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Cell adhesiveness and affinity for limb pattern formation

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ABSTRACT Stage-dependent cell sorting *in vitro* is an intriguing property that mesenchymal cells of a chick limb bud have. We previously proposed that N-cadherin, a cell adhesion molecule, is involved in the sorting process and is likely to be a component of the mechanism of proximal-distal patterning in the developing limb (Yajima *et al.*, (1999) *Dev. Dynam.* 216: 274-284). Here, we present more direct evidence that N-cadherin is one of the molecules responsible for regulation of stage-dependent cell sorting *in vitro*. Our results suggest that N-cadherin, which accumulates in the distal region of the chick limb bud as limb development proceeds, is related to the positional identity that gives rise to the different shapes and numbers of cartilaginous elements along the proximal-distal axis. In this article we also give insights into positional identity which is mediated by *Hox* genes and cell surface property during limb development.

KEY WORDS: *N-cadherin, cell sorting, limb bud, adenovirus, chick*

Introduction

During development, position-specific cell lineage restriction is an important property of pattern formation that often provides boundaries dividing populations of cells and their descendants into domains. These domains, which have distinct characteristic structures of functions, act as restricted developmental compartments. Once each domain is established, cells in a compartment tend not to cross over into neighboring compartments. One mechanism by which these compartments can be generated is division of a fixed area into several small sections, as is seen in the process of segmentation and imaginal disk development in *Drosophila* and in the process of vertebrate brain vesicle formation. In some other cases, new compartments are successively generated one after another, as is seen in somite formation in vertebrates and in proximal-distal (PD) axis formation in developing limb buds in vertebrates.

The final structure of forelimb skeletal elements can be partitioned into three units along the PD axis, stylopod (humerus in the upper arm), zeugopod (radius and ulna in the forearm), and autopod (tursus and digits in the palm and finger) from the most proximal to most distal. As a limb bud grows distally, proliferating mesencymal cells in the distal region, progress zone (PZ), add more distal structures sequentially (Saunders, 1948; Summerbell, 1974; Summerbell and Lewis, 1975) (Fig. 1A). Progenitor cells in the PZ provide different identities along the PD axis progressively, forming three compartments that correspond with Hox gene expressions and future skeletal structures in the limb. Hox genes (*a9*, *a10*, *a11*, *a13*) of a *Hoxa* cluster expressed in a nested pattern along the PD axis during limb development (Dolle *et al.*, 1989; Izpisua-Belmonte *et al.*, 1991; Yokouchi *et al.*, 1991; Nelson *et al.*, 1996; Shubin *et al.*, 1997) (Fig. 1B) are thought to record positional identity. Knock-out of both *Hoxa11* and *Hoxd11* results in limbs lacking both the radius and ulna (Davis *et al.*, 1995). Mice doubly mutant for both *Hoxa13* and *Hoxd13* fail to form an autopod structure (Fromental-Ramain *et al.*, 1996). Misexpession of the *Hoxa-13* gene in limb mesenchymal cells induces homeotic transformation of the proximal (long) cartilage elements to the more distal (short) elements (Yokouchi *et al.*, 1995). Taken together, these results suggest that the nested expression of Hox genes controls the size and identity of cartilage elements along the PD axis in limbs. In other words, Hox genes seem to mediate the formation of compartments, each of which has its own positional identity, resulting in different shapes of skeletal elements (Yokouchi *et al.*, 1991). In addition, *Meis2*, a homeobox gene, has been identified as a proximal determinant that specifies the most proximal compartment, corresponding to the stylopod region (Capdevila *et al.*, 1999; Mercader *et al.*, 1999; Mercader *et al.*, 2000).

However, it is not clear how these combinations of transcriptional factors mediated by homeobox genes produce the actual skeletal pattern. Moreover, we have to consider the fact that each Hox gene -expressing domain still has further detailed skeletal differences in each region (Fig. 1C, see also discussion for detail). This complicated process may consist of many events, including cell proliferation, migration, differentiation and sequential cell-cell interactions. Previous studies have demonstrated that some cellular properties and

Abbreviations used in this paper: AER, apical ectodermal ridge; PD, proximal-distal; PZ, progress zone.

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nuclear transcriptional environments behave in a stage-specific fashion. Mesenchymal cells of chick limb buds show stage-dependent sorting-out in monolayer culture (Ide *et al.*, 1994; Wada and Ide, 1994) and in recombinant limbs (Wada *et al.*, 1993). These findings suggest that regional difference of cell affinity, which sequentially changes along the PD axis, is involved in the cell-cell interactions for establishing different sizes and shapes of cartilaginous elements. Furthermore, Yokouchi *et al.* (1995) demonstrated that misexpression of the *Hoxa-13* gene in limb mesenchymal cells changes cell affinity and the pattern of cell sorting *in vitro*, suggesting relationships between *Hoxa* gene expressions, cell sorting, and cartilage pattern. Retinoic acid, which is an upstream activator of the proximal determinant, *Meis2* (Mercader *et al.*, 2000), changes the positional identity and affinity of distal cells to the proximal one (Tamura *et al.*, 1997), suggesting relationships between *Meis2* and cell affinity. In our previous study (Yajima *et al.*, 1999), we found that N-cadherin, a member of the classic cadherin family, is a key molecule responsible for the establishment of different cell affinities along the PD axis. We have indirectly demonstrated the role of N-cadherin in cell sorting, by showing that an anti-N-cadherin antibody inhibits the sorting-out of PZ cells in monolayer culture and that some manipulations, including retinoic acid application, inhibit the accumulation of N-cadherin proteins in the PZ. Complementing this indirect evidence, we present here the results of experiments using an adenovirus system for misexpression of N-cadherin that more directly show the function of N-cadherin in cell sorting *in vitro* and cell identity *in vivo*. Our findings suggest that N-cadherin has a molecular nature that provides different degrees of adhesiveness in compartments of limb mesenchymal cells.

Results

Position-Dependent Distribution of N-Cadherin Protein in Limb Bud Mesenchyme

We (Yajima *et al.*, 1999) and others (Hatta *et al.*, 1987; Oberlender and Tuan, 1994a; Oberlender and Tuan, 1994b; Hayashi and Ozawa, 1995) have reported that at least two populations of cells in the developing limb bud express N-cadherin protein: limb distal mesenchymal cells and migrating muscle precursor cells. The presence of N-cadherin protein in the latter population prevents us from comparing distributions of N-cadherin protein in the distal and proximal regions of mesenchyme of the limb bud. For that reason, with focus on the distal and proximal regions of mesenchyme in the chick wing bud, we analyzed the position-dependent N-cadherin distribution quantitatively. After dividing stage 24 limb buds into distal portion, proximal-core region which should not contain myoblasts, and proximal-periphery region (shown in the inset of Fig. 1C), Western blot analysis with anti-N-cadherin monoclonal antibody (NCD2) was performed (Fig. 1B). Figure 2C shows relative amounts of N-cadherin protein, demonstrating that the distal region has nearly 2.5-times greater amount of N-cadherin protein than does the proximal-core region, in which there is almost no invasion of muscle precursors. The amount of N-cadherin protein in the proximal-periphery region was nearly 1. 8-times greater than that in the proximal-core region. Based on these and previously reported findings (Hatta *et al.*, 1987; Oberlender and Tuan, 1994a; Oberlender and Tuan, 1994b; Hayashi and Ozawa, 1995; Yajima *et al.*, 1999), we propose a deduced representation of the stage- and position-dependent N-cadherin protein distribution in limb buds as shown in Fig. 2D. The N-cadherin gene expression profile determined by whole-mount *in situ* hybridization (Fig. 2A, and not shown) supports the stage-dependent N-cadherin expression in the distal mesenchyme.

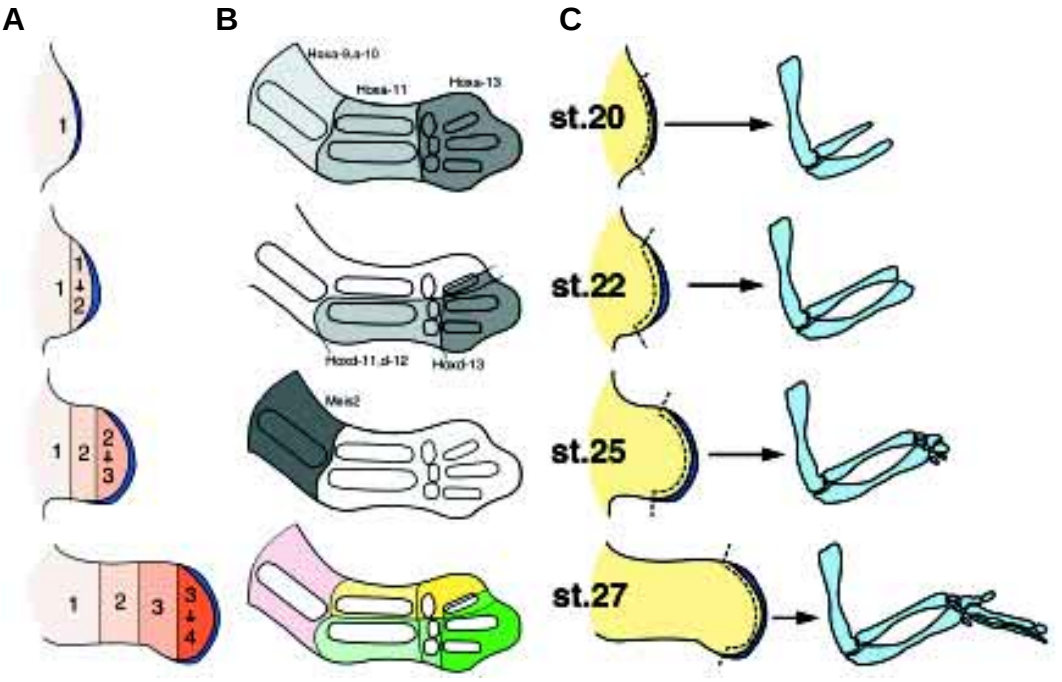


Fig. 1. Limb pattern formation and positional identities. (A) *PZ cells autonomously change their positional identities to make differential proximodistal patterns of the limb as they proliferate under the influence of the AER.* **(B)** *Expression patterns of homeobox genes make significant compartments correlated with skeletal patterning in the limb bud. Almost every compartment correlates with each skeletal element.* **(C)** *AER excision induces truncation of the proximodistal skeletal structure. These truncations do not necessarily correspond to joints or compartments provided by the expression pattern of transcriptional factors.*

Overexpression of N-Cadherin in Limb Bud Mesenchymal Cells

We previously reported that inhibition of N-cadherin by its specific antibody clearly prevent sorting-out of limb mesenchymal cells (Yajima *et al.*, 1999). In order to obtain more direct evidence that N-cadherin plays a role in position-dependent cell sorting, we used a system for overexpression of N-cadherin protein in cultured limb mesencymal cells with an adenovius vector. The adenovirus vector that we used is capable of carrying and driving larger than 2 kb inserts. Furthermore, this vector is advantageous for cell sorting analysis

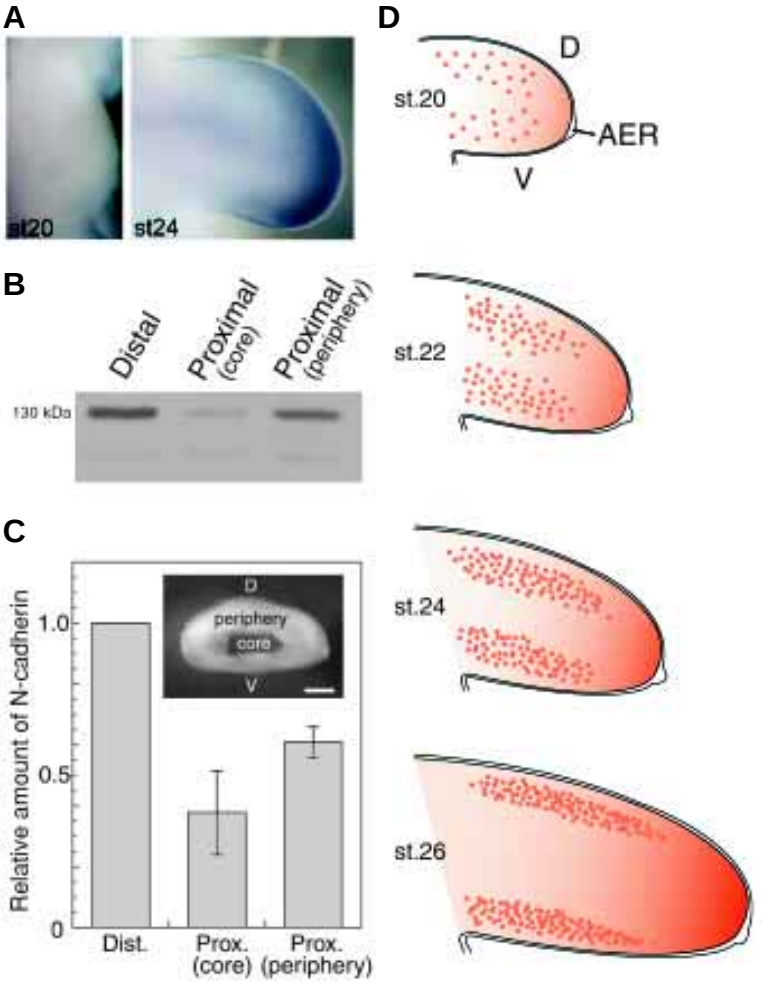
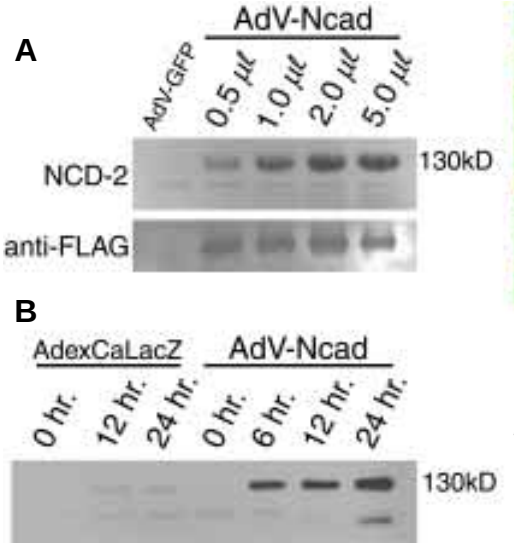


Fig. 2. Distribution of N-cadherin along the proximodistal axis in the limb bud. (A) *Localization of* N-cadherin *mRNA in distal mesenchyme by whole-mount* in situ *hybridization.* **(B)** *Immunoblot detection of N-cadherin protein from lysate of various regions of stage 24 wing buds (shown in the inset of C; scale bar, 200 μm). Each lane contains 30 μg of extracted total protein. NCD-2 detected a 130 kDa N-cadherin band. Lower bands are probably degradation products.* **(C)** *Densitometric analysis of 130 kDa bands in (B). Relative level of N-cadhern protein was calculated by defining the amount in distal mesenchyme as 1.0. Bars represent averages +/- SE (N=3). Scale bar, 200 μm.* **(D)** *Schematic representation of N-cadherin distribution in the developing limb. Myoblasts have significant amount of N-cadherin protein (shown as dots), and the gradient of N-cadherin should be in the mesenchyme along the proximodistal axis.*

because the virus is replication-incompetent and we can mix infected and uninfected cells in a culture dish and recognize them. Using an adenovirus construct containing N-cadherin with a C-terminal FLAG-tag (AdV-Ncad), we infected dissociated stage 20 chick limb mesenchymal cells and cultured them for 24 hours. GFP-adenovirus (AdV-GFP) and LacZ-adenovirus (AdexCaLacZ) were used for controls. Western blot analysis with NCD-2 revealed that the relative amount of N-cadherin protein in cultured cells increased as the amount of introduced AdV-Ncad was increased (Fig. 3A, top row). The detection by anti-FLAG antibody suggests that increased N-cadherin is not a secondary by-product after infection but is derived from the introduced virus (Fig. 3A, bottom row). In the infected and cultured cells, an obvious increase in the amount of N-cadherin protein was observed as early as 6 hours after infection, and the amount of N-cadherin protein continued to increase for up to 24 hours after infection (Fig. 3B). The ectopic N-cadherin was detectable with anti-FLAG antibody on the cell surface (Fig. 3C, and its insert). Figure 3D shows that AdV-GFP, a control virus, was indeed infected with a high frequency. X-gal staining confirmed that another control virus, AdexCaLacZ, was also fully infected (not shown). These results demonstrate that these adenovirus vectors are functional for an *in vitro* culture system of the chick limb bud.

Grafting of N-Cadherin-Overexpressing Cells into the Limb Bud

To examine whether AdV-Ncad-infected cells can participate in normal cartilage formation of the limb bud, a small lump of infected stage 20 PZ cells simultaneously labeled with PKH-26 was grafted into the distal top of a stage 20 forelimb bud. AdexCaLacZ-infected



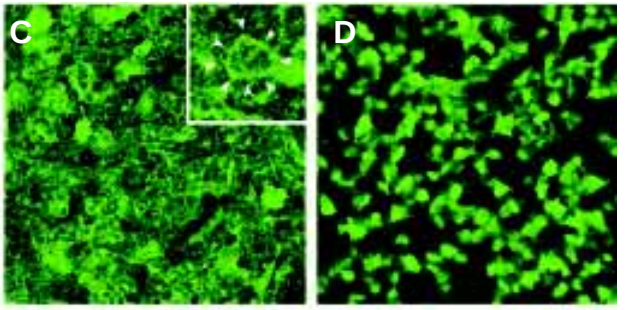


Fig. 3. Misexpression of N-cadherin with an adenovirus vector. (A) *Immunoblot detection of ectopic N-cadherin protein. Stage 20 PZ cells were infected with AdV-GFP as a control or various concentrations of AdV-Ncad and cultured. NCD-2 shows an increase in the amount of ectopic N-cadherin, according to the concentration of AdV-Ncad. Anti-FLAG antibody shows that those N-cadherin proteins were derived from AdV-Ncad.* **(B)** *Temporal profile of misexpression. Stage 20 PZ cells were infected with AdexCaLacZ or AdV-Ncad and cultured. Samples were harvested after various periods of culture and detected by immunoblot analysis with NCD-2.* **(C)** *Localization of ectopic N-cadherin in AdV- Ncad-infected and cultured stage 20 PZ cells was detected with anti-FLAG antibody. Distribution of FLAG-epitope was found on the cell surface. Arrowheads show outline of infected cells at higher magnificaton (inset). For comparison, GFP- AdV-infected cells are shown in* **(D)**.

cells contributed to normal cartilage formation in the host limb bud (Fig. 4 A-C, n=7/7). Grafted cells were distributed from the middle of the zeugopod to the distal apex as a belt, as were non-infected cells (not shown, see Tamura *et al.*, 1997; Mercader *et al.*, 2000). In a similar way, AdV-Ncad-infected cells participated in normal cartilage formation of the host limb (Fig. 4 D-F; n=13/13). Interestingly, in some cases AdV-Ncad-infected cells were distributed only in the autopod region, distal from the wrist level, as if the grafted tissue had been pushed out distally (arrowheads in Fig. 4 E,F; n=11/13), whereas AdexCaLacZ-infected cells were always scattered from the middle of the zeugopod region (arrowheads in Fig. 4 B,C).

Influence of N-Cadherin Overexpression on In Vitro *Cell Sorting*

We next tested whether AdV-Ncad-infected cells show different cell affinity than that of non infected cells. AdexCaLacZ-infected cells (from stage 22 PZ of chick limb bud) were not sorted out from non infected cells (from stage 22 PZ of quail limb bud) and were distributed almost uniformly (Fig. 5A). In comparison, low multiplicity of AdV-Ncad infection did not affect the cell sorting (Fig. 5B, compare with 5A), but as multiplicity of infection increased, infected and non infected cells were sorted out from each other, resulting in the formation of clusters of similar sizes (Fig. 5 C,D).

Although these results suggest that N-cadherin is involved in stage-dependent cell sorting, it is still possible that the cell sorting observed in culture is due to increased adhesiveness as a result of N-cadherin overexpression and has nothing to do with the position-dependent cell affinity in the normal limb bud. To eliminate this possibility and to confirm that N-cadherin-overexpressing cells changed their cell surface affinity from the property of early stage (proximal) to a late one (distal), we mixed the infected cells with different stages of uninfected cells and observed their sorting ability. Interestingly, N-cadherin-overexpressing stage 22 PZ cells were separated from uninfected stage 22 PZ cells (Fig. 6A see also Fig. 5C), but the sorting-out was less in the case with stage 25 PZ cells (Fig. 6B). Infected cells mixed almost uniformly with stage 27 PZ cells, while small clusters of infected cells were still visible (Fig. 6C). These results strongly suggest that N-cadherin-overexpressing stage 22 PZ cells changed their cell surface affinity to a much later one (approximately to stage 27).

Discussion

Differential Cell Affinity and Distribution of N-Cadherin

Cell adhesiveness, which allows cells to bind to each other and to attach to the extracellular matrix, is a key cellular property involved in morphogenesis during development. In particular, direct cell-to-cell interactions mediated by cell adhesion molecules such as cadherin and CAM give rise to change in the forms of cells, tissues, and organs, resulting in a specific shape of each embryonic structure. During later stages of limb development, N-cadherin protein is known to accumulate in the perichondrium (but not in mature cartilage) and to play some roles in chondrogenesis (Oberlender and Tuan, 1994a; Oberlender and Tuan, 1994b; Packer *et al.*, 1997). Inhibition of N-cadherin function by its specific neutralizing antibody resulted in a severe defect of cartilage formation both *in vitro* and *in vivo* (Oberlender and Tuan, 1994a). Rather than the later phase of N-cadherin functions in limb development, we propose here that the early phase of N-cadherin functions is mediation of region-specific cell affinity along the PD axis. As far as only limb mesenchymal cells in early stages are concerned, N-cadherin accumulates in the distal region as shown in Fig. 2C, and it was estimated on the basis of our previous observation by immnoblotting (Yajima *et al.*, 1999) that the amount of N-cadherin proteins in stage 26 PZ is nearly fifteen-times greater than that in stage 20 PZ in the chick limb bud. Stage-dependent distribution of N-cadherin protein is also evident by N-cadherin gene expression (Fig. 2A). Our evaluation of the distribution of N-cadherin protein, as shown in Fig. 2D, implies region- and stage-specific roles of N-cadherin in limb patterning.

Stage specificity of PZ cells appears in segregation of cells when PZ cells derived from different stages are mixed. Similar segregation can be observed also in recombinant *Xenopus* limb mesenchyme (Koibuchi and Tochinai, 1999). Regenerat-

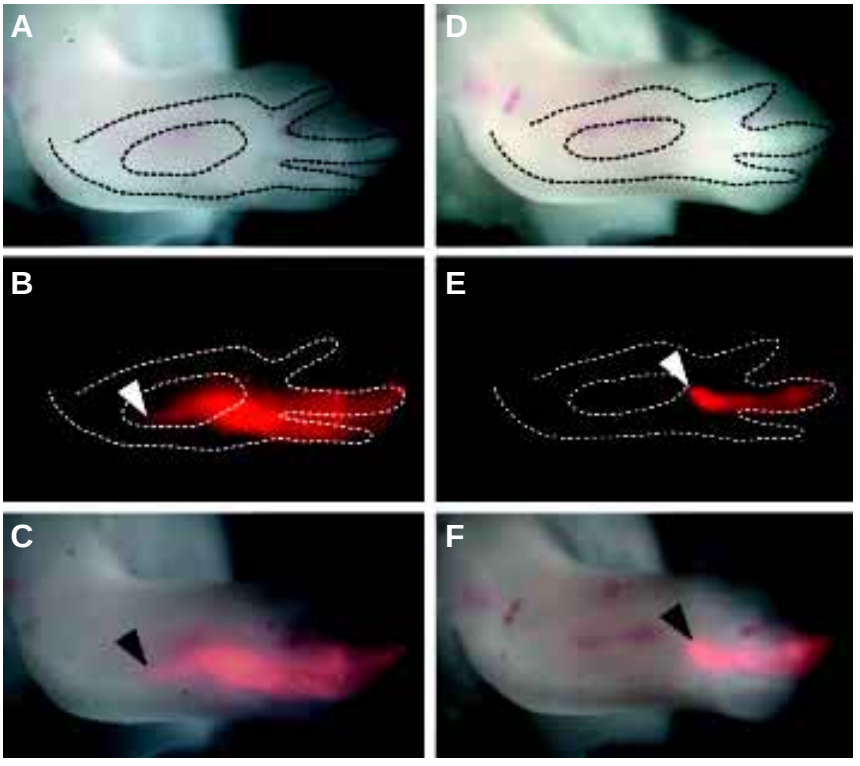


Fig. 4. *In vivo* assay. *Changes in PZ cell affinity by misexpression of N-cadherin* in vivo. *AdexCaLacZ-infected (A-C) or AdV-Ncad-infected (D-F) stage 20 PZ cells were grafted under the AER of a stage 20 wing bud. Dorsal view of the operated limb bud 3 days after the explantation.* **(A,D)** *Bright images.* **(B,E)** *Epifluorescent image shows the distribution of fluorescent dye-labeled PZ cells.* **(C)** *is composed of A and B and* **(F)** *is composed of D and E. Dotted line shows the outline of the pre-cartilaginous region. Arrowheads show the proximal level of infected cells. PZ cells with ectopic N-cadherin were pushed out distally.*

ing limb blastema shows a graded difference in cell surface properties as well. When two blastemas from the wrist and upper arm level are combined and cultured in contact, the distal mesenchyme is engulfed by the proximal one (Nardi and Stocum, 1983), suggesting that the distal property has a higher degree of adhesiveness. Distal displacement of distal-derived blastema implanted into a more-proximal level (Crawford and Stocum, 1988) is also suggestive of the graded difference in cell adhesiveness. Sorting out in cultured mesenchymal cells of the chick limb bud (Ide *et al.*, 1993; Wada and Ide, 1994; Ide *et al.,* 1997) clearly demonstrates that the PZ of each developmental stage has its own cell surface property. The nested expression pattern of Hoxa genes represents a feature of positional identity that provides compartments along the PD axis. However, in addition to this scenario, we must consider more features of positional identity to explain the fact that each of three compartments (stylopod, zeugopod, and autopod) consists of complex components: a positional identity should have a gradual and sequential aspect. Excision of the AER from an early limb bud results in a severe distal truncation (see Fig. 1C). The earlier the stage at which the embryo is operated on, the more severe is the deficiency from proximal parts that the resulting limb has (Saunders, 1948; Summerbell, 1974; Summerbell and Lewis, 1975). The positional identity is thought to stop at the time when the AER is removed, and this causes a lack of a more distal part that ought to be made as more distal identity. Appropriate timing of AER removal results in truncation of two thirds, half, or one third of the distal part of each element (or compartment). The property of positional identity seems to progress gradually and consecutively even in a compartment. The results of cell-sorting experiments using cells derived from different PD levels of the limb bud supports this idea. PZ cells at stage 24 and stage 26 are sorted out from each other (Wada and Ide, 1994), suggesting that they have different cell surface properties, whereas they share the same expression pattern of Hoxa genes and give rise to a part of the autopod structure.

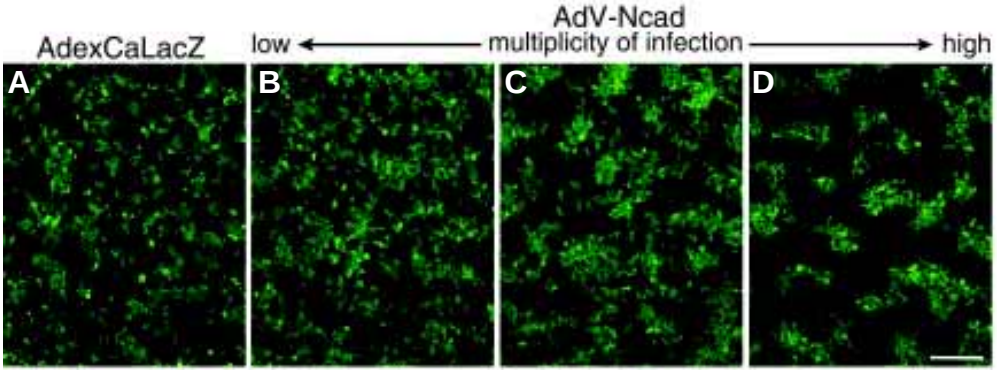


Fig. 5. *In vitro* assay. *Changes in PZ cell affinity by misexpression of N-cadherin* in vitro. *AdexCaLacZ-infected (A) or AdV-Ncad-infected (B-D) stage 22 PZ cells (chick) were mixed with non-infected stage 22 PZ cells (quail) and cultured. The distribution of chick cells was detected with A223, a chick-specific monoclonal antibody (Ide* et al., *1994).* **(A)** *Infection by the a denovirus itself should not induce change in cell affinities and does not affect cell sorting.* **(B-D)** *Misexpression of N-cadherin caused a change in cell sorting as an amount of its proteins. Scale bar, 200 μm.*

We have demonstrated that not only does N-cadherin protein have a temporally and spatially restricted expression pattern but also that it is likely to play a role in making and/or maintaining different positional identities along the PD axis. A large amount of N-cadherin protein overexpressed by an adenovirus vector changed the state of affairs of cell affinity, causing sorting out from cells originating from the same stage. Moreover, these N-cadherin-misexpressing cells are in harmony with later PZ cells, suggesting that those N-cadherin-rich cells a obtained more-distal property. Taken together with the results showing that those N-cadherin-rich cells tend to be pushed out distally on the host limb bud (Fig. 4F), our findings suggest that more amount of N-cadherin protein moves the cell surface property forward from the early character to the later one. We previously demonstrated that inhibition of N-cadherin function by its specific antibody prevents sorting-out of limb mesenchymal cells (Yajima *et al.*, 1999). Thus, a regulated amount of N-cadherin appears to modulate sorting-out of limb mesenchymal cells, and N-cadherin is thought to be an important key player in the emergence of stage-dependent PZ cell propertyes during normal limb development. This idea is supported by the results of some *in vivo* experiments. For example, the AER excision prevents accumulation of N-cadherin proteins (Yajima *et al.*, 1999), suggesting that the accumulation depends on the AER function. Retinoic acid treatment, which proximalizes the PZ cell property (Tamura *et al.*, 1997), also prevents the accumulation of N-cadherin (Yajima *et al.*, 1999). Retinoic acid and FGF competitively control the PD axis through regulation of *Meis* gene, a proximal determinat (Mercader *et al.*, 2000). Endogenous retinoic acid signaling is required to maintain the proximal *Meis* expression in the

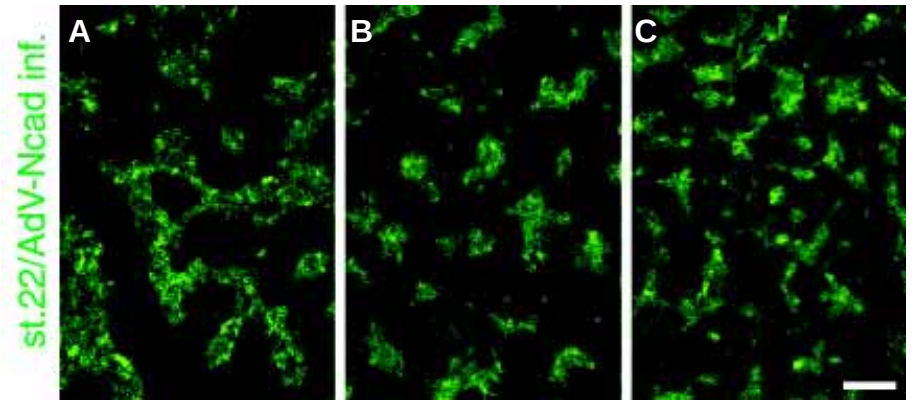


Fig. 6. *In vitro* assay. *Correspondence of ectopic N-cadherin-derived cell affinity with endogenous one. AdV-Ncad-infected stage 22 PZ cells were mixed with stage 22* **(A)**, *25* **(B)** *and 27* **(C)** *PZ cells and cultured. The distribution of infected cells was detected with anti-FLAG antibody. The affinities of N-cadherin-misexpressing cells might be similar to those of later PZ cells. Scale bar, 200 μm.*

limb, and retinoic acid application in the distal region activates *Meis* expression ectopically. FGF, an AER factor, has the opposite effect on *Meis* expression. Thus, down-regulation of N-cadherin by AER excision and retinoic acid might be related to the *Meis* regulation by FGF and retinoic acid. The stage-dependent and gradual accumulation of N-cadherin protein in the distal region corresponds with the positional identity discussed above.

PZ Theory, Positional Identity and Cell Affinity

Based on our results and results of others, we propose a model that outlines the cellular properties that give rise to positional identity in the proximal-distal axis of the limb skeletal pattern (Fig. 7). We can divide the cellular properties into two phases. One is the transcriptional environment that makes compartments and gives identity to each compartment. This includes specific expression patterns of Hoxa, Meis, and other genes. The other is cell affinity that is settled by graded accumulation of N-cadherin protein. The sequential difference of cell adhesiveness brings about distinction of shape and number of cartilage elements along the proximal-distal axis. This, however, does not exclude the contribution of other cell surface molecules. Other cadherin family members have been reported to be expressed region-specifically in the developing limb bud. Cadherin-11, which belongs to the classic cadherin superfamily, is expressed in the PZ of the mouse limb bud, and cadherin-11-positive cells can sort out from negative ones *in vitro* (Kimura *et al.*, 1995). This cadherin has been suggested to contribute to the prevention of undifferentiated limb me senchymal cells from mixing with other cell populations (Kimura *et al.*, 1995). In addition, quantitative and qualitative changes in cell adhesiveness may be involved in the second phase of this model. Wada *et al.* (1998) suggested that glycosylphosphatidylinositol (GPI)-anchored proteins and EphA4, as a candidate of receptors for GPI-anchored protein, play important roles in cell sorting in the limb mesenchyme. Recently, Stadler *et al.* (2001) showed that limb mesenchymal cells that lack functional Hoxa13 protein reduce Eph expressions and change cell affinities. The results of these studies suggest that Hoxa13 directly or indirectly controls cellular properties causing positional identities. It is well known that Eph/ephrin interactions contribute to cell-cell repulsion or attraction in neural development (O'Leary and Wilkinson, 1999; Wilkinson, 2000). In limb development, this mechanism could be important role in giving positional identities to the developing limb mesenchyme or in maintaining these positional identities.

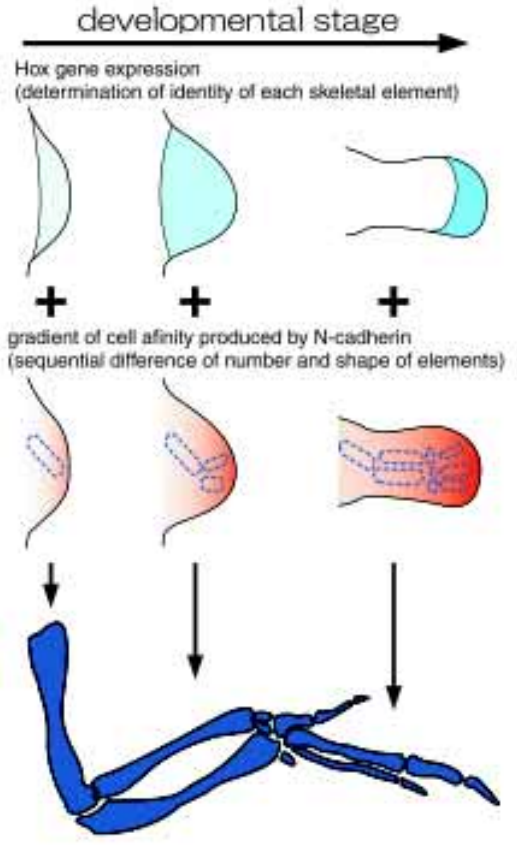


Fig. 7. Hypothesis regarding the formation of the proximodistal skeletal structure. *In limb development, the compartments that are specified by the expression pattern of transcriptional factors, such as* Hox *genes, give identities to each skeletal element and position of junctions. The gradient of cellular adhesiveness along the proximodistal axis, caused by different amounts of N-cadherin, may generate a precartilaginous pattern that gives rise to sequential and global differences in the number and shape of skeletal elements. As a consequence, we find that the proximal region has a few large elements and that the distal region has many small elements divided with joints.*

It is still not clear how the N-cadherin distribution is regulated and what molecules are involved in this pathway. Overexpression of Hoxa13 results in change of cell affinity and distalization in cell identity (Yokouchi *et al.*, 1995). However, our preliminary data revealed that misexpression of Hoxa13 does not change the level of N-cadherin protein in limb mesenchymal cells (Y.H. and K. T. unpublished observation, in preparation), implying that Hoxa13 may regulate the positional identity through a different pathway (e.g. Eph receptor) from N-cadherin. A recent study by Miura and Shiota (2000) has demonstrated that TGF-β2 promotes the expression of N-cadherin protein in the mouse limb mesenchyme and suggested that TGF-β2 is possibly responsible for the cell-sorting phenomenon and plays an activator-like role in the reaction-diffusion model during chondrogenic pattern formation. This supports the possibility that TGF-β2 provides the precartilage pattern via N-cadherin in limb development.

The generation of a chimeric embryo composed of wild-type and N-cadherin-deficient descendent cells by N-cadherin-deficient embryonic stem cells results in the segregation of the mutant cells from wild-type cells in some tissues such as somites, neural tube, and brain (Kostetskii *et al.*, 2001). N-cadherin not only contributes to the recognition of different tissue s but also may supply cells with a capability to distinguish their identity in a morphogenetic field during development. In order to know the detailed effects of this important adhesion molecule, it will be crucial to precisely control the amount and timing of N-cadherin expression.

Material and Methods

Western Blot Analysis

Fertilized chicken eggs were incubated at 38°C, and the embryos were staged according to Hamburger and Hamilton (1951).

Exact tissues were dissected from wing buds, homogenized, and dissolved in a sample buffer, 60 mM Tris-HCl (pH 6.8), containing 0.9 mM Ca2+, 0.33 mM Mg2+ and 2 % sodium dodecyl sulfate (SDS). Cultured cells were washed with phosphate-buffered saline (PBS) and dissolved in the

same sample buffer. The loaded sample was 30 μg per lane, and electrophoresis was carried out with a 7.5 % polyacrylamide SDS gel. The separated proteins were electrophoretically transferred from the gel to a polyvinylidene membrane (Immobilon-P, Millipore). The blotted membrane was blocked with 5 % skim milk in PBS and incubated with rat-monoclonal-anti-N-cadherin antibody (NCD-2 culture supernatant; NCD-2 hybridoma was donated by Dr. M. Takeichi; Hatta and Takeichi, 1986) or rabbit-anti-FLAG antibody (1:750, anti-Octapeptide Epitope Tag, Zymed) at 4°C overnight. After washing in PBS, the samples were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000, rabbit-anti-rat IgG, Zymed; goat-anti-rabbit IgG, Chemicon) for 2 hours at room temperature. After washing, the membranes were incubated with enhanced chemiluminescence (ECL) detection reagents (Amersham) to recognize the signals as chemiluminescence. Quantification of the signals was carried out using a Molecular Imager System (GS-525, Bio Rad) and its software, Molecular Analyst (Bio Rad).

Preparation of Adenoviruses

AdV-Ncad, an adenovirus expressing chicken N-cadherin with FLAG epitope at the C-terminus (Nakagawa and Takeichi, 1998), was a kind gift from Dr. S. Nakagawa at Kyoto University. AdV-GFP and AdexCaLacZ were kindly given by Dr. J.C. Izpusia-Belmonte at Salk Institute (Tavares *et al*., 2000) and by Dr. Saito and Dr. Kanegae at Tokyo University. These recombinant adenoviruses were amplified with 293 cells and purified by $CsCl_2$ step-gradient centrifugation (Kanegae *et al*., 1994).

Cell Culture and Infection of Adenovirus

For cell cultures, distal tips (within 150-200 μm from the AER) were dissected from chick or quail wing buds at various stages, and mesenchymal cells were isolated by methods previously described (Hattori and Ide, 1984; Aono and Ide, 1988). The cells were cultured at the density of $8.8x10^5$ cells/cm^2 in Ham's F12 medium containing 1 % fetal calf serum (F12-1%FCS).

To infect cells with adenovirus, dissociated cells were incubated in F12-1%FCS containing an adenovirus for 1 hour at 38°C. This period was sufficiently long for all cells to be infected (data not shown). The cells were washed with a fresh medium several times to remove non-absorbed virus and used in the following assays.

Cell Sorting and In Vivo *Assay*

For *in vitro* assays, infected cells were mixed with uninfected cells at the ratio of 1:2 to 2:3 and plated on a small area (6 mm in diameter) of a culture dish with the aid of a stainless column, as reported previously (Hattori and Ide, 1984; Aono and Ide, 1988; Ide *et al*., 1994; Wada and Ide, 1994). At 24 hours after the plating, the distributions of infected cells were visualized by immunohistochemistry (see below).

For *in vivo* assays, infected cells were labeled with $4x10^{-6}$ M PKH-26 (Zynaxis) and pelleted by 400Xg centrifugation in F12-10 % FCS. After incubation for 1 hour at 38°C, the pellets were cut into cubes of about 100x200x200 μm in size and inserted into a slit under the wing bud AER. Embryos were allowed to develop further for 3 days, and the grafted cells were detected under an epifluorecence stereomicroscope (Olympus).

Immunohistochemistry and Whole-Mount In Situ *Hybridization*

The cultures were fixed in 4% paraformaldehyde-PBS fixative for 30 min at 4°C, blocked with 0.5 % skim milk-PBS, and incubated with A223, a mouse-monoclonal antibody that specifically recognizes chicken cells (Ide *et al*., 1994), or with rabbit-anti-FLAG antibody (1:250) at 4°C overnight. When using anti-FLAG antibody, samples were treated with 2 N HCl for 20 min at room temperature before blocking. After several washes with PBS, cultures were incubated with goat-anti-mouse IgG conjugated with FITC (1:60, Tago Immunologicals) or goat-anti-rabbit IgG conjugated with FITC (1:100, Jackson ImmunoResearch) for 2 hours at room temperature. Cultures were washed with PBS and sealed with glycerol containing 0.1 % *p*-phenylenediamine and then observed under a laser confocal scanning microscope (Fluoview, Olympus).

Whole-mount *in situ* hybridization was performed as described before (Yonei *et al*., 1995). An antisense RNA probe was synthesized from plasmid containing a 473-bp fragment of chick N-cadherin (X07277, Hatta *et al*., 1988).

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