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Original Article

Involvement of *Frzb-1* in mesenchymal condensation and cartilage differentiation in the chick limb bud

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ABSTRACT In developing limb bud, mesenchymal cells form cellular aggregates called "mesenchymal condensations". These condensations show the prepattern of skeletal elements of the limb prior to cartilage differentiation. Roles of various signaling molecules in chondrogenesis in the limb bud have been reported. One group of signaling factors includes the Wnt proteins, which have been shown to have an inhibitory effect on chondrogenesis in the limb bud. Therefore, regulation of Wnt activity may be important in regulating cartilage differentiation. Here we show that *Frzb-1*, which encodes a secreted frizzled-related protein that can bind to Wnt proteins and can antagonize the activity of some Wnts, is expressed in the developing limb bud. At early stages of limb development, *Frzb-1* is expressed in the ventral core mesenchyme of the limb bud, and later *Frzb-1* expression becomes restricted to the central core region where mesenchymal condensations occur. At these stages, a chondrogenic marker gene, aggrecan, is not yet expressed. As limb development proceeds, expression of *Frzb-1* is detected in cartilage primordial cells, although ultimately *Frzb-1* expression is down-regulated. Similar results were obtained in the recombinant limb bud, which was constructed from dissociated and re-aggregated mesenchymal cells and an ectodermal jacket with the apical ectodermal ridge. In addition, *Frzb-1* expression preceded aggrecan expression in micromass cultures. These results suggest that *Frzb-1* has a role in condensation formation and cartilage differentiation by regulating Wnt activity in the limb bud.

KEY WORDS: *chondrogenesis, mesenchymal condensation, limb development, aggrecan, Frzb-1*

Introduction

During limb development, mesenchymal cells of the limb bud differentiate into chondrogenic cells or fibroblastic cells. Chondrogenesis starts in the proximal central region of the limb bud and proceeds distally. Mesenchymal cells in the central region of the limb bud form cellular aggregates, called "mesenchymal condensations" (Ede, 1983; Hall and Miyake, 1992). Subsequent to condensation formation, cartilaginous primordia become visible in the limb bud. In the condensation, the cells are closely packed, and are of a rounded shape. These cells start to synthesize cartilage-specific extracellular matrix proteins (ECMs), such as aggrecan, which accumulate around the cells (Hall and Miyake, 1992, 1995). In addition to ECMs, cell adhesion molecules are also expressed by these cells (Chuong *et al.,* 1993; Oberlender and Tuan, 1994; Hall and Miyake, 1995). Thus, cell-cell and cell-ECM interaction are indispensable for cellular condensations and to promote chondrogenesis in the limb bud.

Secreted signaling molecules thought to have a role in chondrogenesis are members of the *Wnt* gene family. A number of these genes are expressed in the limb bud, and the roles of their products during limb development have been shown. *Wnt-3a* and *Wnt-7a* are expressed in the ectodermal tissues (Dealy *et al.*, 1993; Riddle *et al.*, 1995; Vogel *et al.*, 1995; Kengaku *et al.*, 1998), and *Wnt-4*, *Wnt-5a* and *Wnt-11* are expressed in the mesenchyme (Dealy *et al.*, 1993; Tanda *et al.*, 1995; Kawakami *et al.*, 1999). Ectopic expression of some *Wnt* genes (*Wnt-1*, *Wnt-5a* or *Wnt-7a*) inhibits chondrogenesis in the limb bud (Zakany and

Abbreviations used in this paper: ECM, extracellular matrix protein; AER, apical ectodermal ridge.

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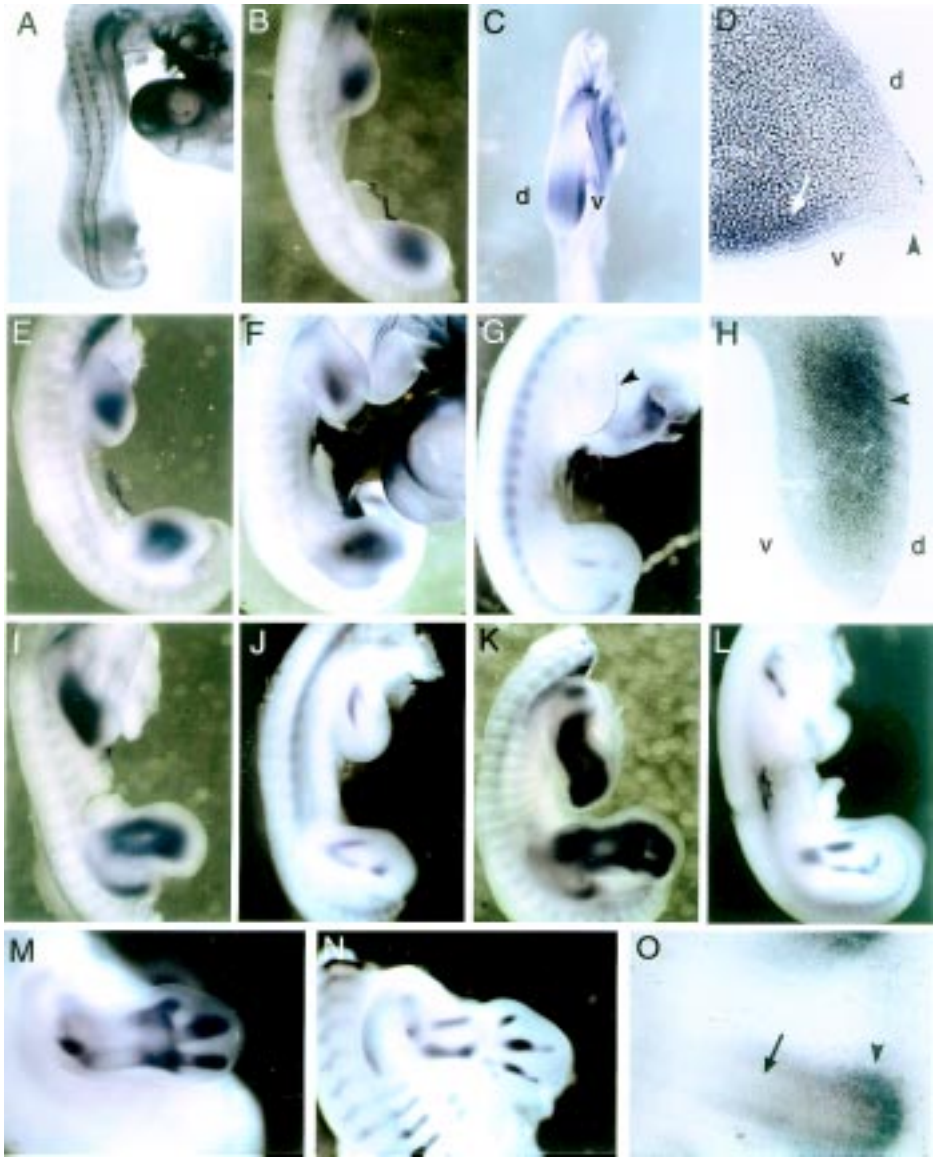

Fig. 1. Expression patterns of *Frzb-1* and aggrecan in the chick limb bud. **(A-F,H,I,K,M,O)** *Frzb-1 expression at stage 18* **(A)**, *stage 20* **(B,C,D)**, *stage 22* **(E)**, *stage 24* **(F,H)**, *stage 25* **(I)**, *stage 26* **(K)**, *and stage 28* **(M,O)**. **(G,J,L,N)** *Aggrecan expression at stage 24* **(G)**, *stage 25* **(J)**, *stage 26* **(L)**, *and stage 28* **(N)**. **(A)** *Frzb-1 is faintly expressed throughout the mesenchymal cells of the limb bud at stage 18.* **(B-D)** *The stage 20 limb bud shows mesenchymal Frzb-1 expression in the central region of the limb bud with dorsoventral polarity (C,D; arrow). No expression is observed in the ectoderm, including the AER (D; arrowhead).* **(E,F)** *Triangular-shaped expression is observed in the limb bud at stage 22 (E) and 24 (F).* **(H)** *Transverse section of stage 24 hind limb bud. Frzb-1 is expressed at the central domains where the mesenchymal condensation has formed (arrowhead). At this stage, polarized expression along dorsoventral axis has ceased.* **(I,K)** *At stage 25 and 26, expression of Frzb-1 marks the cartilage rudiments of the limb.* **(M,O)** *At stage 28, expression of Frzb-1 is detected in the perichondrium of differentiated cartilage tissue, predominantly at the epiphyses (arrowheads), and Frzb-1 expression is excluded from the core region (O; arrow).* **(G,J,L,N)** *Expression of aggrecan to show the developing skeletal elements of the limb bud. Aggrecan is also expressed in the AER at early stages of limb development (G; arrowhead). Abbreviations: d, dorsal; v, ventral.*

Duboule, 1993; Rudnicki and Brown, 1997; Kawakami *et al.*, 1999), and therefore it is suggested that Wnt products in general negatively regulate chondrogenesis in the limb bud.

Recently, a secretory Wnt-binding protein, Frzb-1 (sFrp-3), has been identified in several vertebrates (Hoang *et al.*, 1996; Leyns *et al.*, 1997; Wang *et al.*, 1997). Frzb-1 contains a cysteine-rich domain which is homologous to a region in the extracellular domain of the Frizzled family, the Wnt receptors. Frzb-1 binds to Wnt proteins through the cysteine-rich domain, and antagonizes the biological activity of Wnt proteins by restricting Wnt binding to the Frizzled receptor (Leyns *et al.*, 1997; Wang *et al.*, 1997). In the mouse embryo, *Frzb-1* and the related genes are expressed in various tissues with regional specificity, including the cartilage primordia (Hoang *et al.*, 1998; Leimeister *et al.*, 1998). Also, several molecules that are homologous to *Frzb-1* have been identified (Hoang *et al.*, 1998; Leimeister *et al.*, 1998).

In this report, we show the expression pattern of *Frzb-1* in the developing chick limb bud. *Frzb-1* is first expressed in the ventral region of the early limb bud, and later expression coincides with the pre- and post-mesenchymal condensations. *Frzb-1* expression precedes aggrecan expression, which is observed in the differentiated chondrocyte. At later stages, *Frzb-1* expression is restricted to perichondrial tissues of cartilaginous primordia. Similar results were obtained in recombinant limb buds and micromass cultures. From these results, it is suggested that Frzb-1 is involved in the formation of mesenchymal condensations by regulating Wnt activity in the limb bud.

Results

Expression pattern of Frzb-1 in the developing limb bud

First, we examined the expression pattern of *Frzb-1* in limb buds between stages 18 to 28. At stage 18, *Frzb-1* is faintly expressed throughout mesenchymal cells of the limb field (Fig. 1A). At stage 20, *Frzb-1* expression is observed in the core region of the limb bud (Fig. 1B), predominantly in the ventral mesenchyme (Fig. 1C,D). The expression is restricted to the mesenchyme, and no expression is observed in the ectoderm, including the apical ectodermal ridge (AER) (Fig. 1D). By stage 22, triangular-shaped expression of *Frzb-1* is observed, and the signal becomes intense (Fig. 1E). Expression in the ventral mesenchyme is down-regulated. At stage 24, *Frzb-1* is intensely expressed in the central region of the limb bud where mesenchymal cells form aggregates (Fig 1F,H). By stage 25-26, expression of *Frzb-1* is clearly identical to the prepattern of the cartilage primordia of the limb (Fig. 1I,K). At stage 28, *Frzb-1*

expression is localized to perichondrium of differentiated cartilage tissue, predominantly at the epiphyses (Fig. 1M), although weak expression is observed in the central region of the cartilage (Fig. 1O).

We compared the expression pattern of *Frzb-1* with that of aggrecan, which is primarily expressed by chondrocytes (Oettinger and Pacifici, 1990; Enomoto-Iwamoto *et al.*, 1998). Aggrecan mRNA is not detected in the mesenchyme of the developing limb bud until stage 23, when its expression is restricted to the AER (Fig. 1G). At stage 24, weak expression of aggrecan is detectable in the mesenchyme in the proximal-central region of the limb bud in addition to the AER. In the hind limb bud, the mesenchymal expression is already divided into two domains corresponding to the presumptive tibia and fibula (Fig. 1G). By stage 25-26, bifurcated and segmented expression of aggrecan is observed representing presumptive zeugopodal structures (Fig. 1J,L). Aggrecan expression is restricted within the *Frzb-1* domain. At stage 28, intense expression of aggrecan is detectable throughout the cartilage primordia of the limb (Fig. 1N).

Altogether, *Frzb-1* expression starts prior to the cartilage marker gene expression, and coincided with the region of mesenchymal condensations in the limb bud between stages 24 to 28, whereas aggrecan expression in the limb mesenchyme represents the pattern of differentiating cartilage.

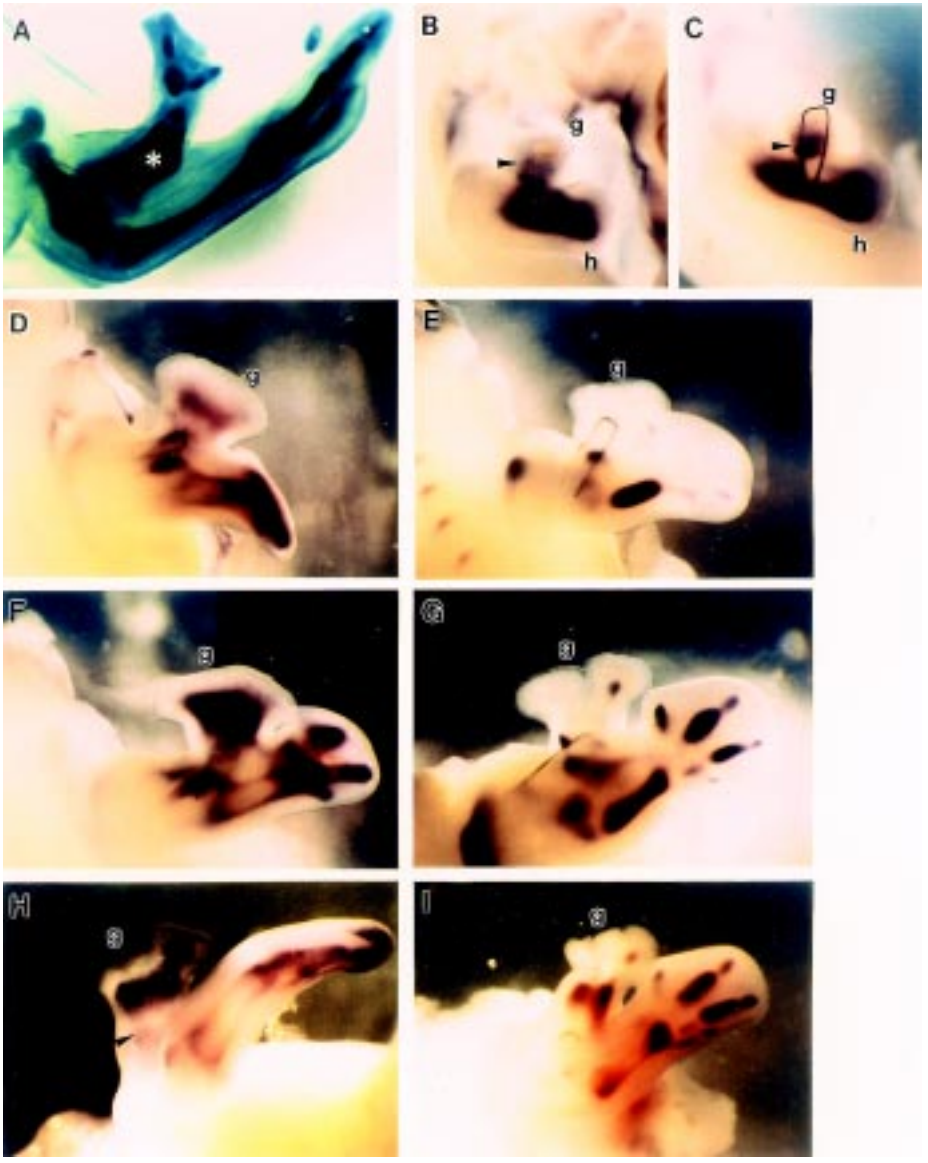

Fig. 2. Expression patterns of *Frzb-1* and aggrecan in the recombinant limb bud. (A) *An example of cartilage elements which developed from the recombinant limb bud (asterisk). Arrowhead indicates a tungsten pin used to fix the recombinant limb bud.* **(B,C,D,F,H)** *Expression patterns of Frzb-1 in the recombinant limb bud. Expression of Frzb-1 was observed within 12 h after grafting (B). The expression was restricted to the central region throughout recombinant development (C,D,F,H). After 60 h grafting, proximal expression became weak (H; arrowhead).* **(E,G,I)** *Expression patterns of aggrecan in the recombinants at 36 h (E), 48 h (G) and 60 h (I) after grafting. Abbreviations: g, grafted recombinant limb bud; h, host limb bud.*

Expression patterns of Frzb-1 in the recombinant limb bud

Next, we investigated the expression of *Frzb-1* and aggrecan in a recombinant limb bud. During development of recombinant limb buds, cell-cell interactions and position specific gene expressions of mesenchymal cells are re-established, and the cells acquire new positional identity in the recombinant limb (Ros *et al.*, 1994; Hardy *et al.*, 1995; Wada *et al.*, 1998b). To prepare the recombinant limb bud, we recombined distal mesenchyme of stage 20 wing buds with stage 22-23 leg ectodermal jackets. The recombinant limb bud was grafted onto the rostral end of the host limb bud, and allowed to develop further. They formed well-shaped cartilage structures with several digits at the distal end, although these structures lost anteroposterior polarity (Fig. 2A).

In the recombinant limb bud, weak expression of *Frzb-1* was already observed at 12 h after grafting (2 out of 2 cases) (Fig. 2B). No polarized expression along dorsal-ventral axis was observed. After 24 h, *Frzb-1* was expressed broadly in the central core region of the recombinant limb bud (2/2 cases) (Fig. 2C). By 36 h, *Frzb-1* was clearly expressed in the proximal core region of the recombinant limb bud, and *Frzb-1* could not be detected in the peripheral region (3/3 cases) (Fig. 2D). The localized expression of *Frzb-1* was maintained during further development of the recombinant limb bud, then the expression in the proximal region became weak after 60 h (3/3 and 2/2 cases at 48 and 60 h, respectively) (Fig. 2F,H; arrowhead).

On the contrary, expression of aggrecan started 36 h after grafting of the recombinant limb buds (3/3 cases) (Fig. 2E). At this

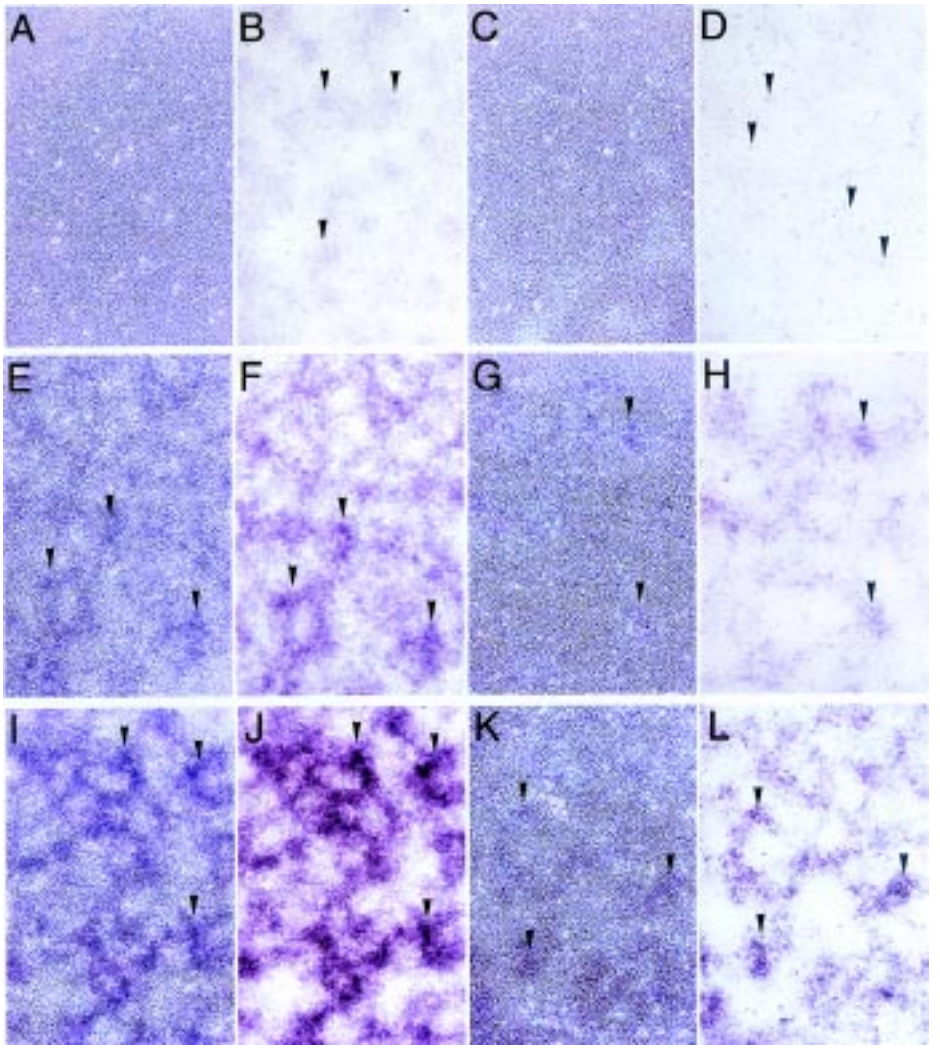

Fig. 3. Expression of *Frzb-1* and aggrecan in cultured limb bud cells. *Phase contrast* **(A,C,E,G,I,K)** *and corresponding bright-field views* **(B,D,F,H,J,L)** *are shown for Frzb-1 expression at 24 h* **(A,B)**, *48 h* **(E,F)** *and 72 h* **(I,J)** *after plating, and aggrecan expression at 24 h* **(C,D)**, *48 h* **(G,H)** *and 72 h* **(K,L)** *after plating. At 24 h, weak and broad expression of Frzb-1 was detected (A,B; arrowheads), whereas aggrecan expression was detected in a few cells (C,D; arrowheads). At 48 h, Frzb-1 was expressed in the condensing cells (E,F; arrowheads). At this time point, aggrecan was expressed in a small patches of cells (G,H; arrowheads). At 72 h, strong expression of Frzb-1 was observed (I,J; arrowheads), and aggrecan mRNA was also detected (K,L; arrowheads). Both genes were expressed in the cellular condensations, although expression of Frzb-1 was more intense and extensive than that of aggrecan.*

stage, aggrecan expression was restricted to most proximal region of the recombinant. By 48 h after grafting, aggrecan was expressed in the proximal core region of the recombinant, and weak expression was detected in the distal region (3/3 cases) (Fig. 2G). Unlike *Frzb-1*, widespread expression of aggrecan was not observed at this stage. After 60 h, aggrecan was strongly expressed in the proximal region, while weak but clear expression was observed in the distal region of the recombinant (2/2 cases) (Fig. 2I).

Expression of Frzb-1 in cultured cells

We next investigated the expression of *Frzb-1* and aggrecan in cultured cells to further examine temporal changes of *Frzb-1* expression during cartilage differentiation. Stage 22-23 limb bud cells were plated at high density so that they undergo chondrogenesis. Results are shown in Figure 3. Weak and broad expression of *Frzb-1* was already observed 24 h after plating (Fig. 3A,B). At this time, we could not detect cellular condensations using a phase-contrast microscopy. Forty-eight hours after plating, when cellular condensations were observed, expression of *Frzb-1* was mainly detected in the condensing cells (Fig. 3E,F). *Frzb-1* expression was more enhanced at 72 h, and intense expression was more widespread (Fig. 3I,J). On the other hand, aggrecan was faintly expressed in a few cells at 24 h after plating (Fig. 3C,D), and started at least 48 h after plating (Fig. 3G,H). Within 72 h, expression was clearly detected in the cellular condensations (Fig. 3K,L). At this time, *Frzb-1* expression was larger than aggrecan expression domain, in agreement with the endogenous expression in the limb bud.

Discussion

In this study, we show the expression pattern of *Frzb-1* in the chick limb bud, and compare its expression to that of the cartilage marker, aggrecan. *Frzb-1* is first expressed in the center of the limb bud, initially in the ventral mesenchyme, but later localizes midway along the dorsoventral axis. During later limb development, *Frzb-1* expression shows the prepattern of skeletal elements of the limb, although *Frzb-1* expression domain is wider than the region of aggrecan expression. Once the cartilage rudiments have formed, expression of *Frzb-1* localizes to the perichondrial tissue. In recombinant limb buds, *Frzb-1* is also expressed in the proximal core region prior to the aggrecan expression, and no expression of *Frzb-1* was observed near the ectoderm. Moreover, *Frzb-1* is expressed in cultured limb mesenchymal cells. Its expression started within 24 h after plating in culture, when aggrecan mRNA was not yet detectable, and later localized to condensing mesenchyme. These expression patterns suggest that Frzb-1 products are involved in the early phase of chondrogenesis in the limb bud.

Mesenchymal condensation is an early event of chondrogenesis. In the limb bud, condensation is formed in the core region when the cells become packed closely (Ede, 1983; Hall and Miyake, 1992). Condensation formation during chondrogenesis has also been reported in culture systems (Ahrens *et al.*, 1977). Condensation in the limb bud is first observed at stage 22 (Ahrens *et al.*, 1977), or slightly later (Ede, 1983). The expression of *Frzb-*

1 starts earlier than the beginning of condensation in regions not associated with chondrogenesis, but later coincides with the area of mesenchymal condensation *in vivo* and *in vitro*. The later expression pattern of *Frzb-1* in the limb bud is similar to the distribution of the peanut agglutinin lectin binding molecule or the chondroitin sulfate proteoglycan, versican (Aulthouse and Solursh, 1987; Shinomura *et al.*, 1990), although the peanut agglutinin lectin binding region seems to be narrower than the *Frzb-1* expression domain. These molecules are predominantly expressed in mesenchymal condensations (Shinomura *et al.*, 1990). Therefore, Frzb-1 may have roles in initiation and maintenance of mesenchymal condensations, eventually promoting cartilage differentiation in the limb bud.

At later stages of development, Frzb-1 may regulate Wnt activity in the central core region of the limb bud, since Frzb-1 can bind to some Wnts, and antagonize their biological activity (Leyns *et al.*, 1997; Wang *et al.*, 1997). In the limb bud, various *Wnt* genes are expressed in the mesenchyme (Dealy *et al.*, 1993; Tanda *et al.*, 1995; Kawakami *et al.*, 1999), and Wnts (Wnt-1, Wnt-5a and Wnt-7a) inhibit chondrogenesis (Rudnicki and Brown, 1997; Kawakami *et al.,* 1999). A similar result was also obtained from the analysis of *Wnt-1* transgenic mice (Zakany and Duboule, 1993). Frzb-1 products may promote condensation formation and cartilage differentiation by antagonizing Wnt activity in the limb bud.

On the other hand, *Frzb-1* is already expressed at early stages of limb development when mesenchymal condensations have not yet formed. At these stages, expression of *Frzb-1* is enhanced in the ventral mesenchyme. Such a polarized expression along the dorsoventral axis has been also reported in the mouse limb bud (Hoang *et al.*, 1998; Leimeister *et al.*, 1998). These results indicate the existence of distinct regulatory pathways of *Frzb-1* expression in early limb buds, and possible involvement of *Frzb-1* in dorsoventral specification of the limb. *Wnt-7a* is expressed in the dorsal ectoderm of the early limb bud (Dealy *et al.*, 1993; Riddle *et al.*, 1995), and has a role in dorsal patterning of the limb by activating *Lmx-1* in underlying mesenchyme (Riddle *et al.*, 1995; Vogel *et al.*, 1995). On the contrary, *En-1* is expressed in ventral ectoderm, and is involved in ventral structure formation (Loomis *et al.,* 1996; Logan *et al.,* 1997). Early expression of *Frzb-1* underlies *En-1* expression in the ectoderm. Thus, at least at early stages of limb development, Frzb-1 products may enhance the ventralizing signal of *En-1* by antagonizing Wnt-7a activity in the ventral region of the limb bud.

In the recombinant limb bud, *Frzb-1* was restricted to the core region, and no expression signal was detected in the mesenchyme near the ectoderm throughout limb development, possibly owing to the ectodermal stage of the recombinant limb bud. We used stage 22-23 leg bud to prepare ectodermal jacket of the recombinant, and no polarized expression of *Frzb-1* was detected in the normal limb bud at these stages. Therefore, *Frzb-1* was expressed only in the core region in the recombinant limb.

In the recombinants, *Frzb-1* was expressed prior to aggrecan expression, and then their domains of expression overlapped. This indicates that the chondrogenesis in the recombinant limb bud proceeds similarly to that of the normal limb bud. The restricted expression of *Frzb-1* was established within 12 h after recombinant grafting, suggesting that *Frzb-1* expression responds rapidly to changing environmental signals. Also, in the normal limb bud, *Frzb-1* is not expressed in the mesenchyme near the ectoderm at chondrogenic stages. These results indicate that expression of *Frzb-1* may be inhibited by the ectoderm. Because mesenchymal expression of *Frzb-1* is observed at a distance from the ectoderm, diffusible factors produced in the ectoderm may inhibit the expression of *Frzb-1* in the peripheral region of the limb bud. Since Frzb-1 possibly promotes mesenchymal condensations as discussed above, inhibition of *Frzb-1* may in turn suppress chondrogenesis. It is known that cartilage differentiation in the limb bud is inhibited by the surrounding ectoderm (Solursh and Reiter, 1988; Gregg *et al.*, 1989), hence ectodermal factors may regulate cartilage differentiation in the limb bud by inhibiting *Frzb-1* expression in the mesenchyme.

Materials and Methods

Animals

Fertilized White Leghorn chicken eggs were used. Eggs were incubated at 38°C and embryos were staged according to Hamburger and Hamilton (1951).

Medium

To culture mesenchymal cells of the limb bud, we used F-12 medium (Nissui) containing 2% FBS (ICN) as the culture medium (Wada *et al.*, 1998a).

In situ hybridization

Whole-mount *in situ* hybridization was performed according to the method of our previous report (Kawakami *et al.*, 1996), using the following chicken cRNAs. An antisense cRNA probe for *Frzb-1* was synthesized with a 1.2 kb fragment encoding part of the 3'-coding region and 3'-noncoding region (Ladher *et al.*, in preparation). Chicken cartilage aggrecan cDNA, obtained from Dr. Iwamoto (Enomoto-Iwamoto *et al.*, 1998), was used as a template for cRNA synthesis. After *in situ* hybridization, the embryos were re-fixed in 4% paraformaldehyde, embedded in 4% agarose (FMC), and sectioned on a vibratome at 50 μm (Logan *et al.*, 1997).

In situ hybridization of cultured cells was performed according to the method of whole-mount *in situ* hybridization with a slight modification. In brief, 2 μg/ml of proteinase K was used in pretreatment of the cells, and hybridization was performed at 50ºC for 16 h.

Preparation of recombinant limb buds

Recombinant limb buds were prepared as reported previously (Wada *et al.*, 1998b). The distal regions of stage wing buds were excised, and mesodermal tissues were dissociated into single cells. The cells were pelleted by centrifugation and packed into an ectodermal jacket prepared from stage 22-23 whole leg buds. The recombinant limb bud was grafted onto the anterior-proximal region of stage 21-23 wing bud with a tungsten pin, allowed to develop, and fixed for either *in situ* hybridization or skeletal analysis.

Skeletal analysis

For the analysis of the skeletal pattern, the recombinants were allowed to develop for five days. Embryos with a limb-like structure were fixed in 10% formalin, stained with 0.1% Alcian green, macerated in 1% potassium hydroxide, and cleared in 50% glycerol solution.

Micromass culture of mesenchymal cells

Limb buds were dissected out from the stage 22-23 embryos, and mesenchymal cells from the distal region of the limb bud were prepared as previously described (Wada *et al.*, 1998a). The cells were suspended in the culture medium, and were seeded into penicillin cups (6 mm diameter) in a 35 mm plate (Falcon 3001) to a final density of 2.2×10^5 cells/well. Three hours after incubation, the penicillin cups were removed and

2 ml of culture medium was added. After incubation, cultured cells were fixed with 4% paraformaldehyde for *in situ* hybridization.

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