

## Differential effects of transforming growth factors $\beta 1$ , $\beta 2$ , $\beta 3$ and $\beta 5$ on chondrogenesis in mouse limb bud mesenchymal cells

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**ABSTRACT** The present study was performed to determine whether mammalian TGF- $\beta$  isoforms and *Xenopus* TGF- $\beta 5$  elicit a differential chondrogenic response on mesenchymal cells during mouse limb development. Results showed that TGF- $\beta$  isoforms produced a distinct chondrogenic pattern depending on embryonic stage. When they were applied to 5 day micromass cultures of limb mesenchymal cells from embryonic stages 19, 20 and 21, a differential response to all four TGF- $\beta$  isoforms assayed was observed. By stage 19 the cells formed a uniform sheet of cartilage cells; by stage 20, mesenchymal cells were more responsive to TGF- $\beta 1$  and TGF- $\beta 5$  than at stages 19 and 21, showing an entire cell layer of chondrogenic cells with higher accumulation of extracellular matrix. The diminished effect of TGF- $\beta 2$  and TGF- $\beta 3$  at stages 20 and 21 was accompanied by a nodular pattern of chondrogenic cells rather than by a uniform sheet, as seen at stage 19. At stage 20 TGF- $\beta 1$  and TGF- $\beta 5$  enhanced the expression of sulfated proteoglycans, type II collagen, cartilage link protein and alkaline phosphatase activity. In contrast, TGF- $\beta 2$  and TGF- $\beta 3$  caused less expression in the same parameters. Only a transient exposure to TGF- $\beta$  isoforms at days 1 and 2 of culture stimulate chondrogenesis, indicating that TGF- $\beta$  isoforms could regulate chondrogenesis at early stages of chondrocyte differentiation. However, when TGF- $\beta$  isoforms were applied to low density cultures of mesenchymal cells, chondrogenesis was enhanced only by 25%, suggesting that TGF- $\beta$  isoforms enhanced cartilage differentiation to higher levels in micromass cultures than in situations in which little or no chondrogenic differentiation normally occurs.

**KEY WORDS:** cartilage, chondrogenesis, limb development, mesenchymal cells, TGF- $\beta$  isoforms

### Introduction

During limb embryogenesis, undifferentiated mesenchymal cells migrate to the limb bud from the lateral plate mesoderm; later they condense to give rise to chondrogenic blastema and begin to synthesize and deposit extracellular matrix (ECM) components characteristic of cartilage. Condensing mesenchyme differentiates further into chondrocytes, which gradually undergo a process of hypertrophy, mineralization and vascular invasion. Then, osteoclasts and osteoblasts invade the cartilage zone forming a bone matrix with a functional bone marrow and a periosteum around the bone. A broad variety of growth factors influence the formation of cartilage during early embryogenesis, such as members of the transforming growth factor-beta (TGF- $\beta$ ) family among others (Solursh, 1989; Carrington and Reddi, 1991; Reddi, 1992, 1994; Elima, 1993; Cancedda *et al.*, 1995).

The TGF- $\beta$  family consists of five proteins with wide-ranging and opposite effects on many cellular processes such as control of cell proliferation and differentiation. The amino acid sequences of

TGF- $\beta$  isoforms show approximately 70% homology among different mammalian species. Chicken TGF- $\beta 4$  and frog TGF- $\beta 5$  present homologies with mammalian TGF- $\beta 1$  of 82% and 76%, respectively, suggesting that TGF- $\beta 4$  and TGF- $\beta 5$  are the chick and frog homologs of mammalian TGF- $\beta 1$  (Kondaiah *et al.*, 1990; Burt and Paton, 1992).

Among the diverse roles of TGF- $\beta$  isoforms in cell differentiation are those related to chondrocyte differentiation. They exert both stimulatory and inhibitory effects on cartilage cells *in vitro*, depending on their developmental origin and stage, as well as culture conditions. In micromass cultures of chick limb mesenchymal cells, many growth factors elicit different responses when added exogenously. Exposure to TGF- $\beta 1$ , TGF- $\beta 2$  and TGF- $\beta 3$  in the presence of serum stimulate chondrocyte differentiation on mesenchymal cells obtained from chick embryos at stages 22/23 and 23/24 (Kulyk *et al.*, 1989; Carrington and Reddi, 1990; Schofield and Wolpert, 1990; Leonard *et al.*, 1991; Roark and Greer, 1994; Tsonis *et al.*, 1994). In the presence of 5% serum, TGF- $\beta 1$  and TGF- $\beta 2$  decrease chondrocyte differentiation at embryonic stages

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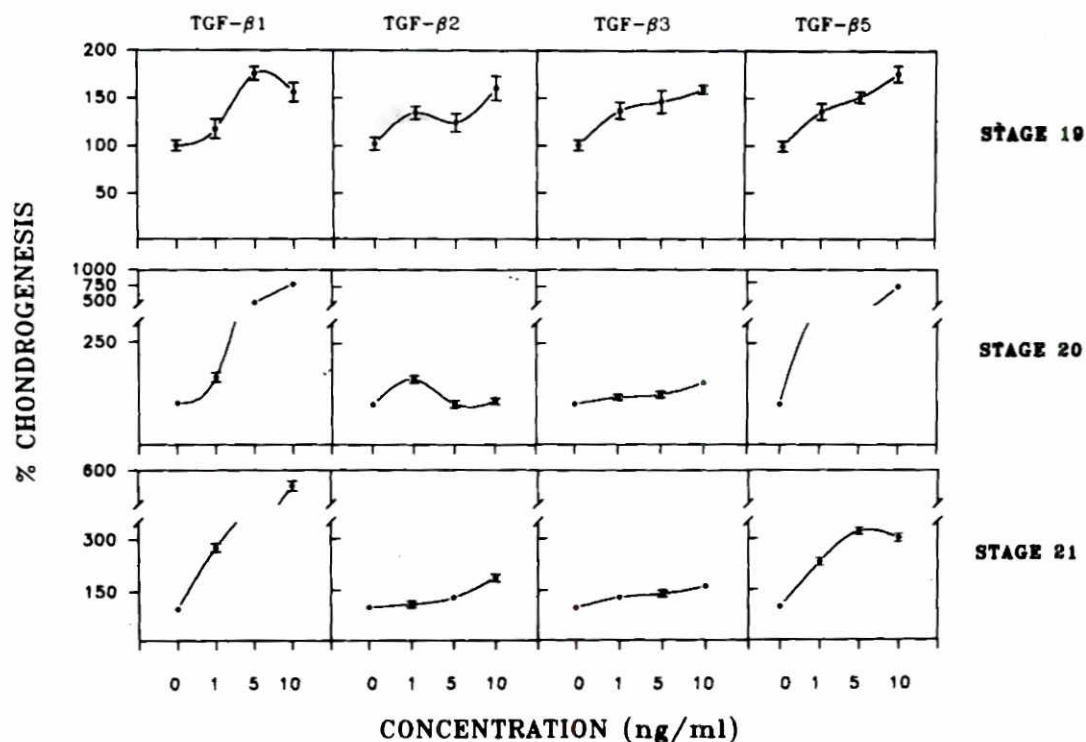


Fig. 1. Dose response effect of TGF- $\beta$  isoforms on cartilage matrix production as measured by Alcian blue extraction from micromass cultures of limb bud mesenchymal cells at embryonic stages 19, 20 and 21. Data are means  $\pm$  standard deviation from 4-5 cultures of 3 separate experiments. All values have a  $P < 0.01$  (vs controls). % chondrogenesis means percentage of chondrogenesis stimulation as compared to controls. For stage 19 controls had an optical density ( $OD_{600}$ ) of  $0.550 \pm 0.038$ ; stage 20,  $0.437 \pm 0.025$ ; stage 21,  $0.358 \pm 0.031$ . Data were represented as % chondrogenesis to make comparable chondrogenesis stimulation among the three different stages.

23/24 and 24/25, but this inhibition is avoided by BMP-3 and BMP-4 (Carrington and Reddi, 1991; Carrington *et al.*, 1991; Chen *et al.*, 1991). These reports suggest that TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 are key regulators of the chondrocyte differentiation of chick embryonic limb bud mesenchymal cells *in vitro* as are BMPs (Carrington *et al.*, 1991; Chen *et al.*, 1991; Roark and Greer 1994). However, TGF- $\beta$  isoforms do not induce cartilage and bone in ectopic sites as do BMPs (Wozney *et al.*, 1988).

Other molecules that regulate chondrocyte differentiation of chick embryonic limb bud mesenchymal cells are activin and inhibin (Chen *et al.*, 1993; Jiang *et al.*, 1993). An increase in the size of precartilaginous nodules and in the expression of N-CAM and tenascin occurs as a consequence of activin administration (Jiang *et al.*, 1993). Similarly, vitamin D enhances chondrogenesis (Tsonis, 1991) and the expression of N-cadherin and alkaline phosphatase in chick limb bud cells (Tsonis *et al.*, 1994); it also downregulates TGF- $\beta$ 2 expression, suggesting that vitamin D upregulates cell adhesion molecules which, in turn, downregulate TGF- $\beta$  (Del Rio-Tsonis *et al.*, 1994). Finally, retinoic acid (RA) is a multifunctional factor with important activities on chondrocyte differentiation. Chondrogenesis in embryonic chick limb mesenchymal cells is stimulated at a concentration of 5 ng/ml, whereas at concentrations up to 50 ng/ml it is inhibited (Paulsen *et al.*, 1988). Its effects on mouse limb bud mesenchymal cells is mediated by RA receptor- $\beta$ 2 (Jiang *et al.*, 1995). In chick craniofacial mesenchyme the inhibition or stimulation of chondrogenesis by RA depends on the developmental stage and origin of tissue (Langille *et al.*, 1989).

In the current study, we have evaluated the effects of mammalian TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 on the chondrogenic differentiation of mouse embryonic limb bud mesenchymal cells and determined whether these effects could be different in embryonic stages 19, 20 and 21. In addition, we evaluated the effects of another TGF-

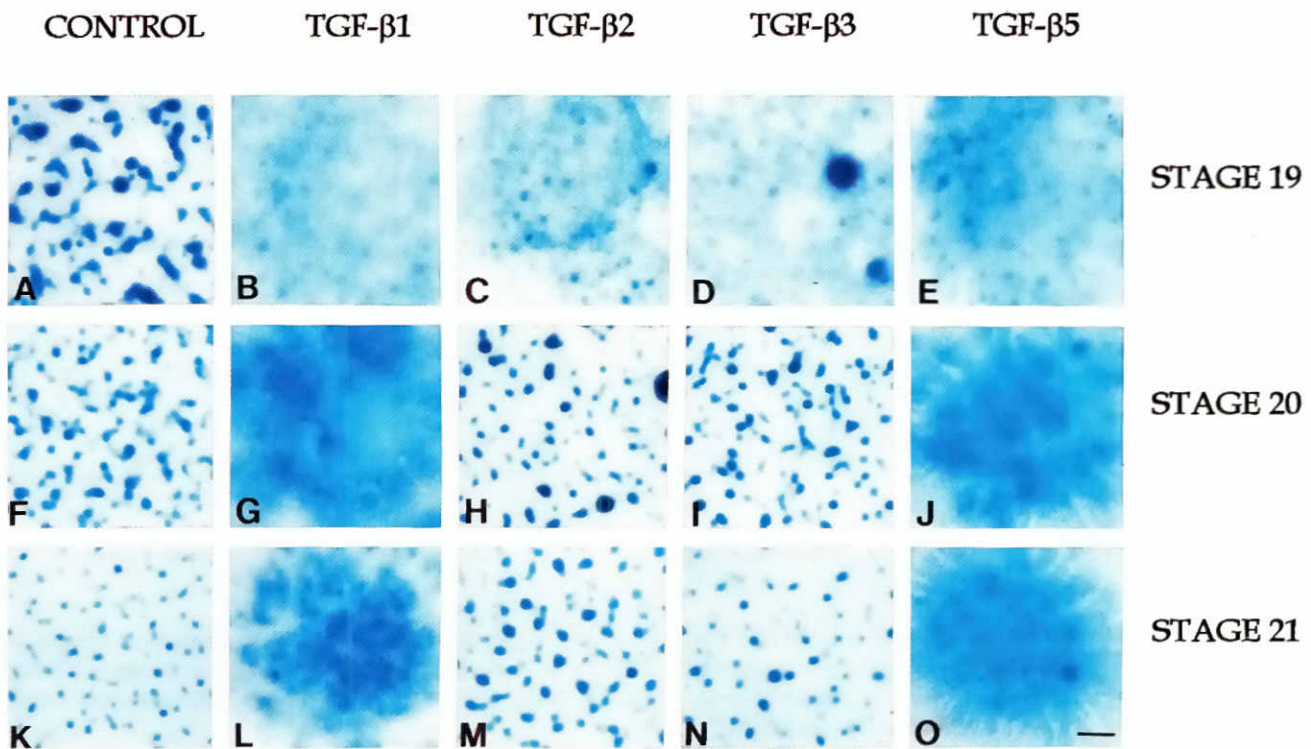
$\beta$  isoform (*Xenopus* TGF- $\beta$ 5) on the same model. The high homology between TGF- $\beta$ 1 and TGF- $\beta$ 5 led us to investigate whether or not TGF- $\beta$ 5 could regulate chondrocyte differentiation in mouse limb bud cells.

Our results showed that all four TGF- $\beta$  isoforms elicited a differential response on mouse limb bud mesenchymal cells depending on the embryonic stage. At stage 20, they differentially regulated the expression of specific cartilage matrix components. When all four TGF- $\beta$  isoforms were administered during the first two days of culture chondrogenesis was enhanced, while at day 5 of culture it was inhibited, and TGF- $\beta$ 2 and TGF- $\beta$ 3 had no effect. In low density cultures, all four TGF- $\beta$  isoforms slightly promoted chondrogenesis, suggesting that they stimulate cartilage differentiation to higher levels in micromass conditions rather than in situations in which little or no chondrogenic differentiation normally occurs. Based on the reported homologies between TGF- $\beta$ 1 and TGF- $\beta$ 5 (Kondaiah *et al.*, 1990; Burt and Paton, 1992), and on the biological activities reported in this work, we support the suggestion that *Xenopus* TGF- $\beta$ 5 is equivalent to mammalian TGF- $\beta$ 1.

## Results

### Effects of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 on *in vitro* chondrogenesis

To test the functionality of the TGF- $\beta$  isoforms used in this work, we evaluated them in chick embryo limb bud mesenchymal cells at stages 23/24 seeded at micromass cultures in the presence of 10% FBS during 5 days. The results obtained indicate that all four TGF- $\beta$  isoforms stimulated the accumulation of cartilage matrix and  $^{35}$ S-sulfate incorporation into proteoglycans. TGF- $\beta$ 1 showed an increase on synthesis of proteoglycans by 250%; TGF- $\beta$ 2 by 230%; TGF- $\beta$ 3 by 220% and TGF- $\beta$ 5 by 240% above controls



**Fig. 2.** Influence of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 on chondrogenesis pattern. Micromass cultures at day 5 of culture were stained with Alcian blue, cells from stage 19 (A-E), stage 20 (F-J), and stage 21 (K-O) were cultured as micromass cultures without TGF- $\beta$  isoforms (A, F and K) or with 10 ng/ml TGF- $\beta$ 1 (B, G and L), 1 ng/ml TGF- $\beta$ 2 (C, H and M), 10 ng/ml TGF- $\beta$ 3 (D, I and N) and 10 ng/ml TGF- $\beta$ 5 (E, J and O). Bar, 0.5 mm

( $P < 0.01$ ). These results are comparable with those obtained by Kulyk *et al.* (1989), Leonard *et al.* (1991), Roark and Greer (1994) and Schofield and Wolpert (1990).

Once the functionality of TGF- $\beta$  isoforms was established, we examined the possible role of TGF- $\beta$  isoforms in the regulation of chondrogenic differentiation on the accumulation of cartilage matrix in mouse limb bud mesenchymal cells, plated in micromass culture conditions. Micromass cultures were prepared from the cells comprising stages 19, 20 and 21 mouse limb buds. All four TGF- $\beta$  isoforms elicited a differential response on chondrogenesis, depending on the developmental embryonic stage (Figs. 1 and 2). At stage 19 we added different concentrations of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 to micromass cultures. A gradual increase in the accumulation of Alcian blue, pH 1.0-positive ECM, became apparent after 5 days of culture in a dose-dependent fashion. In the presence of 5 ng/ml TGF- $\beta$ 1 and 10 ng/ml TGF- $\beta$ 5 the accumulation of Alcian blue-positive ECM was enhanced by 75% above controls, whereas 10 ng/ml TGF- $\beta$ 2 and TGF- $\beta$ 3 increased the accumulation of Alcian blue-positive ECM by 55% above controls (Fig. 1). At stage 20, the response of mesenchymal cells to different TGF- $\beta$  isoforms was dose-dependent, except for TGF- $\beta$ 2. The accumulation of Alcian blue-positive ECM was different for each growth factor; the stimulation was 675% for 10 ng/ml TGF- $\beta$ 1 and 590% for 10 ng/ml TGF- $\beta$ 5 above controls, while for 1 ng/ml TGF- $\beta$ 2 and 10 ng/ml TGF- $\beta$ 3 the stimulation was 55% above controls (Fig. 1). Finally, the dose-dependent effect of TGF- $\beta$  isoforms was maintained at embryonic stage 21, although the accumulation of Alcian blue-positive ECM was decreased when compared to stage

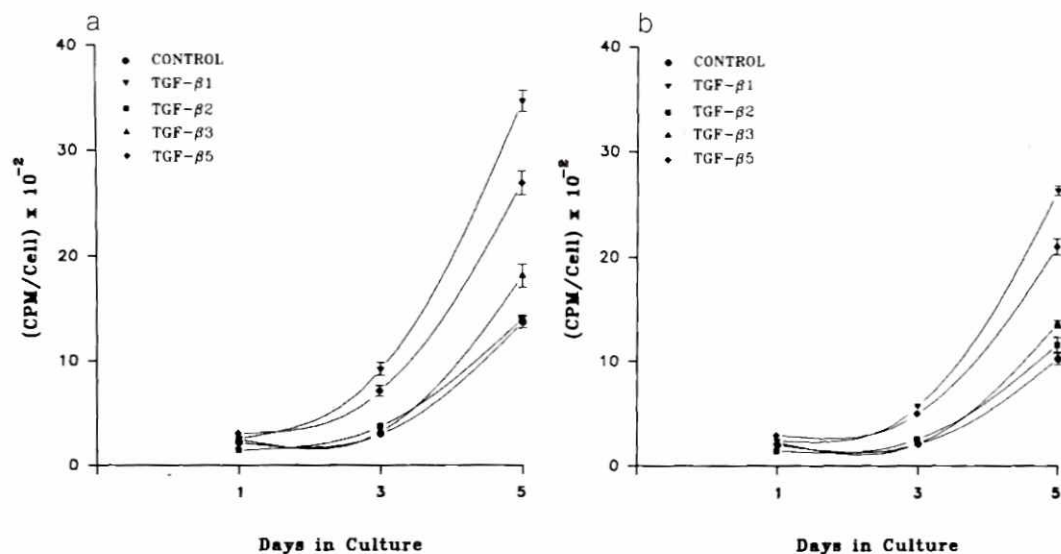
20. In the presence of 10 ng/ml TGF- $\beta$ 1 and 5 ng/ml TGF- $\beta$ 5, the accumulation corresponded to 455% and 211% above controls, respectively. Regarding TGF- $\beta$ 2 and TGF- $\beta$ 3, 10 ng/ml of both factors enhanced accumulation of Alcian blue-positive ECM by 75% above controls (Fig. 1).

By morphological analysis we observed that TGF- $\beta$  isoforms regulate the pattern formation of Alcian blue-positive nodules (Fig. 2). At embryonic stage 19 TGF- $\beta$ -treated cultures promoted the formation of a uniform sheet of Alcian blue stained cartilage matrix (Fig. 2A-E). At embryonic stage 20 mesenchymal cells showed a strong decrease in their response to TGF- $\beta$ 2 and TGF- $\beta$ 3, exhibiting Alcian blue positive cartilage nodules separated by much non-chondrogenic tissue (Fig. 2H and I). Also, TGF- $\beta$ 1 and TGF- $\beta$ 5 promoted a striking increase in the uniform mass of staining cartilage matrix and decreased non-chondrogenic areas in the micromass cultures (Fig. 2G and J). For embryonic stage 21, the fusion of cartilage nodules was still present at 10 ng/ml TGF- $\beta$ 1 and TGF- $\beta$ 5 (Fig. 2L and O), while TGF- $\beta$ 2 and TGF- $\beta$ 3 did not increase the fusion of cartilage nodules (Fig. 2M and N).

Since TGF- $\beta$ 1 and TGF- $\beta$ 5 regulate the fusion of Alcian blue-positive cartilage nodules and promote the accumulation of Alcian blue-positive ECM much better than TGF- $\beta$ 2 and TGF- $\beta$ 3 at embryonic stages 19, 20 and 21, we further analyzed the effects of TGF- $\beta$  isoforms on mesenchymal cells from embryonic stage 20.

#### Metabolism of proteoglycans

TGF- $\beta$ 1 and TGF- $\beta$ 5 enhanced the synthesis of proteoglycans in micromass cultures to higher levels from the third day of culture,



**Fig. 3. Effects of TGF- $\beta$  isoforms on the synthesis of glycosaminoglycans by stage 20 mouse limb bud mesenchymal cells. (a) Time course incorporation of  $^{35}\text{S}$ -sulfate; (b) incorporation of  $^3\text{H}$ -glucosamine. Data represent means  $\pm$  standard deviation from 3 cultures for each time point of 2 separate experiments.  $P < 0.01$  (vs controls) except for TGF- $\beta$ 2 treated cultures.**

compared to TGF- $\beta$ 2 and TGF- $\beta$ 3. At day 5 of culture TGF- $\beta$ 1, TGF- $\beta$ 5 and TGF- $\beta$ 3 at a concentration of 10 ng/ml enhanced the synthesis of proteoglycans by 1.54-, 1.0- and 0.34-fold respectively above controls, while TGF- $\beta$ 2 did not stimulate proteoglycan synthesis (Fig. 3a). The increase in the accumulation of sulfated glycosaminoglycans (GAG) into proteoglycans was not due to oversulfation of GAG chains, since the incorporation of  $^3\text{H}$ -glucosamine into GAG, which occurred in the presence of TGF- $\beta$  isoforms, resembled the incorporation of  $^{35}\text{S}$ -sulfate (Fig. 3b). TGF- $\beta$ 1, TGF- $\beta$ 5 and TGF- $\beta$ 3 enhanced  $^3\text{H}$ -glucosamine incorporation by 1.57-, 1.05- and 0.34-fold respectively above controls. However, TGF- $\beta$ 2 did not promote the incorporation of  $^3\text{H}$ -glucosamine into GAG (Fig. 3b).

#### **$^3\text{H}$ -thymidine incorporation**

The incorporation of  $^3\text{H}$ -thymidine by mesenchymal cells was measured in micromass cultures at days 1, 3 and 5 of culture (Table 1). At day 1 of culture only TGF- $\beta$ 3 decreased  $^3\text{H}$ -thymidine incorporation, whereas TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 5 had no effect. At day 3 of culture TGF- $\beta$ 1 and TGF- $\beta$ 5 presented no significant differences as compared with controls, TGF- $\beta$ 2 increased  $^3\text{H}$ -thymidine incorporation and TGF- $\beta$ 3 showed a slight decrease. Finally at day 5 of culture there were no significant differences between controls and TGF- $\beta$  treated cultures.

#### **Regulation of type II collagen, cartilage link protein and alkaline phosphatase activity**

To determine the specificity of the effects of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 on chondrogenic differentiation, the expression of type II collagen and cartilage link protein was evaluated (Figs. 4 and 5). By immunofluorescence staining all four TGF- $\beta$  isoforms stimulated the expression of type II collagen above controls (Fig. 4a). Nevertheless, with TGF- $\beta$ 1 and TGF- $\beta$ 5, an entire cell layer of chondrogenic cells expressing type II collagen was observed, instead of a nodular pattern as seen with TGF- $\beta$ 2 and TGF- $\beta$ 3 (Fig. 4a). Western-blot analysis showed that all four TGF- $\beta$  isoforms increased type II collagen expression. However, such increase was more pronounced with TGF- $\beta$ 1 and TGF- $\beta$ 5

(Fig. 4b). Scanning densitometry of western-blot showed that TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 enhanced type II collagen expression by 2.57-, 1.34-, 1.59- and 1.86-fold, respectively (Fig. 4c).

The immunofluorescence staining for cartilage link protein showed a pattern similar to that obtained for type II collagen. TGF- $\beta$ 1 and TGF- $\beta$ 5 showed immunoreactivity on the entire cell layer, while in TGF- $\beta$ 2 and TGF- $\beta$ 3 treated cultures a nodular pattern was observed (Fig. 5a). Western-blot analysis showed that all four TGF- $\beta$  isoforms increased cartilage link protein expression (Fig. 5b). Scanning densitometry of western-blot showed that TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 enhanced cartilage link protein expression by 2.40-, 1.21-, 1.48- and 1.93-fold respectively (Fig. 5c).

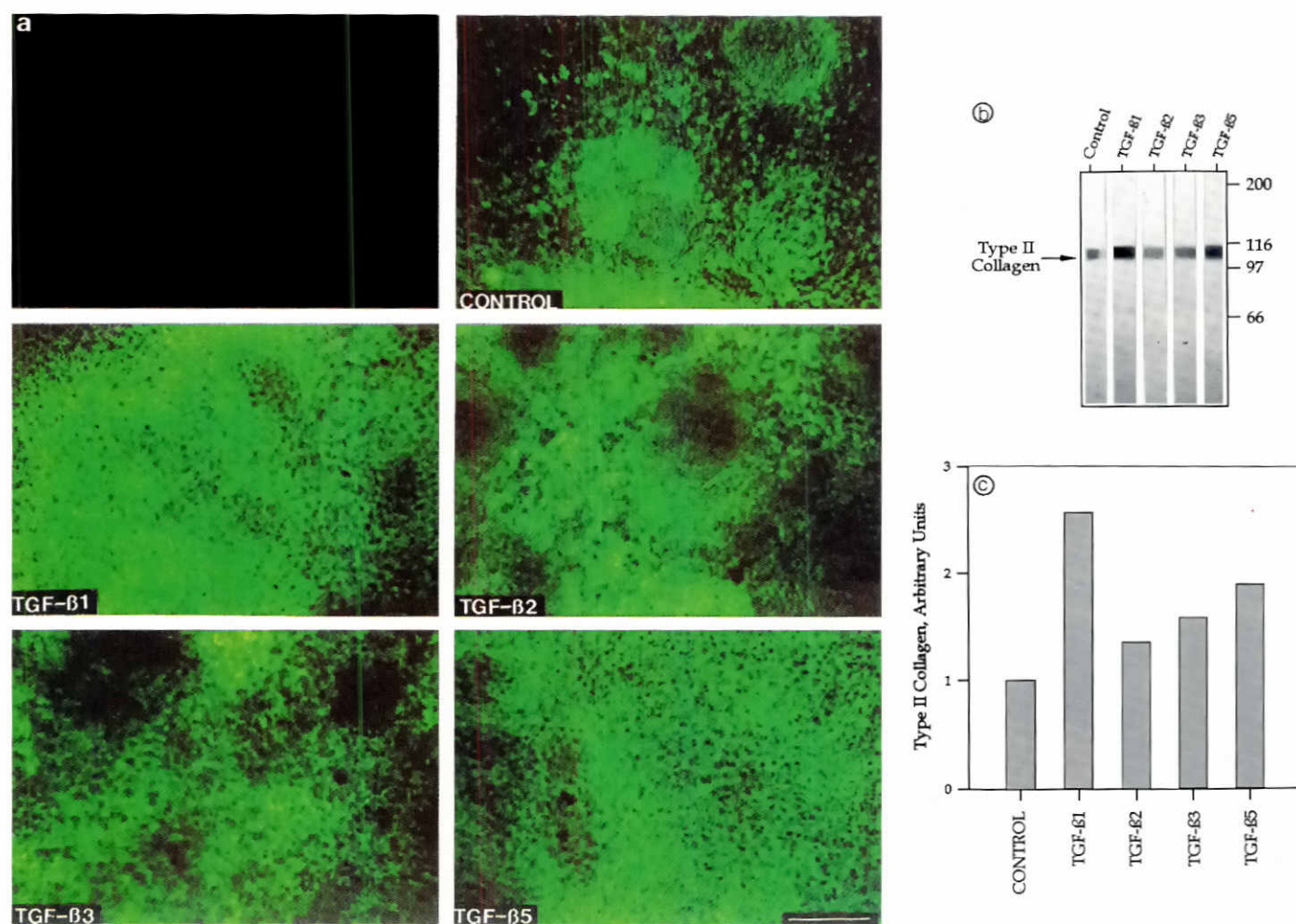
Alkaline phosphatase activity was differentially stimulated by TGF- $\beta$  isoforms. TGF- $\beta$ 2 and TGF- $\beta$ 3 promoted alkaline phosphatase activity in internodular regions, while in control cultures it was only seen in nodular regions. TGF- $\beta$ 1 and TGF- $\beta$ 5 enhanced the activity by forming a uniform sheet of alkaline phosphatase-positive cells (Fig. 6a). Moreover, the activity was lower and similar under all experimental conditions at day 2 of culture (data not

TABLE 1

#### **EFFECTS OF TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, AND TGF- $\beta$ 5 ON INCORPORATION OF $^3\text{H}$ -THYMIDINE BY MOUSE LIMB BUD MESENCHYMAL CELLS IN MICROMASS CULTURES**

	Day $^3\text{H}$ -thymidine incorporation (CPM/ $\mu\text{g}$ DNA)		
	1	3	5
Control	6958 $\pm$ 550 <sup>#</sup>	8725 $\pm$ 747	7917 $\pm$ 346
TGF- $\beta$ 1	6458 $\pm$ 651	8026 $\pm$ 945	7911 $\pm$ 508
TGF- $\beta$ 2	8098 $\pm$ 788	11578 $\pm$ 558*	8520 $\pm$ 530
TGF- $\beta$ 3	4620 $\pm$ 859*	6570 $\pm$ 775*	7053 $\pm$ 802
TGF- $\beta$ 5	6924 $\pm$ 866	7489 $\pm$ 566	7074 $\pm$ 759

<sup>#</sup>Data are given as average CPM  $\pm$  standard deviation of triplicates. \* $P \leq 0.05$  vs controls.



**Fig. 4.** Influence of TGF- $\beta$  isoforms on type II collagen expression. (a) Indirect immunofluorescence for type II collagen in the presence of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5, viewed under confocal microscopy. Mesenchymal cells were cultured as micromass cultures without TGF- $\beta$  isoforms or with 10 ng/ml TGF- $\beta$ 1, 1 ng/ml TGF- $\beta$ 2, 10 ng/ml TGF- $\beta$ 3 and 10 ng/ml TGF- $\beta$ 5 to determine the presence of type II collagen. Bar, 100  $\mu$ m. (b) Immunoblot analysis of type II collagen was obtained from control cultures and TGF- $\beta$  treated cultures at day 5 of culture. Protein load was 40  $\mu$ g/lane in the SDS-PAGE. (c) The expression of type II collagen was used as cartilage marker and is given in arbitrary units, as measured by scanning densitometry of western blots.

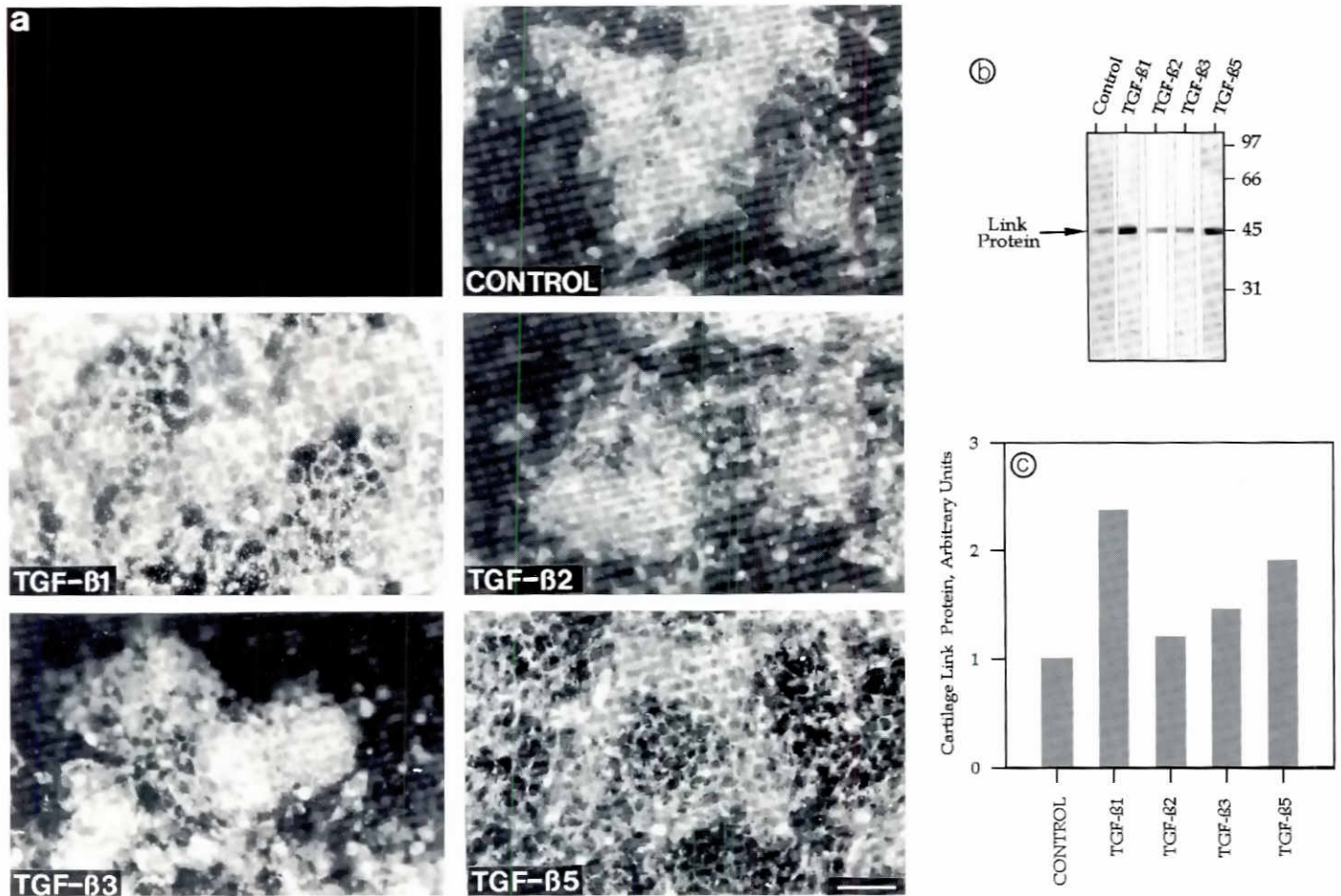
shown). A detergent extraction was performed to quantitate alkaline phosphatase activity. TGF- $\beta$ 1 increased alkaline phosphatase activity by 100% above controls, while TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 by 35%, 35% and 60% respectively (Fig. 6b).

#### TGF- $\beta$ isoforms regulate chondrogenesis depending on the stage of cartilage differentiation

To determine the time when TGF- $\beta$  isoforms affect cell differentiation of limb mesenchymal cells, we exposed stage 20 micromass cultures to growth factors for a 24 h-period at different days of culture. The effects on the accumulation of Alcian blue-positive ECM and synthesis of sulfated proteoglycans were evaluated on the fifth day of culture (Fig. 7). Exposure to TGF- $\beta$ 1 and TGF- $\beta$ 5 for 0-24, 24-48 and 48-72 h periods had an enhancing effect on the accumulation of Alcian blue-positive ECM (Fig. 7B-D and Q-S). However, no uniform staining of cartilage matrix was observed, but nodules were seen and a diffuse mass of staining was present in the internodular regions (Fig. 7B-D and Q-S). When TGF- $\beta$ 1 and

TGF- $\beta$ 5 were added after 72-96 and 96-120 h a decrease in the stimulation of chondrogenesis was observed (Fig. 7E-F and T-U). Exposure to TGF- $\beta$ 2 during 0-24 and 24-48 h produced a slightly diffuse mass staining of cartilage matrix (Fig. 7G-H). For TGF- $\beta$ 3 the enhancement effect on cartilage matrix was at the pulse of 24-48 h (Fig. 7L-M). When TGF- $\beta$ 2 and TGF- $\beta$ 3 were added at 48-72, 72-96 and 96-120 h there was no effect on the stimulation of cartilage matrix formation (Fig. 7I-K and N-P).

To quantitate chondrogenesis enhancement by growth factors we determined the synthesis of sulfated proteoglycans at day 5 of culture. Cultures without growth factors were considered as controls. Exposure to TGF- $\beta$ 1 for 0-24, 24-48, and 48-72 h promoted 70%, 80% and 70% respectively as compared to cultures exposed along a 5 day period. When cultures of 72-96 h were exposed to TGF- $\beta$ 1, the incorporation of  $^{35}$ S-sulfate on proteoglycans was very similar to controls, while at 96-120 h period the incorporation of label was inhibited when compared to controls (Fig. 8). Exposure to TGF- $\beta$ 2 for 0-24 and 24-48 h promoted the incorporation of  $^{35}$ S-



**Fig. 5. Influence of TGF- $\beta$  isoforms on cartilage link protein expression.** (a) Indirect immunofluorescence cartilage link protein in the presence of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5. Mesenchymal cells were cultured as micromass cultures without TGF- $\beta$  isoforms or with 10 ng/ml TGF- $\beta$ 1, 1 ng/ml TGF- $\beta$ 2, 10 ng/ml TGF- $\beta$ 3 and 10 ng/ml TGF- $\beta$ 5 to determine the presence of cartilage link protein. Bar, 100  $\mu$ m. (b) Immunoblot analysis of cartilage link protein was obtained from control cultures and TGF- $\beta$  treated cultures at day 5 of culture. Protein load was 40  $\mu$ g/lane in the SDS-PAGE. (c) The expression of cartilage link protein was used as another cartilage marker and is given in arbitrary units, as measured by scanning densitometry of western blots.

sulfate by 30% and 60% above values of cultures exposed along the 5 day period. Thereafter, the levels of incorporation of  $^{35}$ S-sulfate during subsequent times were very similar to controls (Fig. 8). TGF- $\beta$ 3 stimulated the incorporation of  $^{35}$ S-sulfate by 30% above 5 days TGF- $\beta$ 3 treated cultures, when it was administered for 24-48 h of culture. For the 0-24 h period the levels of stimulation were very similar to 5 days, TGF- $\beta$  treated cultures and exposure to TGF- $\beta$ 3 for 48-72, 72-96 and 96-120 h reduced the chondrogenic enhancing effect (Fig. 8). Finally, exposure to TGF- $\beta$ 5 for 0-24, 24-48 and 48-72 h produced similar effects to those observed with TGF- $\beta$ 1 for the same periods. When TGF- $\beta$ 5 was added for 72-96 and 96-120 h a decrease was determined in  $^{35}$ S-sulfate incorporation reaching levels below the controls (Fig. 8).

**Effects of TGF- $\beta$  isoforms on  $^{35}$ S-sulfate on low density cultures**

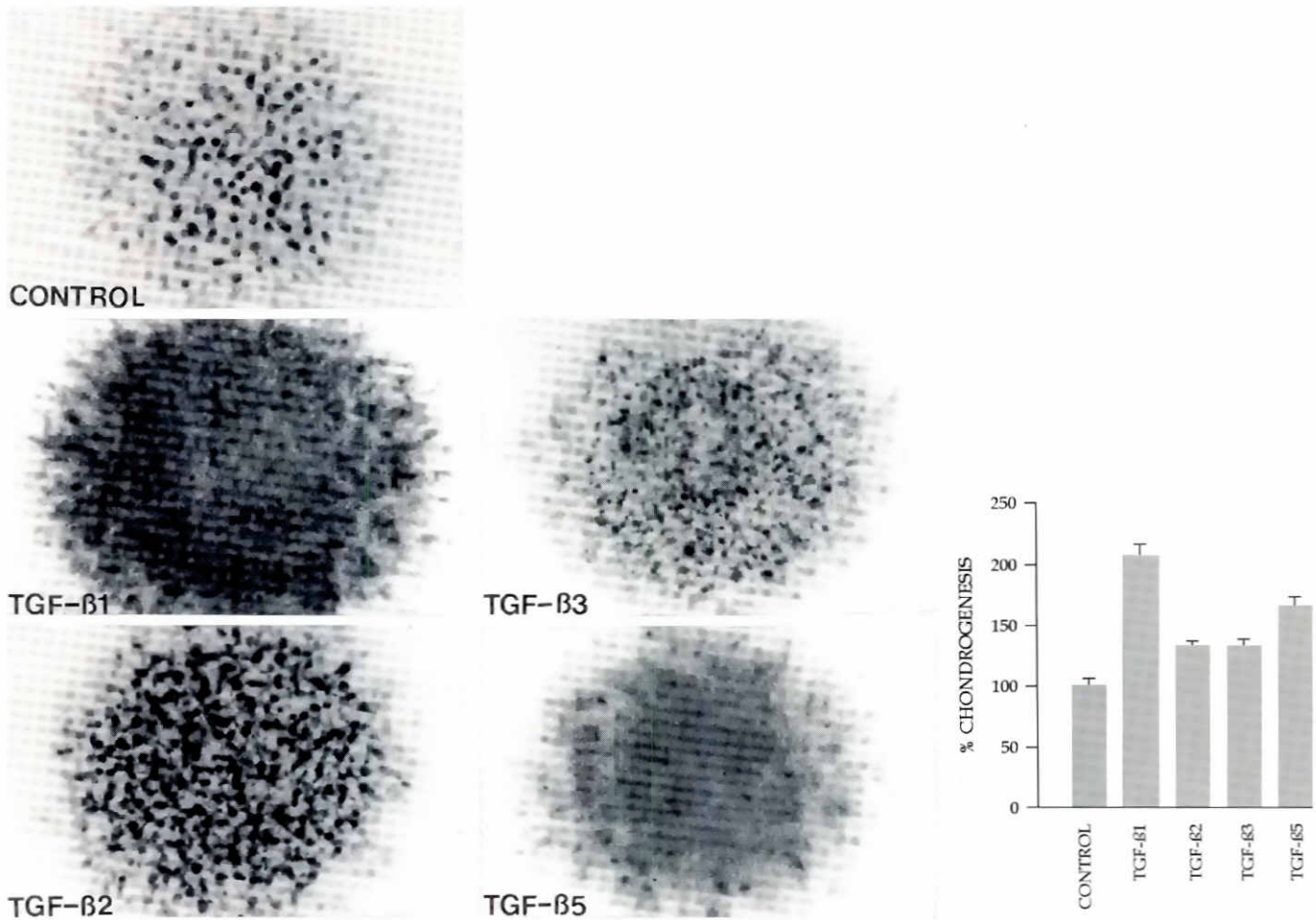
The results described above showed that all four TGF- $\beta$  isoforms differentially enhanced chondrogenesis in micromass cultures. It is also known that these culture conditions allow chondrogenic differentiation spontaneously. Thus, we evaluated the effect of all

four TGF- $\beta$  isoforms on proteoglycans by using low density cultures with 2% FBS, a condition in which mesenchymal cells undergo minimal chondrogenic differentiation. The incorporation of  $^{35}$ S-sulfate was about 25% for TGF- $\beta$ 1, 12% for TGF- $\beta$ 2, 21% for TGF- $\beta$ 3 and 23% for TGF- $\beta$ 5 (Fig. 9).

**Discussion**

The aims of the present study were: 1) to determine whether mesenchymal cells of mouse whole limb bud embryonic stages 19, 20 and 21 elicited a differential response to TGF- $\beta$  isoforms and 2) to evaluate whether TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 5 elicited a differential stimulus on mesenchymal cells of mouse embryonic limb buds.

The chondrogenesis process exhibited by mesenchymal cells in the micromass cultures of mouse embryonic stages 19, 20 and 21 in the absence of growth factors occurs in a similar way to that described by Ahrens *et al.* (1977) and Owens and Solorsh (1981). The histogenic potential of mesenchymal cells to originate chondrogenic nodules is lost as developmental stages progress.



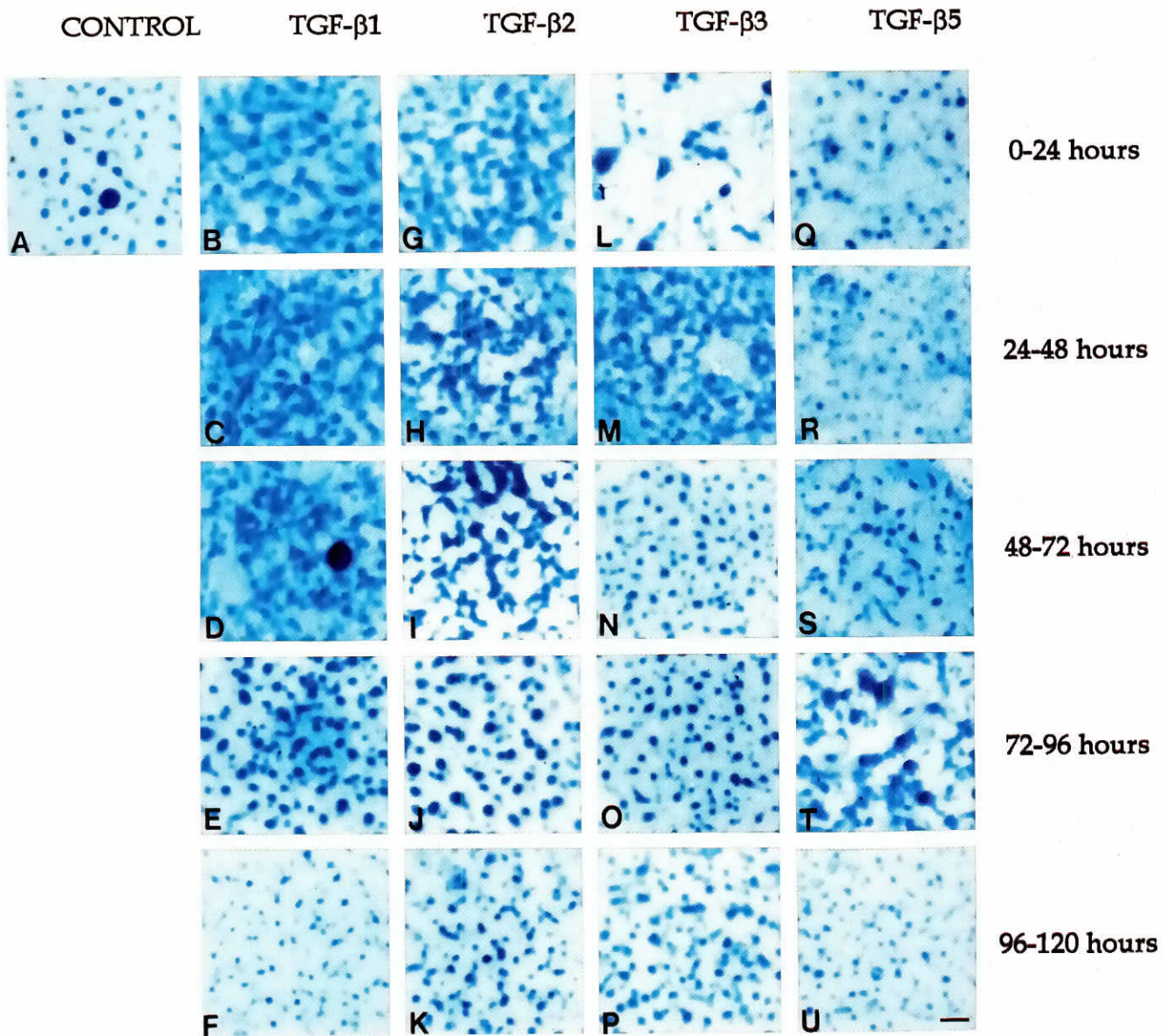
**Fig. 6. Effect of TGF- $\beta$  isoforms on alkaline phosphatase activity.** (a) Mesenchymal cells were cultured as micromass cultures without TGF- $\beta$  isoforms or with 10 ng/ml TGF- $\beta$ 1, 1 ng/ml TGF- $\beta$ 2, 10 ng/ml TGF- $\beta$ 3 and 10 ng/ml TGF- $\beta$ 5 to determine alkaline phosphatase activity. Bar, 0.5 mm. (b) Quantitation of alkaline phosphatase activity was achieved in 3 cultures of 2 separate experiments after extraction with 10% SDS. Data are represented as % chondrogenesis to indicate the proportion of chondrogenesis stimulation; control cultures had an  $OD_{400}$  of  $0.230 \pm 0.015$ ; TGF- $\beta$ 1,  $0.468 \pm 0.032$ ; TGF- $\beta$ 2 and TGF- $\beta$ 3,  $0.352 \pm 0.029$  and  $0.360 \pm 0.037$ ; TGF- $\beta$ 5,  $0.372 \pm 0.031$ .

In this study mouse whole limb cells showed differential responses to TGF- $\beta$  isoforms, according to their developmental stages, suggesting that competence for TGF- $\beta$  isoforms by mesenchymal cells changed during development. There are three types of receptors which bind TGF- $\beta$  with different affinities; TGF- $\beta$  receptors I and II are involved in the binding and signal transduction (Lin *et al.*, 1992; Wrana *et al.*, 1992). Receptor III modulates the binding of TGF- $\beta$  to receptors I and II (López-Casillas *et al.*, 1993, 1994) and the affinity of receptors I and II for TGF- $\beta$ 2 is reduced when receptor III is absent from the cellular membrane (Ohta *et al.*, 1987; Cheifetz *et al.*, 1990; López-Casillas *et al.*, 1993, 1994).

The low response of limb bud mesenchymal cells at stages 20 and 21 to TGF- $\beta$ 2, compared with stage 19, could be explained by the absence of receptor III, as suggested in other systems (Ohta *et al.*, 1987; Cheifetz *et al.*, 1990; López-Casillas *et al.*, 1993, 1994; Chai *et al.*, 1994). A second possibility is that the expression of both TGF- $\beta$  receptors I and II would not occur at same time, since coexpression of both receptors is required for TGF- $\beta$ 2 binding (Rodríguez *et al.*, 1995). Another molecule with ability to bind TGF- $\beta$ 2 is the  $\alpha$ 2-macroglobulin present in serum, which binds to TGF-

$\beta$ 2 better than to TGF- $\beta$ 1 or TGF- $\beta$ 3. The experiments in this study were performed in the presence of serum, showing that mesenchymal cells at stage 19, even in the presence of serum, are far more sensitive to chondrogenesis enhancement by TGF- $\beta$ 2 than at stages 20 and 21, suggesting that responsiveness to distinct TGF- $\beta$  isoforms is strongly influenced by cell-specific receptors. The levels of enhancement of cartilage matrix by TGF- $\beta$ 3 were very similar in the three stages as compared to controls. However, the chondrogenesis pattern is different since fusion of cartilage nodules observed at stage 19 was not seen at stages 20 and 21. Similar observations have been found in other experimental systems and this depends on different affinities of TGF- $\beta$ 1 and TGF- $\beta$ 3 to TGF- $\beta$  receptors (Cheifetz *et al.*, 1990; ten Dijke *et al.*, 1990; Graycar *et al.*, 1989). The responsiveness of mouse mesenchymal cells to TGF- $\beta$ 5 is important because it demonstrates that TGF- $\beta$ 5 influences mammalian cells as mammalian TGF- $\beta$  isoforms affect amphibian cells (Rosa *et al.*, 1988), suggesting that TGF- $\beta$  isoforms are functionally conserved through evolution.

In other experimental systems it is clear that cells respond in a distinct way to different amounts of TGF- $\beta$  isoforms; TGF- $\beta$ 1 is a

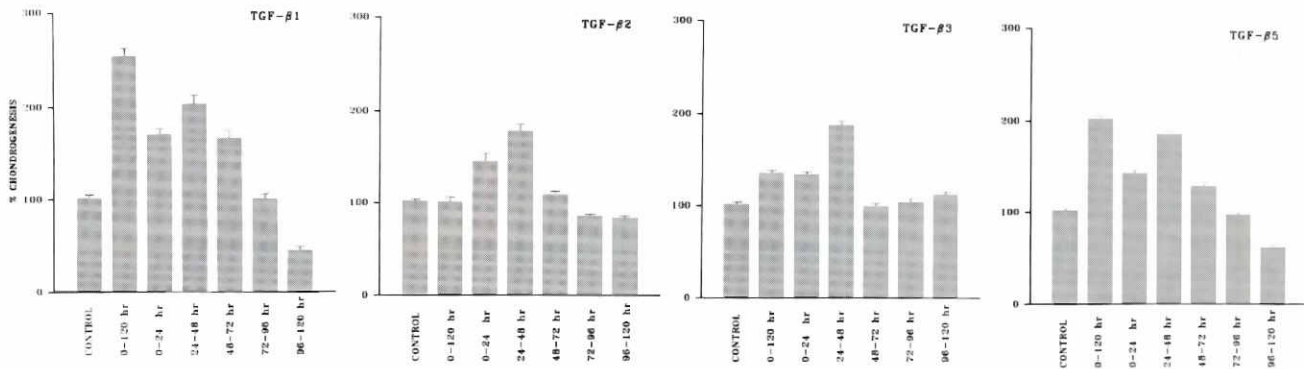


**Fig. 7. Time-dependent effects of addition of TGF- $\beta$  isoforms on chondrogenic pattern formation.** Mesenchymal cells were cultured as micromass cultures with 10 ng/ml TGF- $\beta$ 1 (B-F), 1 ng/ml TGF- $\beta$ 2 (G-K), 10 ng/ml TGF- $\beta$ 3 (L-P) and 10 ng/ml TGF- $\beta$ 5 (Q-U). Growth factors were added to micromass cultures at the following times of culture: 0-24 h (B, G, L and Q), 24-48 h (C, H, M and R), 48-72 h (D, I, N and S), 72-96 h (E, J, O and T) and 96-120 h (F, K, P and U). A representative culture without growth factors is shown in A. All cultures were fixed and stained at 120 h. Bar, 0.5 mm.

more potent inhibitor than TGF- $\beta$ 2 for endothelial cell growth. This difference may be due to a central region of TGF- $\beta$ 1 not present in TGF- $\beta$ 2 (Qian *et al.*, 1992). Also, receptor II presents an extracellular domain with binding affinity for TGF- $\beta$ 1 and TGF- $\beta$ 3, but not TGF- $\beta$ 2 (Lin *et al.*, 1995). Consequently, the presence of receptor III is necessary to allow the availability of TGF- $\beta$ 2 to receptor II, since that molecule can be found in a soluble form inhibiting cell signaling or in a membrane-anchorage form that enhances TGF- $\beta$ 2 binding to type II receptor, eliciting a signaling response (López-Casillas *et al.*, 1993). For that reason, further experiments are necessary to determine whether TGF- $\beta$ 1 and TGF- $\beta$ 3 present different affinities to TGF- $\beta$  receptors and to determine whether

receptor III regulates the availability of TGF- $\beta$ 2 to TGF- $\beta$  receptors during mouse limb bud development. Likewise, there are other molecules that can bind TGF- $\beta$ s, like decorin and biglycan (Yamaguchi *et al.*, 1990; Fosang and Hardingham, 1992; Kresse *et al.*, 1994). We speculate that downregulation of decorin and biglycan levels may enhance the effects of TGF- $\beta$ s, or that upregulation of both molecules may decrease the effects of TGF- $\beta$ s, since they may inhibit TGF- $\beta$ s action. So, the increased expression of these ECM molecules during mouse limb bud development at stages 20 and 21 may therefore cause a reduction in the pool of active TGF- $\beta$ 2 and TGF- $\beta$ 3 in mesenchymal cells, thus producing a distinct pattern formation or a differential re-





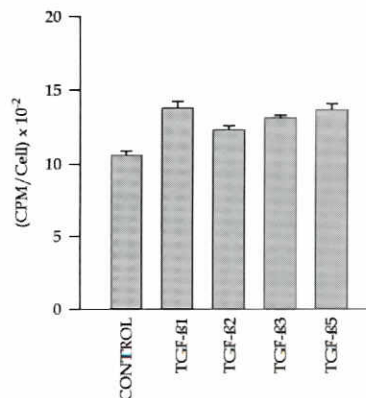
**Fig. 8. Time-dependent effects of TGF- $\beta$  isoforms on the synthesis of sulfated proteoglycans.** Growth factors were added to micromass cultures at the following times of culture: 0-120 h, 0-24 h, 24-48 h, 48-72 h, 72-96 h and 96-120 h. All cultures were incubated with  $^{35}\text{S}$ -sulfate during 6 h at day 5 of culture. Data represent the means  $\pm$  standard deviation of 3 cultures for each time point treatment of 2 separate experiments.  $P < 0.01$  (vs controls). Data are represented as % chondrogenesis to compare the proportion of stimulation or inhibition in the TGF- $\beta$  treated cultures in relation to controls for each TGF- $\beta$  isoform. The values for control cultures and TGF- $\beta$  treated cultures during 5 days were very similar to those obtained and represented in Figure 2.

sponse in the stimulation of specific cartilage molecules as compared with TGF- $\beta$ 1 actions.

Here we showed morphogenetic differences between stages 19, 20 and 21 in response to TGF- $\beta$  isoforms. By stage 19 all four TGF- $\beta$  isoforms promote the formation of a continuous sheet of cartilage cells, while in mesenchymal cells from limb stage 20, only TGF- $\beta$ 1 and TGF- $\beta$ 5 produce a continuous sheet of cartilage cells, and TGF- $\beta$ 2 and TGF- $\beta$ 3 produce a nodular pattern of cartilage. This suggests that mesenchymal cells from stage 19 and 20 present different signals that stimulate or inhibit chondrogenesis pattern. We demonstrated that TGF- $\beta$ 2 and TGF- $\beta$ 3 promoted the expression of proteoglycans, type II collagen, cartilage link protein and alkaline phosphatase activity at significant levels as compared to controls during chondrogenesis at stage 20. However, they did not promote a continuous sheet of cartilage cells, suggesting that TGF- $\beta$ 2 and TGF- $\beta$ 3 may regulate morphogenesis and cell differentiation through different pathways, depending on the developmental stage.

On the other hand, in developing chick embryos the cartilage pattern in leg mesenchyme is different from that obtained with wing mesenchyme, switching from a continuous sheet of cartilage cells to a nodular pattern at stage 24. This suggests that intrinsic morphogenetic differences are present in leg and wing mesenchymal cells from stage 24 chick embryos (Downie and Newman, 1994). The production of fibronectin in leg mesenchyme is higher than in wing mesenchyme both *in vivo* and *in vitro*. Also, the interaction between cells and fibronectin in wing mesenchymal cells but not in leg mesenchymal cells, is mediated by the amino-terminal domain of fibronectin (Downie and Newman, 1995). Taking together the results from Downie and Newman (1994, 1995) and ours we suggest that mesenchyme from distinct stages express differentially TGF- $\beta$  receptors, including the ECM molecules decorin and biglycan (Yamaguchi *et al.*, 1990; Fosang and Hardingham, 1992; Kresse *et al.*, 1994). Likewise, it is possible that signal transduction elicited by TGF- $\beta$  isoforms could be different, evoking regulation of the ECM molecules involved in cartilage formation, causing distinct morphogenetic patterns.

On the basis of the differential responsiveness of mesenchymal cells from stage 20 to TGF- $\beta$  isoforms, we evaluated the action of all four TGF- $\beta$  isoforms on the metabolism of proteoglycans, expression of type II collagen and link protein and alkaline phosphatase activity during chondrogenesis. TGF- $\beta$  isoforms elicited a differential regulation of them. This effect was not mediated by cell proliferation, because the quantity of DNA,  $^3\text{H}$ -thymidine incorporation and cell number in all conditions assayed were similar. However, TGF- $\beta$ 3 at days 1 and 3 decreased  $^3\text{H}$ -thymidine incorporation and TGF- $\beta$ 2 increased  $^3\text{H}$ -thymidine incorporation at day 3. Hence, the TGF- $\beta$  isoforms regulate specific-cartilage matrix molecules as suggested by Kulyk *et al.* (1989), Carrington and Reddi (1990), Leonard *et al.* (1991), Schönfeld *et al.* (1991) and Roark and Greer (1994). Nevertheless, in micromass cultures from chick embryo mesenchymal cells, the three mammalian TGF- $\beta$  isoforms enhance chondrogenesis at similar levels for all parameters examined. In the current study, we found that in mouse mesenchymal cells cultured in similar conditions to chick cells, TGF- $\beta$  isoforms regulate the expression of specific-cartilage matrix components in a distinct manner for each factor, showing that TGF- $\beta$ 1 and TGF- $\beta$ 5 are more potent than TGF- $\beta$ 2 and TGF- $\beta$ 3. Therefore, mouse limb cells provide a suitable experimental system to evaluate the effects of different TGF- $\beta$  isoforms on mouse chondrogenesis.



**Fig. 9. Influence of TGF- $\beta$  isoforms on the synthesis of proteoglycans by low density cultures from stage 20 mouse limb mesenchymal cells.** Growth factors were added to low density cultures for 5 days. All cultures were incubated with  $^{35}\text{S}$ -sulfate during 6 h at day 5 of culture. Data represent the means  $\pm$  standard deviation of 3 cultures of 2 separate experiments.  $P < 0.01$  (vs controls).

The activity of TGF- $\beta$  on ECM production in different systems is well known since it increases the synthesis of ECM components and diminishes matrix degradation. Furthermore, it stimulates the expression of specific receptors for ECM components (Massagué, 1990). However, the mechanisms by which TGF- $\beta$  isoforms regulate chondrogenesis of mouse limb bud mesenchymal cells is unknown. The fact that all four TGF- $\beta$  isoforms promoted chondrogenesis when added during the first two days of culture, suggests that TGF- $\beta$  isoforms action occurs at early stages during the process of chondrogenesis, as suggested by Kulyk *et al.* (1989), Carrington and Reddi (1990), Leonard *et al.* (1991) and Roark and Greer (1994). However, TGF- $\beta$ 1 and TGF- $\beta$ 5 inhibit chondrogenesis when administered on day 5 of culture, suggesting that TGF- $\beta$  isoforms may be acting in a sequential way to control the process of cartilage differentiation.

This study showed that TGF- $\beta$  isoforms are better promoters of the chondrogenic differentiation in micromass cultures than in low density cultures. Kulyk *et al.* (1989) demonstrated that TGF- $\beta$  increased by two to three times  $^{35}\text{S}$ -sulfate incorporation in low density cultures, while in our study we obtained only 25% increase in  $^{35}\text{S}$ -sulfate incorporation. This could be explained perhaps by species differences between chick and mouse embryos or because we used whole limb bud mesenchymal cell whereas Kulyk *et al.* (1989) used distal subridge mesenchymal cells.

We conclude that the action of each member of the TGF- $\beta$  family may be somewhat different, as occurs with the BMP family. The fact that mouse limb bud mesenchymal cells differentially respond to TGF- $\beta$  isoforms indicates that these factors play a critical role in chondrocyte differentiation during limb morphogenesis. TGF- $\beta$  isoforms may have a direct effect on chondrogenic cells; however, an indirect effect mediated by other cell types cannot be excluded. Finally, based on the reported homologies between TGF- $\beta$ 1 and TGF- $\beta$ 5 (Kondaiah *et al.*, 1990; Burt and Paton, 1992), and on the biological activities observed in the current study, we support the suggestion that *Xenopus* TGF- $\beta$ 5 is equivalent to mammalian TGF- $\beta$ 1.

## Materials and Methods

### Cell cultures

Balb/c mice were mated overnight and females with a vaginal plug the following morning were considered to be at day 0 of gestation. Pregnant mice were killed by cervical dislocation and the uteri were dissected out and placed in Petri dishes. The embryos were removed and staged according to Theiler (1972). Mesenchymal cells were obtained by dissociating fore-limb buds of stages 19, 20 and 21 according to Ahrens *et al.* (1977). Cell suspension was adjusted to a concentration of  $2 \times 10^7$  cells/ml for micromass cultures, and a concentration of  $2.5 \times 10^6$  cell/ml of DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc.) for low density cultures. A drop of 10  $\mu\text{l}$  was plated in the center of each of the 24 central wells of a 48 well-plate (Costar) and these were left for cellular attachment during 1 h in a 37°C humidified incubator, and then flooded with 1.0 ml culture medium with 10% FBS for micromasses and 2% for low density cultures, and cultured at 37°C and 5%  $\text{CO}_2$ /95% air during 5 days. Human TGF- $\beta$ 1, porcine TGF- $\beta$ 2, recombinant human TGF- $\beta$ 3 and recombinant amphibian TGF- $\beta$ 5 (R&D Systems) were added to the medium at concentrations of 1, 5 and 10 ng/ml.

### Alcian blue staining

To obtain a quantitative measurement of differentiated cartilage matrix, 5-day cultures were stained overnight with 1% Alcian blue in 3% acetic acid, pH 1.0. Cultures were rinsed in 3% acetic acid and distilled water. Bound

stain was extracted overnight with 0.3 ml 4.0 M guanidine hydrochloride in agitation and quantitated using an ELISA reader at a wavelength of 600 nm.

### Biosynthesis of glycosaminoglycans

Synthesis of proteoglycans and glycosaminoglycans (GAGs) by differentiating limb bud mesenchymal cells was evaluated by  $^{35}\text{S}$ -sulfate and  $^3\text{H}$ -glucosamine incorporation, respectively. Cells were double labeled with 5  $\mu\text{Ci/ml}$   $^{35}\text{S}$ -sulfate and 10  $\mu\text{Ci/ml}$   $^3\text{H}$ -glucosamine (carrier free; Amersham, UK) at days 1, 3 and 5 of culture for 6 h in serum free medium and in the presence of growth factors. Cultures were then washed 6 times with PBS. Cells were resuspended in 50 mM Tris-HCl, pH 8.0, with 3% ethanol. Pronase (1 mg/ml; Sigma) was added and incubated at 50°C for 48 h. The pronase-digested material was centrifuged for 20 min at 13000xg. The supernatant was adjusted to a final 3N NaOH and incubated overnight at room temperature. Thereafter, 5% TCA final concentration was added and overnight incubation performed at 4°C. The mixture was centrifuged for 20 min at 13000xg and the supernatant was dialyzed against distilled water. Glycosaminoglycans were precipitated with 3 volumes 96% ethanol/1.3% potassium acetate and by overnight incubation at -20°C and centrifuged at 10000xg. Precipitate was vacuum dried and resuspended in 200  $\mu\text{l}$  distilled water (Oohira *et al.*, 1977). Aliquots were taken and  $^{35}\text{S}$ -sulfate and  $^3\text{H}$ -glucosamine incorporated into GAGs were counted in a liquid scintillation counter.

### $^3\text{H}$ -thymidine incorporation

Cultures were rinsed with medium without serum and  $^3\text{H}$ -thymidine (Amersham, UK) was added at 10  $\mu\text{Ci/ml}$  and incubated at 37°C for 2 h at days 1, 3 and 5 of culture. Cultures were washed 6 times with PBS, and incorporated radioactivity was measured by the method of Stein and Stein (1989).

### DNA quantitation

The method of Labarca and Paigen (1980) was used to determine the amount of DNA in micromass cultures. The assay was performed using duplicate 50  $\mu\text{l}$  aliquots of each sample in a total volume of 4 ml of reaction mixture. The resulting fluorescence was measured and the quantity of DNA was determined by comparison to a standard curve.

### Immunofluorescence staining

To evaluate the presence of type II collagen and cartilage link protein, immunostaining was performed as described in Jiang *et al.* (1993). Micromass cultures were fixed in 2.5% paraformaldehyde in PBS for 30 min. Micromass cultures were incubated overnight with anti-type II collagen (Chemicon) and anti-cartilage link protein (Gift of Dr. Koji Kimata). Micromass cultures were visualized under a Bio-Rad Model MRC 600 confocal microscope using the krypton-argon laser and COMOS program for image analysis. Controls were incubated without primary antibodies.

### Western-blot analysis

To obtain collagen, proteins were precipitated with 35%  $(\text{NH}_4)_2\text{SO}_4$ , redissolved in 0.5 M acetic acid and digested with pepsin (1 mg/ml; Sigma) for 24 h at 4°C. Pepsin insoluble material was centrifuged at 500xg for 1 h. Then, the supernatant was lyophilized and dissolved in electrophoresis sample buffer. For cartilage link protein, micromass cultures were washed with PBS and treated with a lysis buffer consisting of 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 1% SDS, and 2 mM EDTA containing a cocktail of phosphatase inhibitors (2 mM PMSF, 0.2 TIU/ml aprotinin, 1  $\mu\text{g/ml}$  leupeptin, 50  $\mu\text{g/ml}$  soybean trypsin inhibitor and 30 mM sodium phosphate) according to Lightner *et al.* (1994). After treatment the extract was also mixed with electrophoresis sample buffer. Samples of type II collagen and cartilage link protein were loaded onto 7.5% and 10% SDS-polyacrylamide gels respectively and electrophoresed for 1 h (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes (BIORAD) by overnight electroblotting (Burnette, 1981). Blots were developed for chemiluminescence (Amersham) according to the manufacturers' protocol.

**Alkaline phosphatase activity**

To determine alkaline phosphatase activity by histochemical analysis, micromass cultures were fixed for 10 min with 3.7% formaldehyde at room temperature. Cells were washed with PBS several times and incubated for 20 min with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% *N,N*-dimethylformamide, 2 mM MgCl<sub>2</sub>, and 0.6 mg/ml of fast blue BB salt (Sigma) in 0.1 M Tris-HCl, pH 8.5, at room temperature. To quantitate alkaline phosphatase activity, samples were extracted with 10% SDS dissolved in PBS by overnight incubation and read on a spectrophotometer at 400 nm.

**Statistical analysis**

Data are presented as the means  $\pm$  standard deviation. Comparisons of experimental groups with controls were carried out by one-way analysis of variance, and statistical differences between groups were assessed by Student's *t* test (SigmaStat Statistical Analysis System, Jandel Corporation). Significant differences were accepted at the *P* < 0.05 level.

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