Sorting out of limb bud cells in monolayer culture

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ABSTRACT To examine differences in the surface properties of limb bud cells, we mixed cells from the progress zone at different stages or from different positions along proximodistal axis of chick and quail wing limb buds. To identify the origin of cells, a chick-specific antibody was used in a mixed culture of chick and quail cells, or cells from one of the stages were labeled with a fluorescent dye, PKH-26. Within 18 hours in mixed culture, cells segregated from each other and formed patches of various sizes. The process zone cells at early developmental stages mixed homogeneously with the cells from proximal region of old limb buds and the progress zone cells at late stages mixed homogeneously with the cells from distal region of the old limb buds. These results suggest that surface properties of cells in progress zone change during limb bud development and vary along the proximodistal and anteroposterior axes of the limb bud and that these differences in surface property may correspond to the positional values for limb pattern formation.

KEY WORDS: *limb bud, pattern formation, cell sorting, positional value*

Introduction

The cartilage pattern of chick limb is specified in the distal region of the limb bud, the progress zone (PZ), where the mesodermal cells remain in an uncommitted state under the influence of the apical ectodermal ridge (Summerbell *et al.*, 1973). The cells in the PZ are thought to receive anterior-posterior positional values which are specified by the polarizing region (ZPA) at the posterior margin of the limb bud, and proximal-distal positional values which change during limb bud development (Summerbell, 1974). Thus, cells in the PZ seem to be different from each other both temporally and spatially.

Recent findings on homeobox gene expression in the mouse and chick limb buds support the idea of positional heterogeneity in the PZ (Izpisua-Belmonte *et al.*, 1991; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991b). This heterogeneity in homeobox gene expression might be reflected in cell surface differences since local cell-to-cell interaction may be necessary for limb pattern formation. However, little experimental work on heterogeneity of PZ cells has been carried out.

We have already reported the sorting out between chick PZ cells at different developmental stages *in vitro* and *in vivo* (Wada *et al.*, 1993) and between cells from different regions of limb bud along the anteroposterior and proximodistal axis *in vitro* (Ide *et al.*, 1994). In the present study, we have investigated the sorting out further with special reference to the positional values of the limb bud.

Results

Sorting out between the progress zone cells at different stages

In the mixed cultures of PKH-labeled stage-24 quail PZ cells and non-labeled stages-20, -22, -24 and -26 quail PZ cells, sorting out occurred within 18 h of plating except in the combination of labeled and non-labeled stage-24 PZ cells where the cells were mixed homogeneously (Fig. 1). The degree of segregation appeared to be higher, and the number of large patches greatest when the stage difference between the mixed cells was maximal.

No segregation occurred between the stage-22 wing and leg PZ cells (Fig. 2), suggesting that the positional values of PZ cells at the same developmental stages are similar between the wing and leg.

Sorting out between cells from different positions of limb bud

Similar sorting out was observed between the cells from different positions of the limb buds. When the chick and quail limb buds at stage-24 were respectively divided into 6 regions as shown in Fig. 3, and the mesodermal cells were mixed and cultured for 18 h, various degrees of segregation occurred (Fig. 4). The cells from the same positions along the proximodistal and anteroposterior axes showed homogeneous mixing, but those from different positions along the proximodistal or anteroposterior axes showed moderate segregation. Maximal segregation occurred between the cells from different positions along both anteroposterior and proximodistal axes. These results suggest differences in cellular affinity along the anterior-posterior and distal-proximal axes. The degree of the segregation was estimated by counting the cell number in the patches of chick cells in the mix culture (Fig. 5). The limb bud at stage-24 was divided into three regions along proximodistal axis and anteroposterior axis, respectively. The number of clusters with large numbers of cells increased when the cells from different regions were mixed. Maximal clusters were formed when the cells of anterior one-third and of posterior one-third were mixed or those of distal end and proximal regions were mixed.

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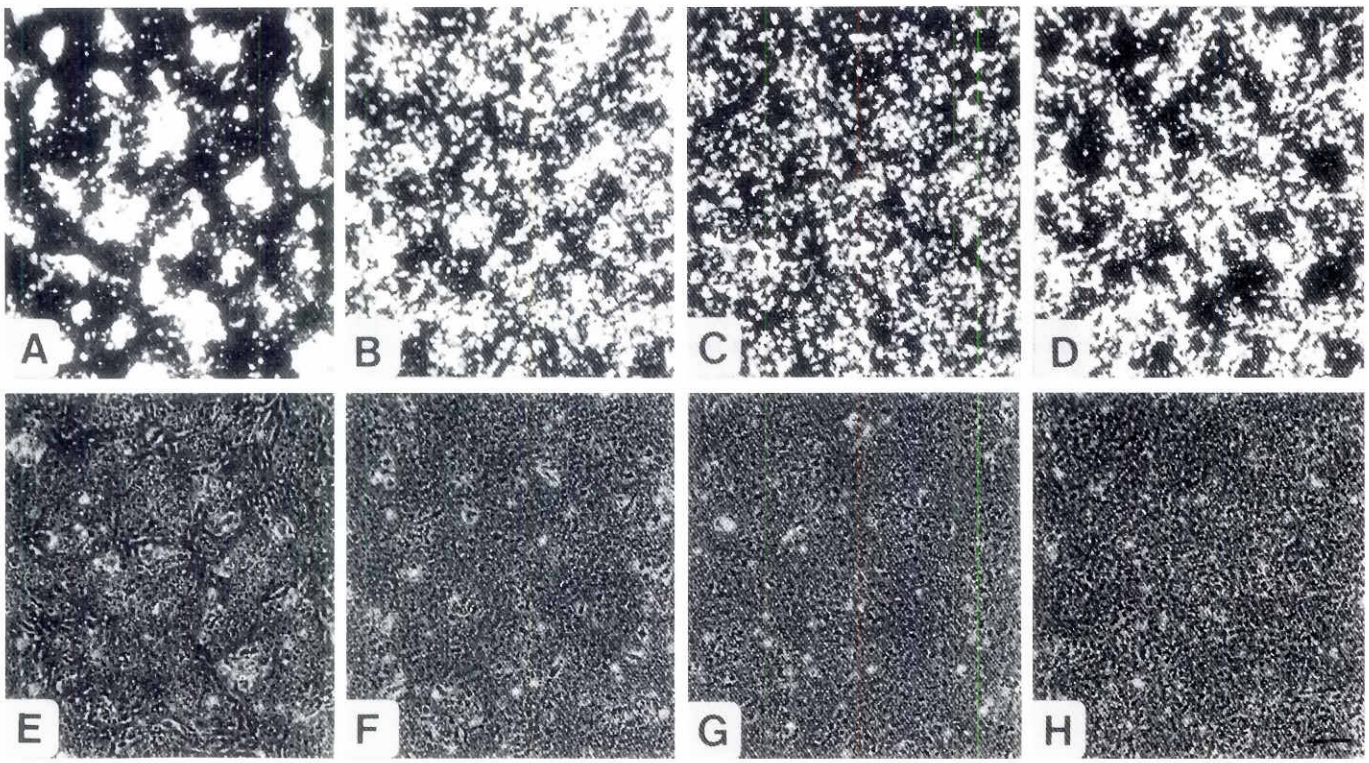

Fig. 1. Sorting out between PZ cells at different developmental stages. *PKH-labeled quail PZ cells at stage 24 were segregated from non-labeled stage 20* **(A,E)**, *22* **(C,F)** *and 26* **(D,H)** *quail PZ cells but not segregated from stage 24* **(B,G)** *quail PZ cells;* **(A-D)** *fluorescence.* **(E-H)** *phase contrast. Bar, 100 μm.*

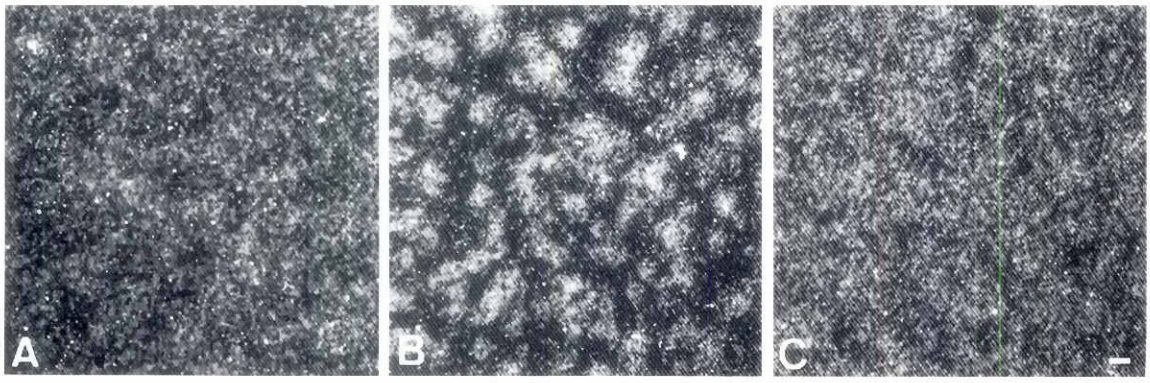

Fig. 2. Sorting out between PZ cells of wing and leg buds. *PKH-labeled PZ cells of wing bud at stage 22 were mixed with non-labeled PZ cells of wing bud at stage 22* **(A)**, *of wing bud at stage 20* **(B)** *and of leg bud at stage 22. Bar, 100 μm.*

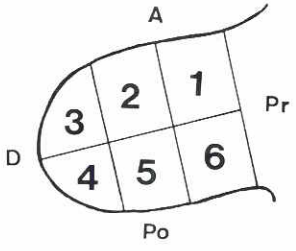

Fig. 3. A diagram of the division of limb bud. *Wing bud at stage 24 was divided into six regions along proximodistal and anteroposterior axes.*

The relation between the change in affinity of PZ cells at different stages and the change in affinity of cells along proximodistal axis

When the chick PZ cells at various developmental stages were mixed with the cells from various positions of stage-26 quail limb bud, a relation between the developmental stages of progress zone cells and proximodistal position of cells was observed (Fig. 6). The PZ cells at stage 20 mixed homogeneously with the cells from proximal regions. Stage-22 PZ cells mixed homogeneously with the cells both from distal (PZ) and subdistal region. Stage-26 PZ cells mixed homogeneously with the cells only from distal (PZ)

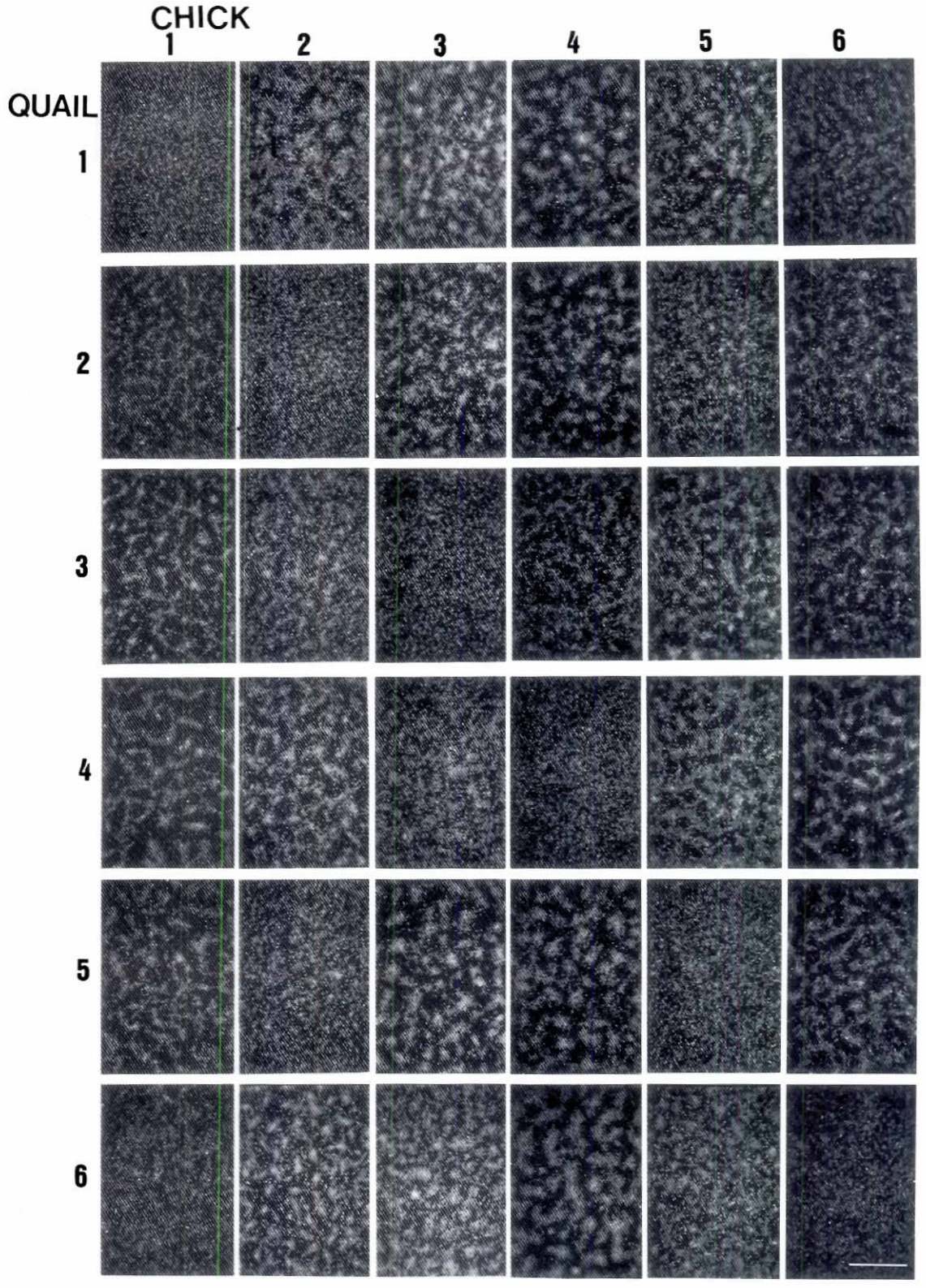

Fig. 4. Sorting out between the cells from different positions of wing buds at stage 24. *Chick and quail wing buds were divided into six regions as shown in Fig. 3 and chick cells from one region were mixed with quail cells from various regions. Bar, 500 μm.*

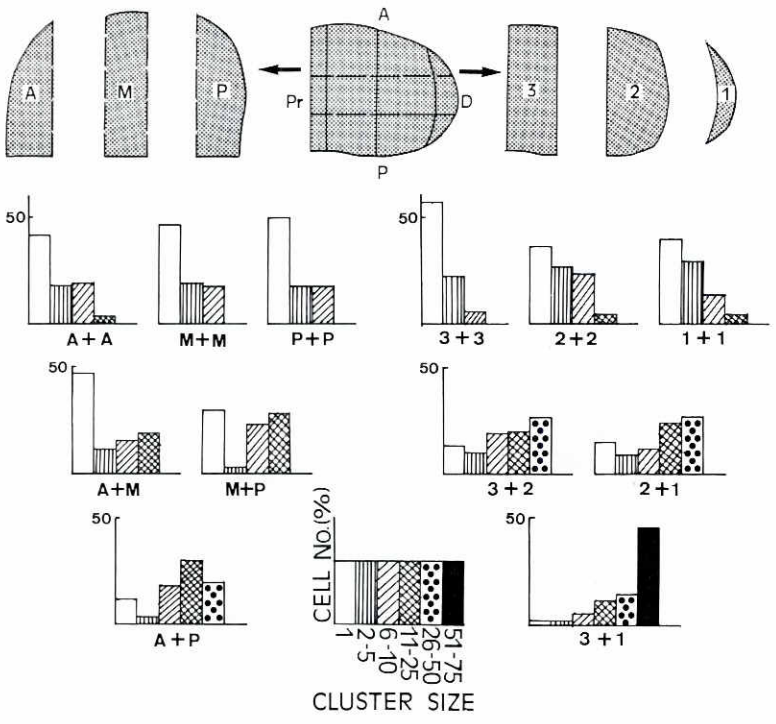

Fig. 5. A quantitative analysis of the sorting out between the cells from different regions. *Chick and quail wing buds at stage 23 were respectively divided into anterior (A), medial (M) and posterior (P) regions or divided into PZ (1), subdistal (2) and proximal (3) regions. The cells of two regions, one from chick and the other from quail, were mixed and cultured for 18 h and the number of cells in the cluster was counted. The values show the percentage of cells in the cluster with various sizes.*

region. Sorting out occurred in the other combinations. These results suggest that stage-20 PZ cells may have similar characteristics in affinity to stage-26 proximal cells and stage-22 PZ cells to stage-26 subdistal and stage-24 PZ cells to stage-26 distal, as the positional values along the proximodistal axis were progressively allotted to the PZ cells at each developmental stage.

Changes in cell affinity of cultured PZ cells

These results enabled to us examine the change in surface property of cells in culture. When quail PZ cells at stage 21 were cultured for 24 h, and then chick limb bud cells from different positions along proximodistal axis at stage 24 were added, segregation between the cultured and newly-added cells occurred within 18 h (Fig. 7). The cultured quail PZ cells mixed homogeneously with the cells from chick subdistal region (distal one third of non-PZ, Fig. 7B) and medial region (central one-third region of non-PZ, Fig. 7C), but segregated the cells from PZ (Fig. 7A) and proximal region (proximal one-third on non-PZ). Thus, the quail PZ cells cultured for 24 h have a surface property which seems to be similar not to that of PZ, but of subdistal region. When the quail PZ cells were cultured in the medium containing retinoic acid (RA), the surface property changed and the cells began to mix with those of proximal cells (Fig. 7H) and segregated from those of subdistal region (Fig. 7E).

Discussion

In both our previous (Ide *et al.*, 1994; Wada *et al.*, 1993) and present papers, we have shown the sorting out between PZ cells at different developmental stages and between the cells from different positions of the limb bud along the proximodistal and anteroposterior axes. These results suggest the possibility that PZ cells change their surface properties sequentially, the surface property seems to differ also in the non-PZ proximal regions along the proximodistal and anteroposterior axes and the surface property of PZ is common between leg and wing buds.

Further, the surface properties of the early and late PZ cells were similar to those of proximal and distal cells of late limb bud respectively. This spatiotemporal change in surface property seems to correspond to the changes of positional values in the PZ theory (Summerbell *et al.*, 1973).

Recent findings on homeobox gene expression in the mouse and chick limb buds support the idea of positional heterogeneity in the PZ (Izpisua-Belmonte *et al.*, 1991; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991b). The expression pattern of the homeobox genes coincides at least partially with the positional value. This pattern of homeobox gene expression might be reflected in cell surface differences since local cell-to-cell interaction may be necessary for limb pattern formation.

The spatio-temporal relationship on the surface property between the PZ cells and more proximal cells supports the idea that the surface property changes according to the positional values and homeobox. In the amphibian regenerating limb, cocultured fragments of blastema, which had been taken from different levels along the proximodistal axis, showed sorting out and engulfment of one fragment by the other (Nardi and Stocum, 1983). This supports the relation between the positional values and the surface properties of blastema cells at different proximodistal positions. Our segregation assay in monolayer culture will be used to measure cell adhesiveness in different positions of the regeneration blastema.

In the present culture conditions, the surface property on sorting out seems not to change. Since the quail embryo at stage 21 become stage 24/25 after 24 h, the PZ cells cultured for 24 h must mix homogeneously with those of stage 24. However, the cultured cells mixed with the cells of subdistal and medial regions

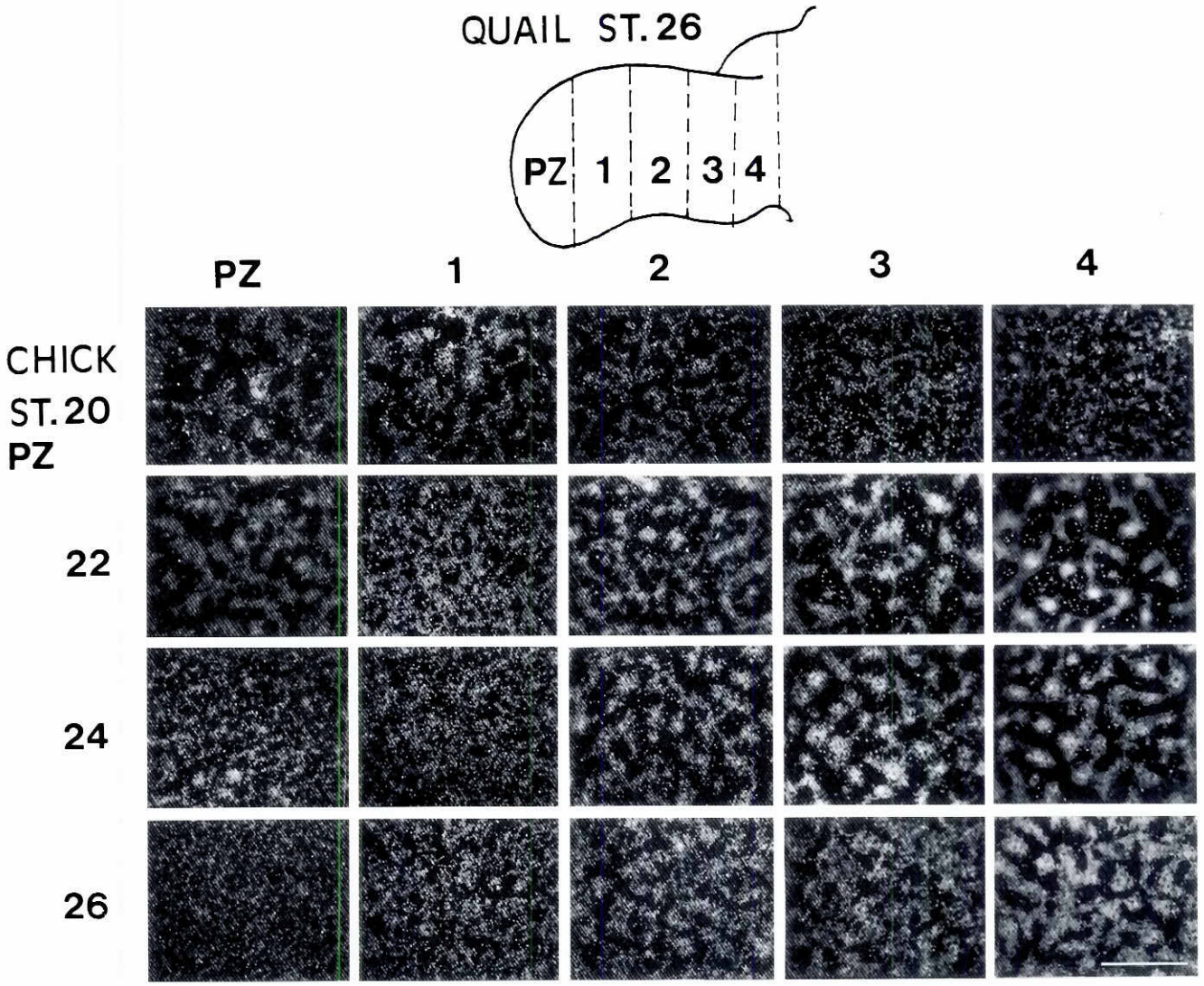

Fig. 6. Sorting out between the PZ cells at different stages and the limb bud cells from different positions. *Quail wing buds at stage 26 were divided into five regions along proximodistal axis and chick PZ cells at stages 20-26 were mixed with the cells from the different regions of the quail wing buds. Bar, 500 μm.*

and rather segregated from those of stage 24 PZ. Thus, the cells seem to retain the surface property of PZ at stage 21 at least for 24 h in culture. To change the surface property to that of advanced stages, AER may be necessary.

The PZ cells which had been cultured in the medium containing retinoic acid (RA) changed their surface property and mixed homogeneously with the cells of more proximal origin. RA is known to proximalize positional value of the regeneration blastema (Clawford and Stocum, 1988).

We have recently shown that the RA-induced proximalization occurs in the chick limb buds when the tissue fragment of distal region was treated with RA-beads and transplanted into young limb buds (Tamura and Ide, 1993). The change in surface property may correspond to the proximalization of the positional value.

Materials and Methods

Cell culture

Various regions of chick and quail wing buds at stages 20-26 were dissected out and mesodermal cells were isolated by the method described previously (Ide and Aono, 1988). The cells were suspended at 2.5×10^5/0.3 ml in F12 medium containing 1% fetal calf serum. 0.15 ml suspensions of chick and quail cells or dye-labeled (see below) and non-labeled quail cells were mixed. In culture of dye-labeled cells, dissociated quail cells were stained with 4×10^{-6} M PKH-26 (ZYNAXIS Cell Science Inc.) for 2 min. After the addition of serum to prevent further staining and centrifugation, the cells were suspended in culture medium and mixed with unlabeled quail PZ cells. After ascertaining that the cell suspension was of single-cells, suspension containing 1.25×10^5 cells each was mixed and plated on a small area of culture dish (6 mm in diameter) with the aid of a stainless column as reported previously (Hattori and Ide, 1984). After 4 h, the column was removed and 2 ml of culture medium was added.

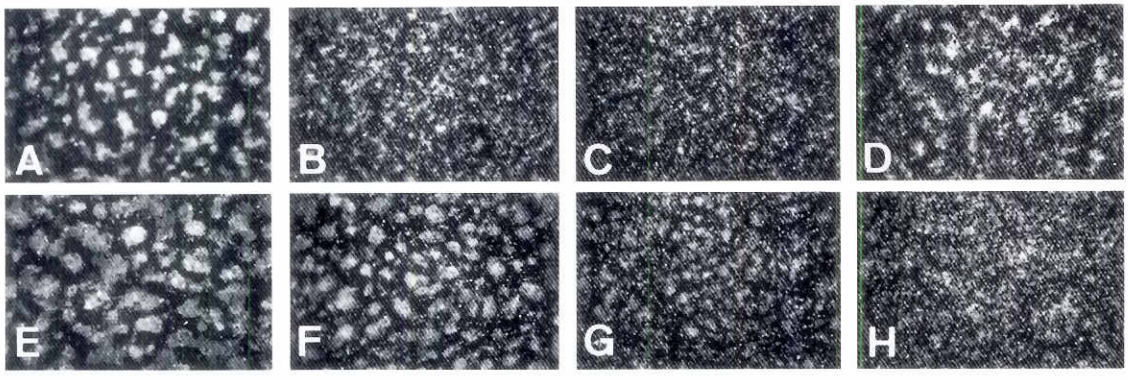

Fig. 7. Sorting out between the precultured PZ cells and the wing bud cells from different positions along proximodistal axis. *Quail PZ cells at stage 21 were cultured for 24 h in the medium containing 0* **(A-D)** *or 200* **(E-H)** *ng/ml RA. After washing with RA-free medium, cells from PZ* **(A and E)** *and subdistal (distal one-third of non-PZ,* **B and F**), *medial (central one-third of non-PZ,* **C and G**), *proximal (proximal one-third of non-PZ,* **D and H**) *regions of chick wing buds at stage 24 were added and further cultured for 18 h. Bar, 500 μm.*

Immunohistochemistry

For the detection of chick cells in the chick-quail mixed cultures, the culture was fixed in periodate-lysine-paraformaldehyde fixative (PLP, McLean and Nakane, 1974) at 4°C for 30 min, treated with 0.1% Triton X-100 for 5 min and stained with A223 mouse monoclonal antibody (Yokouchi *et al.*, 1991a) and FITC-labeled anti-mouse IgG (Cappel). The A223 antibody reacted with the cytoplasm and nucleus of chick cells, but did not react with quail cells.

Quantitative analysis of sorting out

The number of cells in the A223-positive patches was counted on photographs of mixed cultures, and the size distribution of the patches was examined. One to two thousand cells each in four to six randomly selected areas of the culture were counted.

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