from data linking *5' HoxD* genes and control of proliferation (Goff and Tabin, 1997), we decided to examine the rate of proliferation in our cultures as mesenchymal cells progress from an early stage of condensation to a later stage of differentiation to chondrocytes. For this, we treated cultures at different stages of differentiation with ^{3}H-Thymidine and examined the profiles of cell proliferation. After one day in culture we observed a uniform rate of proliferation, but as nodules were formed (3rd or 4th day) proliferating cells were found only in the undifferentiated mesenchymal cells or on the base of established chondrogenic nodules, but not in the rest of the nodules (Fig. 3).

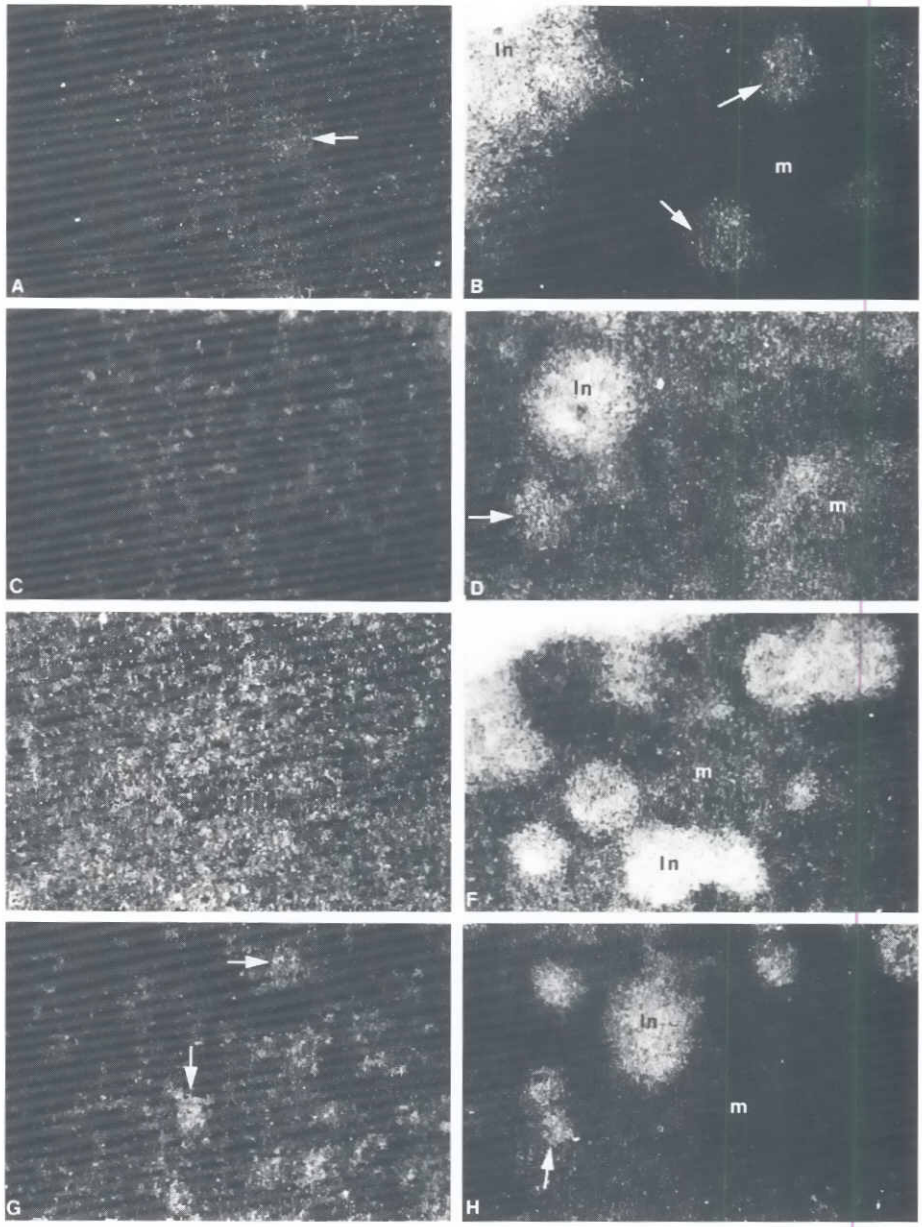

Fig. 2. Higher magnification photographs under dark field showing the expression of the 5' *HoxD* genes to better visualize the two different patterns of expression. (A,B) *HoxD9;* **(C,D)** *HoxD10;* **(E,F)** *HoxD11;* **(G,H)** *HoxD13. (A,C,E,G) 1-day cultures; (B,D,F,H) 3-day cultures. Note that HoxD9 and HoxD13 expression is restricted in patches of mesenchymal cells in 1-day cultures (arrows in A,G) and only in cartilage nodules in 3-day cultures (B,H; In, late mature nodule; arrows indicate earlier nodules; m, mesenchymal cells. Expression of HoxD10 and HoxD11 is widespread in all mesenchymal cells (m) in 1-day cultures* **(C,E)** *and both mesenchymal cells and nodules in 3-day cultures* **(D,F).** *The photographs were taken with the same conditions to better visualize the intensity of the grains and the expression patterns.*

Antisense ODNs inhibit chondrogenesis

To further define the role of *5' HoxD* genes we undertook experiments where their expression was inhibited by ODNs. This could directly link their expression to regulation of chondrogenesis. As mentioned in Materials and Methods, ODNs were applied in two different concentrations and combinations. Their effects were judged from the number of cartilage nodules as indicated by staining with Alcian blue, during the course of the experiment. In control cultures, the mesenchymal cells deposited a virtually continuous sheet of cartilage matrix that stained more uniformly and intensely with Alcian blue. In contrast, when these cells were cultured in the presence of ODNs inhibition of nodule formation was resulted as indicated by the deposition of Alcian blue in cells. When the cultures were treated with ODNs for any individual *HoxD* gene at the concentration of 55 μg/ml no inhibition resulted. However, when cultures were treated with individual ODNs at the concentration of 110 μg/ml they were capable of inhibiting deposition of GAGs in the center of the nodules (Fig. 4). Figure 4A shows the center of a control culture treated with the sense oligo. The area is occupied almost entirely with nodules stained with Alcian blue. In Figure 4B-D we can observe inhibition of nodule formation in cultures treated with ODNs to *HoxD10*, *HoxD11* and *HoxD13* respectively. The effect of *HoxD12* ODN was the same (not shown). When the experimental group and the control group were compared (see Methods) the effect on chondrogenesis was found statistically significant (Fig. 5). We did not observe inhibition when the *HoxD9* ODN was used. We believe that this is due to the fact that unlike all other ODNs the *HoxD9* antisense sequences were not derived

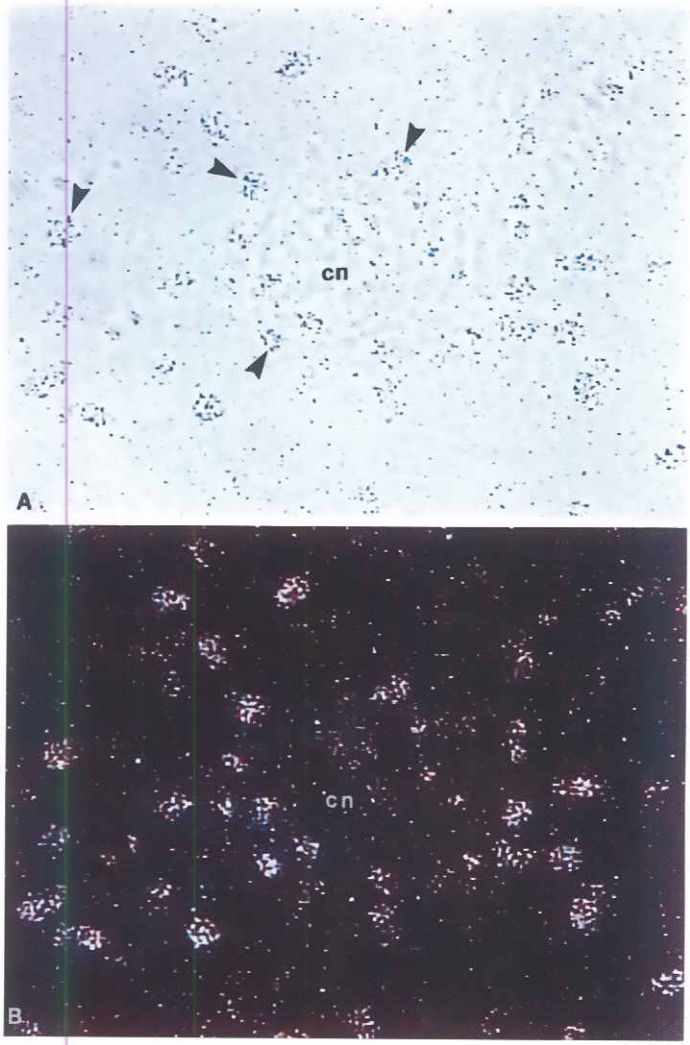

Fig. 3. Proliferation during chondrogenesis. (A) *Proliferation profile in mesenchymal cell cultures three days after plating taken with phase. Note that most of the label (arrowheads) is found in undifferentiated mesenchymal cells and at the base around the cartilage nodule (cn). The nodule is characterized by the presence of extracellular matrix space. This picture was taken focused on the grains.* **(B)** *Dark field of the area appeared in A.*

from the initiation codon but from internal sequences due to the unavailability of 5' sequences. It has been reported before that ODN treatment is most effective when 5' sequences starting with the initiation codon are used (Faiella *et al.*, 1994).

Discussion

Our results establish that *5' HoxD* genes are expressed during *in vitro* chondrogenesis of limb mesenchymal cells. Among the different members the patterns were somewhat different. *HoxD9* and *HoxD13* were found to be expressed mostly in patches of mesenchymal cells and later in the chondrogenic nodules only, whereas expression of the other members was widespread in all cells. Our data suggest that *HoxD9* and *HoxD13* are essential in regulating the growth and differentiation of mesenchymal cells to chondrocytes. *HoxD10*, *HoxD11* and *HoxD12* might be important in maintaining the growth of mesenchymal cells and chondrocytes.

Our proliferation data showed that as chondrogenesis progressed proliferating cells were restricted at the base of the nodule. We believe that this might indicate that the base of a nodule is composed of proliferating chondrocytes and that the rest are pre-hypertrophic, a situation which is reminiscent of the growth pattern of cartilage elements *in vivo*. In this case and since *HoxD9* and *HoxD13* expression was seen in the nodules they may control the rate of cartilage growth, while *HoxD10*, *HoxD11* and *HoxD12* may regulate the growth of undifferentiated mesenchymal cells. Indeed, in misexpression experiments (Goff and Tabin, 1997) it has been shown that *HoxD13* reduces the size of cartilage elements by reducing proliferation. On the other hand misexpression of *HoxD11* was shown to affect the growth of condensations. These misexpression results are consistent with the expression and proliferation patterns observed in the present study and indicate that the *in vitro* system of chondrogenesis could be applied to the study of chondrocyte differentiation and maturation as well.

In culture, the limb bud mesoderm undergoes a process of differentiation very similar morphologically and biochemically to that *in vivo* (Chen *et al.*, 1993). One difference between the *in vivo* and the *in vitro* systems could be the hypertrophy stage. Nodules differentiated in serum-containing media do not express type X collagen (by day 3) which is a hypertrophic cartilage marker (Solursh *et al.*, 1986). It could be that *in vitro* chondrogenesis stops at a pre-hypertrophy stage. This matter needs to be investigated in more detail.

The fact that partial inhibition of chondrogenesis occurred when antisense oligonucleotides were added in the cultures indicates that these *Hox* genes play a role in the control of chondrogenesis *in vitro*. The fact that complete inhibition was not observed could also be due to other factors involved. Other *Hox* genes have been identified that are specific to cartilage (Zhao *et al.*, 1993). Similarly, interaction with the *HoxA* complex could also be vital in this process as is in the developing limb. The fact that all *HoxD* genes examined were able to affect chondrogenesis, despite their mode of expression, suggests that they all might be important but act at different stages of mesenchymal cells or chondrocytes. A model has been recently proposed (Goff and Tabin, 1997) championing the idea that all *Hox* genes regulate the expression of the same targets with some of them exerting such an effect more efficiently. Such a scenario indicates that the overall growth and differentiation of a tissue is the result of the combined action of all *Hox* genes as they compete for the same target genes. This model can in fact account for the results seen in our system. The antisense data in conjunction with the expression data mentioned above suggest that the *5' HoxD* genes are essential regulators of chondrogenesis and needed possibly in an orchestrated fashion. This interaction is reminiscent of their expression pattern *in vivo* during the establishment of the limb skeleton.

5' Hox genes are transcriptional factors and might regulate down-stream cartilage specific molecules, and specifically extracellular matrix, important for cartilage differentiation. For example, a neural cell adhesion molecule, NCAM, is a mesenchymal adhesion molecule that mediates precartilaginous mesenchymal condensations and enhances chondrogenesis. When antibodies to NCAM were added, chondrogenesis is inhibited in micromass

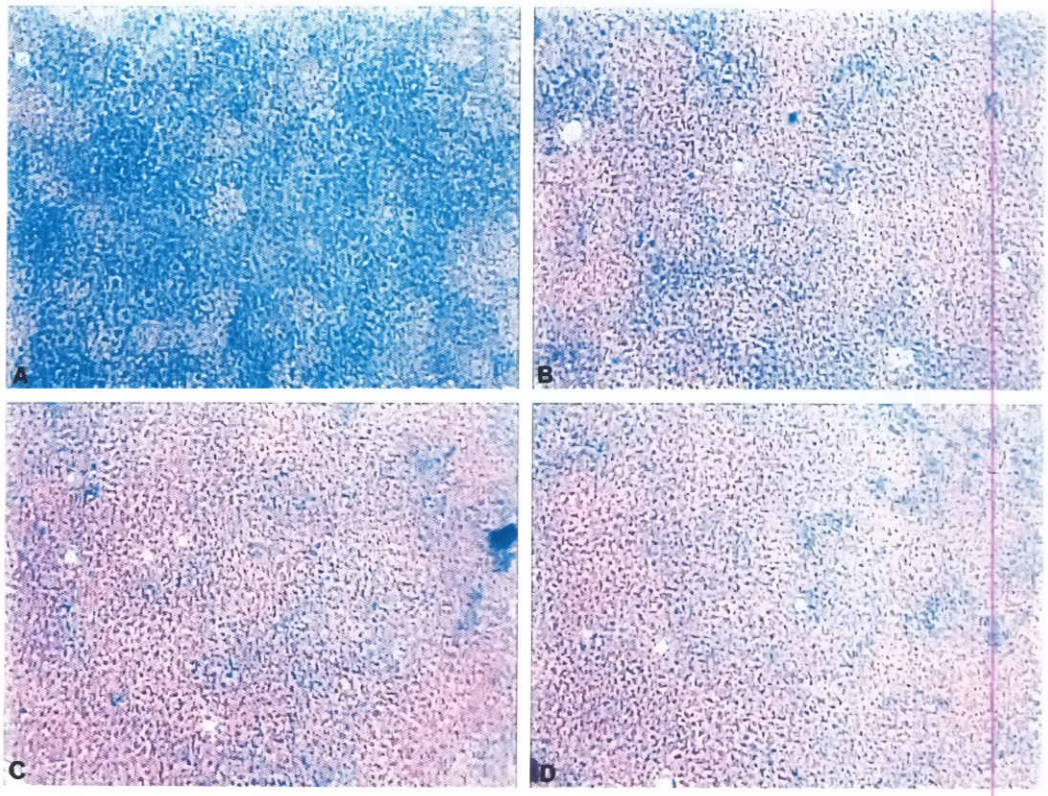

Fig. 4. Effects of antisense ODNs on chondrogenesis of the limb bud mesenchymal cells *in vitro*. **(A)** *Control micromass culture three days after plating treated with a sense ODN at the concentration of 110 μg/ml. Note widespread formation of nodules stained blue with Alcian blue.* **(B,C,D)** *Cultures treated with 110μg/ml antisense ODN to HoxD10, HoxD11 and HoxD13 respectively. Note inhibition of nodule formation as indicated by the lack of Alcian blue staining. All photographs have been taken from the center of the cultures where chondrogenesis is most prominent.*

cultures (Chuong, 1990; Widelitz *et al.*, 1993). The discovery that *Hox* gene binding sites are present in the 5' region of NCAM and that the expression of transfected *Hox* genes in cell lines alters the expression of NCAM suggests that NCAM is one of the likely downstream candidates of *Hox* genes (Jones *et al.*, 1992; Widelitz *et al.*, 1993). We believe that in our system, up-regulation of cell adhesion molecules and possibly other molecules by *5' HoxD* genes and subsequent effects on cell regulation and differentiation is an important manifestation leading to differentiation of chondrocytes. Finally, as more players of chondrogenesis come into the scene, such as *Indian hedgehog, patched, Gli* (Vortkamp *et al.*, 1996), the *in vitro* analysis of chondrogenesis could prove valuable in elucidating the molecular cascade involved in the control of mesenchymal cell differentiation to chondrocytes and their subsequent maturation.

Materials and methods

Animals

Fertilized white Leghorn chicken eggs were obtained from Conway hatchery (Indianapolis, IN).

Cell cultures

Mesenchymal cells were dissociated from the distal tips of Hamburger-Hamilton stage 24 chick embryo limb buds and micromass culture was carried out as described by Ahrens *et al.* (1977). Briefly, the cells were suspended in Ham's F-12 medium with 10% fetal calf serum, 1% penicillin/streptomycin, and the cell density was adjusted to $2x10^7$ cells/ml. The cells were applied in 10 μl (I drop) on cover glass (Gold Seal) in 35-mm Corning culture dishes. After attachment of the cells, the dishes were filled with medium. The media were changed daily. The cultures were maintained for 3 days. Cartilage nodules usually started appearing after 2 days.

Quantitative Alcian blue staining of cultures

Cultures were rinsed with phosphate-buffered saline (PBS) and fixed in 3% acetic acid adjusted to a pH of 1.0 with HCl for 10 min, and stained in 0.5% Alcian blue in 3% acetic acid at a pH of 1.0 overnight. Cultures were destained in 3% acetic acid and stored in 95% glycerol. For quantification, Alcian blue-stained cells were lysed in 4M guanidinium HCl.

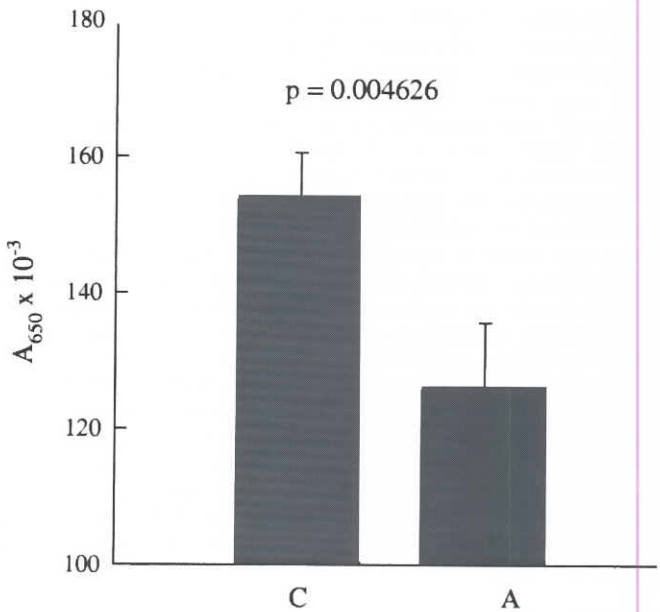

Fig. 5. Statistical comparison of antisense-treated cultures (A) with untreated cultures (C). *The values are absorbance units at A650nm.*

Synthetic deoxyoligonucleotides (ODNs) and treatment

Antisense S-oligonucleotides were purchased from Genemed Biotechnologies, Inc (San Francisco, CA). Sequences of the S-oligonucleotides (21-mers) corresponding to the translation initiation site of chick *HoxD10*, *D11*, *D12*, and *D13*) mRNA were used in the oligonucleotide coculture experiments. The *HoxD9* ODN sequence(30-mers) was also used, but it is important to note that its sequence was made downstream from the start codon, due to the unavailability of more 5' sequence (Nelson *et al.*, 1996).

The oligonucleotide antisense sequences are as follows: HOXD9 : 5'-CTC/TCG/GGA/CGA/GGA/GCA/CTC/AGT/CCT/TTT-3'; HOXD10 : 5'-AGA/GCT/GTT/GGG/AAA/GGA/CAT-3'; HOXD11: 5'GCA/ATC/GTC/AAA/CTC/GGT/CAT-3'; HOXD12 : 5'-GTA/GAG/ACT/GCG/ATC/ACA/CAT-3'; HOXD13 : 5'-GTC/GCC/GCG/CAG/TCC/GTC/CAT-3'; To examine whether any effects of the oligos on chondrogenesis were due to their presence, a sense oligo designed from HOXD13 was used: 5'-ATG/GAC/GGA/CTG/CGC/GGC/GAC-3'. Cells were treated in 24-well culture dishes with the antisense ODNs (individually and in combination) at either 55 μg/ml or 110 μg/ml concentration at time 0. A second dose was added 24 h later and cells were cultured until 72 h without changing the media. Control cells were plated without ODN exposure, or with the HOXD13 sense ODN to exclude the possibility of toxic effects of the oligos. This range of concentration of antisense ODNs has been successfully used in other studies, including those with *Hox* genes (Zamecnik and Stephenson, 1978; Gewirtz and Calabreta, 1988; Faiella *et al.*, 1994). After staining with Alcian blue to visualize the degree of chondrogenesis and after photographing, the cells were lysed and the extracts were analyzed by a spectrophotometer at A650nm to quantify the effect of antisense oligonucleotides on chondrogenesis. For this, we pooled all the treated samples as the experimental group and the sense treated or untreated samples as the control group. The two groups were then compared statistically using Sigma Plot's Student's t.

In situ Hybridization

For these experiments, the cells (day-1 and day-3 cultures) were rinsed with phosphate-buffered saline, fixed in fresh 4% paraformaldehyde for 10 min, and stored in 70% EtOH at 4°C until ready for use. Briefly, the cells were brought to room temperature and immersed in 50% ethanol for 2 min and in PBS (including 0.5% Triton X-100, 5 mM $MgCl_2$) for 10 min. The cells were then immersed in triethanolamine (TEA) buffer for 2.5 min and in TEA buffer plus 0.25% acetic anhydride for another 10 min. After rinsing the cells in 2X standard saline citrate (SSC) for 2.5 min twice, they were dehydrated to 100% ethanol through a graded series and then air dried for 2 h *HoxD9-13* (gift from Dr. C. Tabin) riboprobes (antisense and sense) were made using T7, T3, or SP6 RNA polymerase and [^{35}S]thio]UTP. After purification of the probe through Stratagene's Nuctrap columns, 1X107 cpm/ml of [^{35}S]thio]UTP was added to hybridization buffer that contained 1.2 M NaCl, 20 mM Tris/HCl (pH 7.5), 4 mM EDTA, 2X Denhardt's solution, 20% dextran sulfate, 40% formamide, 1 mg of yeast tRNA per ml, and 50 mM DTT. About 200 μl of the hybridization solution was added to each coverslip, which were then placed in a moisture chamber container at 50-55°C overnight . The coverslips were then washed in 2X SSC for 5 min twice and then placed at the corresponding hybridization temperature in a solution (Solution 1) containing 50% formamide, 1X SSC, and 0.1%-mercaptoethanol for 12 min twice. The coverslips were then transferred to a solution containing 0.5 M NaCl, 10 mM Tris/HCl (pH 8.0), and 20 μg of RNase A per ml for 30 min at 37°C. Subsequently, the coverslips were washed again at the corresponding hybridization temperature in solution 1 for another 12 min twice and then they were incubated twice in another solution (solution II, 37°C) containing 1X SSC, and 0.1%-mercaptoethanol for 12 min. The coverslips were then dehydrated through a graded series of ethanol and allowed to air dry for about 2 h.

Proliferation assay

1.0 μCi [^{3}H]thymidine was added to the media of 1 and 3-day cultures. After incubating for 6 h, the cells were processed for autoradiography.

Autoradiography

Air-dried coverslips were dipped in Kodak NTB-2 emulsion and left in complete darkness to dry overnight. Coverslips were stored in airtight containers at 4°C for exposure times ranging from 5 days to 2 weeks. Exposed coverslips were developed for 2.5 min in D-19 developer (Kodak), rinsed in water and then fixed for 5 min in regular fixer (Kodak). After rinsing for 10 min in distilled water, coverslips were mounted in Permount. Cells were viewed with a Olympus microscope fitted with a dark field illumination system. Photography was performed by printing video images (Sony), and in some cases through regular negative film.

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