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Source: Zoological Science, 13(2) : 207-212

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.207

ZOOLOGICAL SCIENCE **13**: 207–212 (1996)

[Review]

Cell-Cell Interactions in the Mesenchyme of Limb Bud and Blastema

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INTRODUCTION

Cell-cell interaction is known to be important for the various processes of morphogenesis. In limb bud morphogenesis, cell-cell interaction of mesenchyme is crucial (Fig. 1). Cell differentiation of the limb bud mesenchyme proceeds from the proximal to the distal region. In the distal-most region, the progress zone (PZ), within 250–350 μm from tip, the cells are in an undifferentiated state under the control of the tip ectoderm, apical ectodermal ridge (AER) (Summerbell *et al.*, 1973). The mesenchymal cells in the PZ are relatively isolated from each other and surrounded by extracellular matrix molecules such as Type I collagen, fibronectin and proteoglycan M (versican) (Shinomura and Kimata, 1990). Since the limb bud grows distalward and the influence of AER is limited, the cells emerge from the PZ during limb development and undergo differentiation to cartilage or soft connective tissue. This differentiation is thought to be caused by the removal of extracellular matrix which has previously prevented cell-cell interaction. In the core region, the mesenchymal cells condense, which promotes cell-cell interaction and thus, chondrogenic differentiation occurs to form the humerus in the forelimb and the femur in the hindlimb. In the peripheral region, the cell density remains relatively low, and the formation of soft tissues such as dermis and tendon occurs. Thus, the mesenchymal condensation is crucial for the cell differentiation of the limb bud.

Another type of cell-cell interaction occurs in the limb morphogenesis. The mesenchymal tissue of the chick limb bud, especially in the PZ, has a high regulation capacity, and removal or addition of the mesenchyme results in normal development. When a square piece of mesenchymal tissue is removed from the center of the young chick limb bud to produce a hole from ventral to dorsal surface, a limb of normal size and with a normal cartilage pattern is formed (Summerbell, 1981). Thus, the limb bud cells are necessary to recognize their positions and adjacent cells to recover the missing area. This type of interaction seems to depend on "positional values".

The limb cartilage pattern is specified in the PZ (Summerbell, 1974, 1981), in which cells are thought to receive anteroposterior positional values which are specified by the polarizing region at the posterior margin of the limb bud; proximodistal positional values which change autonomously under the influence of AER during the limb bud development are also specified (Summerbell *et al.*, 1973; Fig. 1). Thus, cells in the PZ seem to be different from each other both temporally and spatially. Recent findings on homeobox gene expression and targeting in the limb bud supports the notion that homeobox genes are closely related to positional values (Nohno *et al.*, 1991; Yokouchi *et al.*, 1991; Izpusia-Belmonte *et al.*, 1991; Davis *et al.*, 1995). Since local cell-cell interaction is necessary for limb morphogenesis, the heterogeneity in homeobox gene expression might be reflected in cell surface differences.

Recently, by sorting-out experiments we have found such cell surface differences in the chick limb bud cells which are related to the positional values along the limb bud axes (Wada *et al.*, 1993; Ide *et al.*, 1994; Wada and Ide, 1994). Similar positional value-related cell-cell interaction has been reported in amphibian limb regeneration (Nardi and Stocum, 1983; Crawford and Stocum, 1988).

In this paper, I will review briefly those experiments on the cell surface difference in the limb bud and regenerating blastema from the aspect of positional value-related cell-cell interaction in limb morphogenesis. Cell-substratum interaction in the limb mesenchyme is also important for limb morphogenesis and is closely related to cell-cell interaction, because inhibition of cell-substratum attachment often prompts the cell-cell attachment. Epithelio-mesenchymal interaction is also important for limb morphogenesis (Ide, 1990). However, in this paper, I will focus on the cell-cell interaction of the mesenchyme.

Sorting-Out Between Limb Bud Mesenchymal Cells

The sorting-out between the chick mesenchymal cells in culture was first found by Cottril *et al.* (1987a, b). To examine the mechanisms of cartilage differentiation, they mixed chondrogenic distal limb bud cells with non-chondrogenic

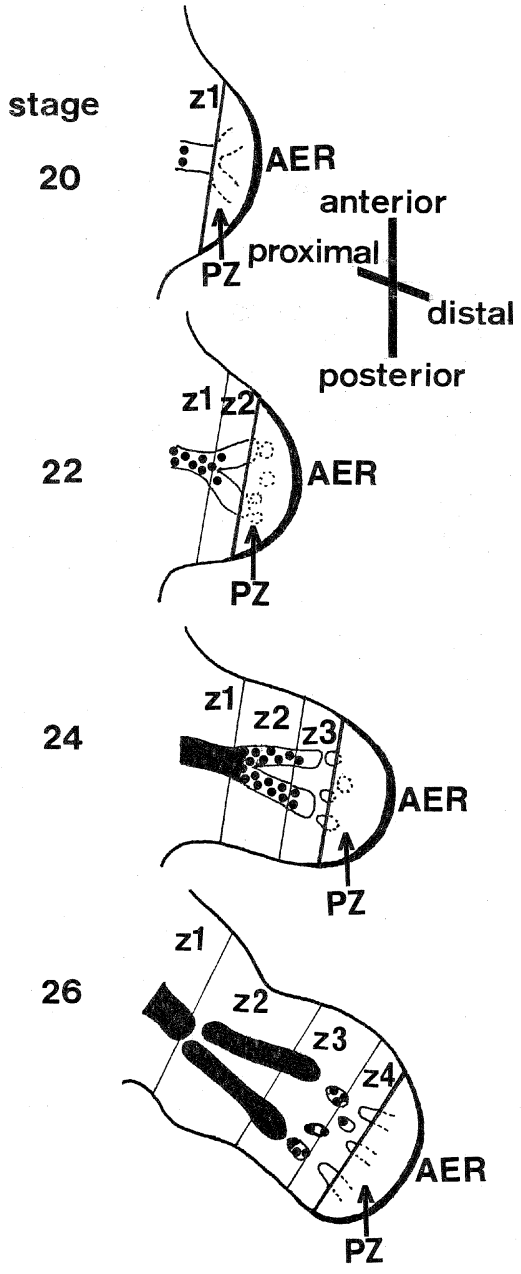

Fig. 1. Development of chick wing bud. Distal-most region of the wing bud mesenchyme, progres zone (PZ), is covered by thickened ectoderm, AER, and positional specification occurs in the progress zone. A stage 20, the cells in the PZ are temporarily in the state of specification at proximal radius-ulna level (indicated by dotted lines), while those in the proximal region (z1) have been specified to form humerus level. At stage 22, the cells emerged from the PZ are locating between the PZ and z2. The cells in z2 have been specified to form proximal elements of radius-ulna. The cells remaining in the PZ are temporarily in the state of specification at distal radius-ulna level. At stage 24 and 26, the PZ cells are temporarily specified to form carpus and metacarpus level, respectively. The cells of areas z3 and z4 emerged from the PZ at stages 22-24 and 24-26, respectively. Chondrogenesis (black area, Type II collagen synthesis) occurs from proximal to distal and is preceded by mesenchymal chondensation (heavily dotted area, PNA biding).

proximal-peripheral cells. The former sorted out from the latter within 2 days and both formed aggregates depending on the proportion of cells in the culture. Cottril *et al.* (1987a, b) explained this result as the sorting out between the chondrogenic mesenchymal and non-chondrogenic mesenchymal cells, and urged that the mesenchymal condensation in culture was the consequence of cell sorting, but not of directed cell migration (Ede, 1983). It remains uncertain whether such cell sorting occurs for mesenchymal condensation in normal development, since mixing of the cells with different properties is rare.

Recently, we have examined the sorting-out of the chick limb bud cells from the aspect of positional values. To examine differences in surface properties of mesenchymal cells of avian limb buds, we mixed cells from the PZ at different stages or from different positions along the proximodistal and anteroposterior axes (Ide *et al.*, 1994; Wada and Ide, 1994). To identify the origin of cells, a chick-specific antibody was used in a mixed culture of chick and quail cells; otherwise cells from one of the stages were labeled with a fluorescent dye, PKH-26. Within 3 hours of the attachment, the cells atarted to segregate, and within 15 hours, patches of various sizes were formed. The segregation occurred independently of mesenchymal condensation and chondrogenic aggregation, which occurred only later in the central region of the patches (Fig. 2). A low concentration of retinoic acid was added to this culture of PZ cells to clear the chondrogenic region. Retinoic acid is known to promote chondrogenic differentiation in PZ cultures (Ide and Aono, 1988).

The area of mesenchymal condensation was identified also by PNA (peanuts agglutinin) binding which was shown to precede the expression of cartilage markers (Aulthouse and Solursh, 1987). Within 48 hours, PNA-positive areas appeared in the central region of both patches. These results strongly suggest that the surface properties of cells in the limb bud change during development, and vary along the proximodistal and anteroposterior axis of the limb bud. No sorting out has yet been detected between the cells of ventral and dorsal halves. In further-developed wing buds of 5-day embryos, a similar sorting out occurred among the cells of presumptive PZ regions of digit II, III and IV (Omi and Ide, in preparation). Thus, the surface properties of PZ cells may also change sequentially along the anteroposteror axis. However, this type of monolayer segregation could be caused by the difference in the affinity to the substratum. To exclude this possibility, we cultured the cell mixture on agar, where cells formed aggregates within 18 hours. The cell mixture at different stages formed independent clusters of cells at the same stage in the aggregate (Fig. 3). Thus, the segregation seemed to be caused by the difference in cell-cell affinity not in cell-substratum affinity.

It seems unlikely that this type of cell sorting occurs in normal development since the surface properties will change gradually in the limb bud. However, the cell sorting can be used as a marker for detecting cell surface difference, which

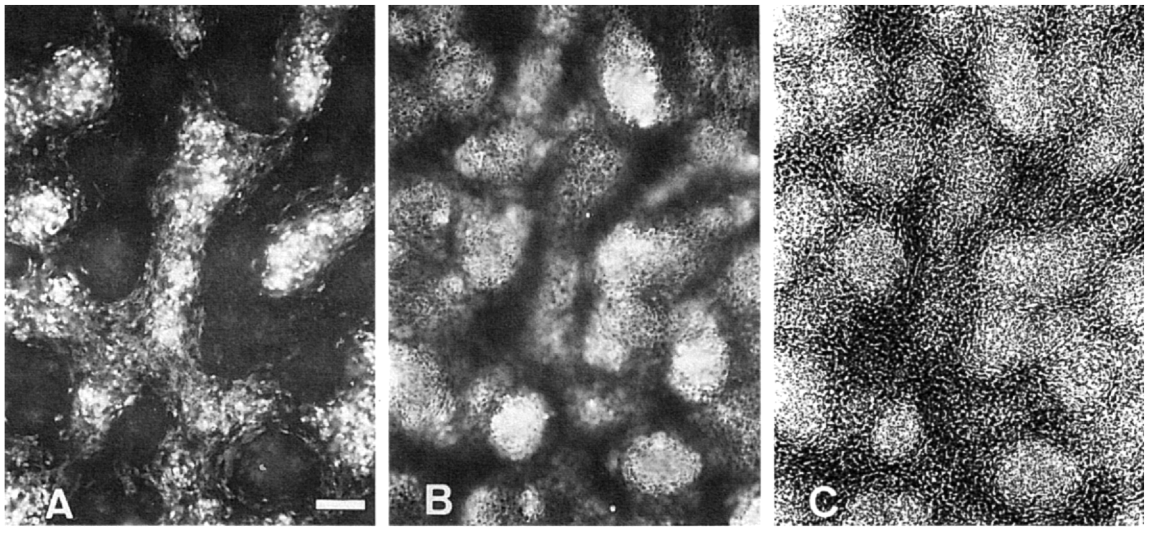

Fig. 2. Chondrogenesis in the segregated area. Stage 20 chick PZ cells were mixed with stage 24 quail PZ cells and cultured for 4 days in the medium containing 20 ng/ml retinoic acid. (A) A223 staining. (B) Proteoglycan H staining for cartilage. (C) phase contrast. Chondrogenesis occurred in the central region of both segregated areas of stage 20 origin and of stage 24 origin. Non-chondrogenic region were localized near the boundary of both segregated areas. Bar=100 μm.

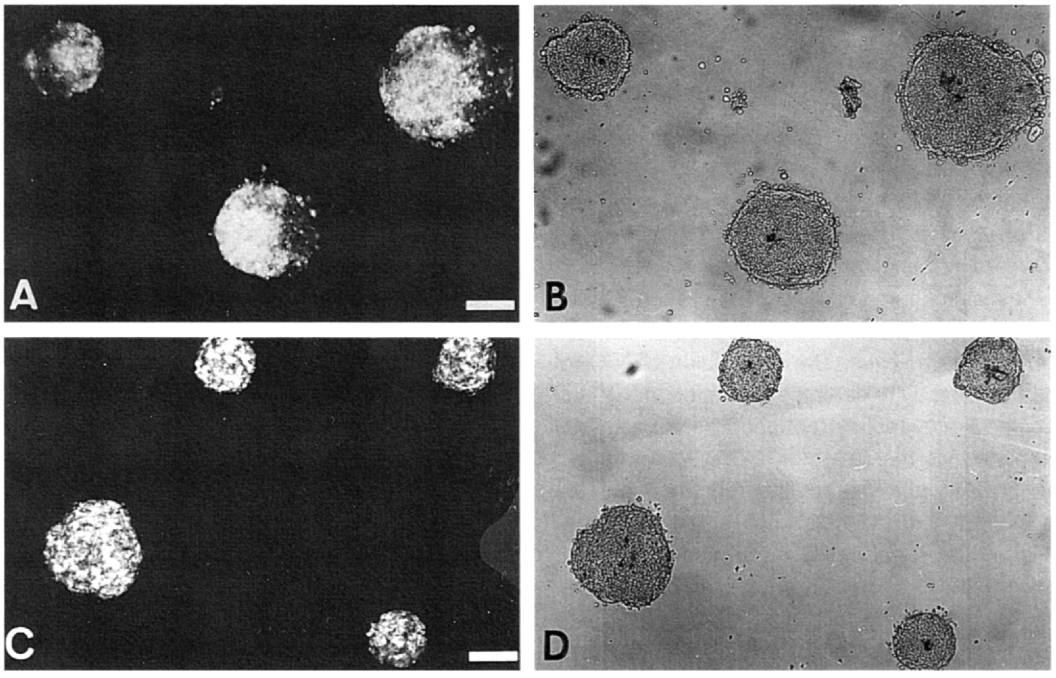

Fig. 3. Sorting out on agar between the PZ cells of chick wing buds at different developmental stages. PZ cells at stage 24 were labelled with PKH-26 and mixed with unlabelled PZ cells at stage 20 (A and B) or stage 24 (C and D). A and C, fluorescent light. B and D, phase contrast. Bar=100 μm.

will relate to the positional values.

Cell Sorting And Positional Values

When the PZ cells of young limb bud (stage 20) were mixed with the cells of old limb bud (stage 26) at various proximodistal levels, sorting-out occurred between stage 20 PZ and stage 26 distal cells, but homogeneous mixing occurred between stage 20 PZ and stage 26 proximal cells (Wada and Ide, 1994). Thus, the surface property of the stage 20 PZ cells appeared to be resemble that of stage 26 proximal cells. Similarly, the surface property of the stage 22 PZ cells resembled that of stage 26 subdistal cells. This corresponds to the positional values along the proximodistal

axis specified in the PZ during the limb bud development.

The process of chondrogenic differentiation includes aggregate formation. Thus, it is possible that the sorting out observed in these experiments occurred between the cells with both high and low chondrogenic activity, independent of stage or position-related characteristics. PZ cells at stage 20 were in fact more chondrogenic than those at stage 25 (Wada *et al.*, 1993). Further, spatial differences in chondrogenic activity in the limb buds have been found (Ide *et al.*, 1991). In the present experiment, however, sorting-out occurred within 18 hours of plating when no aggregate formation was observed. Further, rather homogeneous chondrogenesis occurred within 4 days in the culture of PZ without retinoic acid, although stage-related cell sorting occurred. Thus, observed segregation seems to be independent of chondrogenic activity.

However, it is possible that chondrogenic activity is related to cell sorting. If the PZ cells at different stages with different chondrogenic rates were mixed, the cells with high chondrogenic rate would aggregate precociously, even if cells at both stages had chondrogenic activity. The positional values would accordingly relate to the chondrogenic activity.

Cell Adhesion Molecules In The Limb Bud

The presence of some cell adhesion molecules has been reported in the limb bud (Table 1). N-CAM, a calcium-independent adhesion molecule, is expressed in the mesenchymal tissues of stage 22 limb bud (Crossin *et al.*, 1985). N-CAM is known to enhance mesenchymal condensation and chondrogenesis in culture (Widelitz *et al.*, 1993). Anti-N-CAM antibody conversely inhibits mesenchymal condensation (Widelitz *et al.*, 1993). Thus, The expression of N-CAM seems to be related directly to cartilage formation in the limb bud. It is suggested that stimulation of chondrogenic activity by activin is mediated via regulation of N-CAM (Jiang *et al.*, 1993). Further, N-CAM activity may be regulated by Hox gene products (Jones *et al.*, 1992). However, the participation of N-CAM in pattern specification through positional values remains uncertain.

Table 1. Cell adhesion molecules in the limb bud

Cell adhesion molecules	Localization	Proposed function
N-cadherin	mesenchyme perichondrium AER (mouse, transient) ectoderm except AER (chick)	mesenchymal condensation
Cadherin 11 (mRNA)	distal and peripheral mesenchyme, interdigit	loose association in mesenchyme
N-CAM	mesenchyme, AER ectoderm except AER (transient)	mesenchymal condensation
L-CAM	ectoderm	

N-cadherin, a calcium-dependent adhesion molecule, is present in all limb bud tissues of the 4.5-day embryo except the AER (Hatta *et al.*, 1987). N-cadherin is also shown to play a role in the mesenchymal condensation. Exogenous Ca^{++} stimulates chondrogenesis in limb bud mesenchymal cultures (San Antonio and Tuan, 1986). The condensation and resulting cartilage formation are inhibited by the anti-N cadherin antibody. When the anti-N cadherin antibody was injected into limb buds *in vivo*, inhibition of cartilage and perturbation of limb pattern formation was observed (Oberiender and Tuan, 1994). Furhter, stimulation of chondrogenic activity by vitamin D may be mediated via regulation of N-cadherin (Tsonis *et al.*, 1994). The timing of N-cadherin and N-CAM expression in limb bud cell culture suggested that N-cadherin initiates mesenchymal condensation, thereafter stabilized by N-CAM (Tavella *et al.*, 1994).

Recently, a novel cadherin, cadherin 11, has been found, which is expressed in the progress zone at early stages and in the interdigital region at later stages (Kimura *et al.*, 1995). This expression pattern seems to be similar to *Msx* gene expression (Suzuki *et al.*, 1991), and relate to the undifferentiated state of the cells in the distal region. The cells in the interdigital region may be also in an undifferentiated state since they can differentiate into cartilage by removal of overlying ectodermal tissues; in normal develpment, however, cell death occurs (Hurle *et al.*, 1989). Cadherin 11-positive cells were segregated from negative cells. Thus, cadherin 11 may be one of the molecules controlling segregation of the proximal and distal mesenchymal cells. The distribution of N-CAM and N-cadherin also changes spatiotemporally in the limb development and thus, these molecules also participate in cell sorting, although the antibodies to these proteins failed to block sorting. Other calcium-dependent but low-molecular weight adhesion molecules have been isolated from stage 23 chick limb buds, although their distribution remains unknown (Bee and von der Mark, 1990).

Sorting-Out In Reconstituted Limb Bud

In the reconstituted chick limb bud, similar sorting out occurs *in vivo* using dissociated and reconstituted mesoderm cells. When the PZ cells of chick limb bud at stage 20 and of quail limb bud at stage 25 were mixed and packed into the limb bud ectoderm sack, and the reconstituted limb bud was grafted onto a host limb stump, limb-like structures with bifurcated and segmented cartilage patterns were formed. The anteroposterior polarity of the limb, however, was lost by the dissociation of polarizing region, ZPA. In the limb-like structures, the PZ cells at stage 25 distributed preferentially in the distal region, although those at stage 20 distributed over the whole region (Wada *et al.*, 1993). This distrubution pattern coincides with the lineage of the PZ cells. The PZ cells at stage 20 form all limb elements distal to the zeugopodium, although those at stage 25 form only the autopodium. After dissociation, the PZ cells retain posi-

tional memory to distribute in the reconstituted limbs.

Cell-Sorting In The Regeneration Blastema

Similar memory has been found in the regenerating limbs. In the limb blastema of axolotls, a graded difference has been reported along the proximo-distal axis in the cell surface. Nardi and Stocum (1983) amputated the forelimb at various levels along the preximodistal level such as upper arm, elbow and wrist, and allowed to regenerate. One group of regenerates was radiolabeled by the injection of labeled thymidine before isolation of the regeneration blastema. When these blastemas were cocultured *in vitro* for 4 days, proximal blastema always surrounded distal ones. Similar results was obtained in the hindlimb experiment. This behavior of blastema cells has been interpreted by the graded distribution of surface properties along the proximodistal axis, with adhesiveness increasing distally.

Differences in surface property of the regeneration blastema cells seem to relate to the positional values for pattern specification. Crawford and Stocum (1988) have found the difference in cell surface of the blastema *in vivo*. When a fragment of wrist blastema was grafted onto the hindlimb blastema at thigh level, the graft displaced distally from the host thigh level to corresponding level, ankle, and the fully-developed wrist regenerate articulated with the host ankle. Such displacement did not occur when a fragment of the wrist blastema was grafted onto an ankle blastema. The wrist regenerates articulated with the host ankle. This difference in cell surface property can be altered by retionoic acid treatment which is known to proximalize the positionla values in the axolotl limb blastema. Retinoic acid proximalizes the cell property of the blastema. When forelimb blastema at wrist level was grafted onto the hindlimb blastema at ankle level of retinoic acid-treated host, the displacement of the graft occured, and the fully-development wrist blastema articulated with the distal ankle of proximodistally duplicated hindlimb (Crawford and Stocum, 1988). These results demonstrate the positional value-related cell surface property in the regeneration blastema.

N-CAM is present in mesenchymal cells and the wound epidermis of regenerating newt limb (Maier *et al.*, 1986). Anti-N-CAM antibody delays the regeneration processes (Maier *et al.*, 1986). The expression of N-CAM disappeared from the cartilage tissue of the regenerate (Maier and Miller, 1992). Thus, N-CAM seems to play a role in cell-cell interaction between the blastema cells to form cartilage, as it did in the case of limb development, rather than the axon-mesenchyme interaction.

Conclusions

Aggregate formation is one of the most conspicuous behavior of mesenchymal cells. Cartilage formation in the limb bud and blastema is a suitable system for the analysis of mesenchymal tissues in morphogenesis. Thus, temporal and spatial specification of the mesenchymal condensation region and its control mechanisms are fundamental problems for pattern formation.

The findings of sorting-out of cultured chick limb bud cells and of axolotl limb blastema in culture and *in vivo* suggest the presence of positional value-related surface differences. The resemblance of cell sorting and mesenchymal condensation, however, makes the anlaysis difficult. At present we do not know how many such molecules are concerned with cell sorting, but the sorting out occurs between the cell populations with different amounts of adhesion molecules (Steinberg and Takeichi, 1994), which will diminish the number of the molecules necessary for the process.

ACKNOWLEDGMENT

Our study in this paper was supported by the Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and Yamada Foundation.

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(Received December 22, 1995 / Accepted December 23, 1995)