

Self-organization of periodic patterns by dissociated feather mesenchymal cells and the regulation of size, number and spacing of primordia

Ting-Xin Jiang^{1,‡}, Han-Sung Jung^{2,*‡}, Randall B. Widelitz¹ and Cheng-Ming Chuong^{1,§}

¹Department of Pathology, School of Medicine, University of Southern California, 2011 Zonal Avenue, Los Angeles, CA 90033, USA

²Department of Anatomy and Developmental Biology, University College London, Gower Street, London, UK

*Present address: Developmental Biology Programme, Institute of Biotechnology, Biocentre 1, PO Box 56, FIN-00014, University of Helsinki, Finland

‡These two authors made an equal contribution to the paper

§Author for correspondence (e-mail: chuong@pathfinder.hsc.usc.edu)

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SUMMARY

Periodic patterning is a fundamental organizing process in biology. Using a feather reconstitution assay, we traced back to the initial stage of the patterning process. Cells started from an equivalent state and self-organized into a periodic pattern without previous cues or sequential propagation. When different numbers of dissociated mesenchymal cells were confronted with a piece of same-sized epithelium, the size of feather primordia remained constant, not the number or interbud spacing, suggesting size determination is intrinsic to dissociated cells. Increasing bone morphogenetic protein (BMP) receptor expression in mesenchymal cells decreased the size of

primordia while antagonizing the BMP pathway with Noggin increased the size of primordia. A threshold number of mesenchymal cells with a basal level of adhesion molecules such as NCAM were sufficient to trigger the patterning process. The process is best visualized by the progressive restriction of β -catenin transcripts in the epidermis. Therefore, feather size, number and spacing are modulated through the available morphogen ligands and receptors in the system.

Key words: Pattern formation, Feather, Size regulation, BMP, β -catenin

INTRODUCTION

In animals and plants, elements are often repeated at relatively regular intervals. This constitutes a periodic pattern (Held, 1992) and can be seen in somites/vertebrae (Gossler and Hrabe de Angelis, 1998; Tajbakhsh and Sporle, 1998), limb skeletal patterns (Cohn and Tickle, 1996; Johnson and Tabin, 1997), teeth (Theslef and Pispas, 1998) zebra stripes (Bard, 1981), etc. How are the location, number and size of these repetitive elements determined? Through tissue interactions, cells are distinguished from their neighbors and set on an altered developmental track. Groups of cells are selected at intervals to form a repeated pattern.

Many models for periodic patterning have been proposed to address this fundamental process in biological development (Turing, 1952; Wolpert, 1971; Newman and Frisch, 1979; Steinberg and Poole, 1981; Held, 1992; Newman and Comper, 1990; Steinberg and Takeichi, 1994). Inductive models suggest that substances, appearing in the right place and at the right time, induce each element. In these models, the environment or position of a cell, interpreted through cues such as molecular gradients, cause each cell to adopt a particular determination state. Other proposals suggest that cells with different properties

such as differential adhesiveness can sort themselves out into specific organizations. These models rely heavily on predetermined differences in cells or the environment to generate periodic patterns. Still other models suggest that cells have self-organizing abilities. Cells start from an equivalent state from which periodic patterns gradually emerge and sharpen (Newman and Comper, 1990; Kopan and Turner, 1996). Since all cells have equivalent opportunities to contribute to either the element itself or interelement spacing, these models have a probabilistic nature. However, since the location of cells with respect to the forming pattern also influences their cell fate, there may also be a deterministic nature to the process. Within this class of models, the reaction-diffusion mechanism proposes that random stochastic fluctuations are amplified into peaks and troughs by means of chemicals or mechanical forces that propagate at different rates. In non-living chemical models, a reaction-diffusion mechanism has been shown to form stripes and dots from a uniform state (Ouyang and Swinney, 1991; Lee et al., 1994; Dulos et al., 1996). In biological patterning, it was suggested that cells can be distinguished from adjacent cells with a similar mechanism by interpreting the concentrations of morphogens, which can be activators or inhibitors, or mechanical differences (Oster et al., 1983; Nagorcka and

Mooney, 1985; Tsonis et al., 1989; Koch and Meinhardt, 1994). Finally, selective mechanisms suggest the initial formation of redundant elements, with the final pattern resulting from competition and survival. For example, the formation of a functional neural circuit (Edelman, 1987, 1992) and the formation of periodicity of the ocular dominance column are modulated by neural activity and mediated by synaptic strength (Miller et al., 1989; Goodhill and Lowel, 1995).

Feather patterning has been a major model for the analysis of pattern formation because it provides a distinct two-dimensional layout and accessibility for experimental manipulation (Sengel, 1976; Chuong et al., 1996; Widelitz et al., 1997; Chuong and Widelitz, 1998). In vivo, feather development occurs sequentially across the tract (the region with feathers) from the midline to the flanking regions (Stuart and Moscona, 1967; Sengel, 1975; Mayerson and Fallon, 1985). Morphogenetic stripes propagating from the mid-dorsal line toward the lateral edges of the feather tract field (a region competent to form many individual feather primordia) was proposed to account for the progressive and orderly appearance of feather buds (Davidson, 1983a,b). It was suggested that the previous feather row is required for the formation of the next (lateral) row and models based on propagation were proposed (Cruywagen et al., 1992).

The formation of skin appendages requires the interaction of epithelium and mesenchyme (Saunders, 1958; Dhouailly, 1984; Chuong, 1993, 1998). The mesenchyme determines the number, size, location and structural identity of the appendage, while the epithelium determines the orientation and competence state (Novel, 1973; Chuong et al., 1996). However, the molecular basis of the signals remains largely unknown. Using in situ hybridization and ectopic expression, we and others showed that activation of the FGF and SHH pathways favor the formation of feather primordia, while activation of the BMP pathway favors the formation of interbud regions (Widelitz et al., 1996; Song et al., 1996; Ting-Berretth and Chuong, 1996; Jung et al., 1998; Morgan et al., 1998; Noramly and Morgan, 1998). SHH appears in discrete regions in accordance with the hexagonal feather pattern. We have continued to search for upstream molecules and found that another group of molecules such as Wnt-7a (Widelitz et al., 1999) showed a homogeneous expression pattern, which then progressively became restricted to locations where the feather primordia formed. This suggested that we should focus on the morphogenetic zone to understand the initial events of feather induction and periodic patterning.

Here we have developed an in vitro reconstitution system (Widelitz et al., 1999) in which all mesenchymal cells are reset to an equivalent state and have the same probability to become primordia or interprimordia. Using this model, we were able to analyze the periodic patterning process further and to manipulate several variables that can alter the primordial size and spacing.

MATERIALS AND METHODS

Materials

Pathogen-free chicken embryos were obtained from SPAFAS, Preston, CT and staged according to Hamburger and Hamilton (1951).

RCAS-BMP receptor 1a (BRK-1) and 1b (BRK-2) were kindly provided by Dr Nohno (Kawakami et al., 1996). RCAS-Noggin was from Dr Johnson (Capdevila and Johnson, 1998). RCAS-BMP2 was from Dr Francis-West.

Reconstituted feather explants

Reconstitution of feather buds using intact epithelium and dissociated mesenchyme were made from stage 29-35 developing skin. Dorsal skins containing the spinal tract were incubated at 4°C in 2% trypsin for 15-20 minutes and then washed in medium containing 10% fetal calf serum. Under a dissection microscope, the epithelium and the mesenchyme were separated using a fine needle. The epithelia were trimmed to be of equal size using graph paper beneath the transparent dish as a guide. The epithelia stayed intact in the media. The mesenchyme was pooled and gently triturated to single cells by drawing them through pipettes with decreasing bores. Cells were filtered through nitex netting when necessary. The viability of cells and completion of dissociation were checked microscopically with Trypan blue inclusion. The dissociated cells were counted, repelleted by mild centrifugation (6,500 revs/minute for 4 minutes) and allowed to reaggregate at specific cell densities for 1 hour at 37°C on culture insert dishes (Falcon). The epithelium was then placed on top of the mesenchyme and the reconstituted explants were cultured. At designated times, explants were observed and photographed.

For RCAS transduction, retrovirus was prepared as described (Morgan and Fekete, 1996). The dissociated mesenchymal cells and intact epithelium were incubated at 4°C for 2 hours with retroviral-containing medium. The cells were then pelleted, reaggregated and used to form reconstituted explants as described above. The reconstituted explants were then cultured in the presence of retroviral-containing medium. This procedure was used to analyze the function of Wnt-7a in feather formation (Widelitz et al., 1999).

DiI labeling

The lipophilic dye, DiI (Molecular Probes, UK; 2 µg/ml in dimethyl formamide), was used in skin as described (Chuong et al., 1996). DiI was injected into either epidermal placodes or dermal condensations in vitro at required stages. Label was retained by cells in either the epithelium or mesenchyme following separation. The mesenchyme was then dissociated to a single cell suspension, reassociated and recombined with intact epithelium. At different times, the explants were examined under epifluorescence microscopy to visualize the labeled cells.

We have noted a decrease in the levels of DiI over time, particularly in the reconstituted explants. Some unbound dye was lost after a day in culture. Since the formation of feather buds is dependent on interactions between epithelium and mesenchyme, some mesenchymal cells not overlain with epithelium will simply become fibroblasts in the reconstituted cultures and will be lost to the feather-forming assay.

In situ hybridization and immunostaining

In situ hybridization was carried out as described (Jiang et al., 1998). Probes to *FGF4*, *Wnt-7a*, *Shh*, *BMP2* and *BMP4* were kindly provided by Drs G. Martin, T. Brown and C. Tabin. β-catenin is from this laboratory (Lu et al., 1997). Whole-mount antibody staining was carried out according to Widelitz et al. (1997) using anti-NCAM Fab' (Chuong and Edelman, 1985).

Quantitative measurement of feather primordia in reconstituted explant

The contours of feather primordia were traced and analyzed using the Mocha image analysis program (Jandel Scientific, San Rafael, CA). The average bud size (representing over 100 primordia per data point in Figs 4, 5) and standard deviation were determined. The total size of the explant was also determined. Bud density was obtained by dividing the number of feather primordia by the total explant area.

Total bud area represents the average bud size times the total bud number. Interbud size was determined as the total explant area minus the total bud area.

For the pseudocolor images, image intensity levels were fractionated into 5 equal-sized groups. Each group was assigned colors representing high to low intensity, from red (high), to orange, yellow, green and blue (low).

RESULTS

Dissociated dermal mesenchymal cells can reform periodic patterns in reconstituted explants

The different models for periodic patterning may have arisen because investigators examined different stages of the patterning process, the models describing different steps along the patterning process. Therefore, we wished to analyze the initial event, so that all cells start from equivalent states and have the same probability of becoming the periodically arranged element or non-element. We have previously dissociated epithelium and mesenchyme and recombined them for culture. In this case, epithelia were completely reset and different areas of the epithelia became either placode or interplacode. However, the mesenchyme was not reset in this procedure as the location of newly formed feather buds was set according to the sites of the previous dermal condensations (Novel, 1993; Chuong et al., 1996). Earlier work used dissociated cells to reform feather and hair follicles (Moscona and Moscona, 1965; Lichti et al., 1995), although cellular aggregates were formed making it impossible to address spacing issues. Also, since later-staged cells were used, the findings were a demonstration of cell sorting (Steinberg and Takeichi, 1994), rather than induction.

We have utilized different *in vitro* culture systems to study the initial events of periodic feather patterning. Here we developed a three-dimensional culture system that permits detailed and direct assessment of the interactions involved in periodic feather patterning. Dissociated mesenchymal cells from H&H (Hamburger and Hamilton, 1951) stage 30 chicken dorsal skin were plated at high cell density and then overlain with an intact piece of epithelium (Fig. 1A-C). At first, the explants appeared homogeneous (Fig. 1D). At day 1, dermal condensations with even spacing formed (Fig. 1E). At 2 days, morphologically normal feather buds of similar size to the control formed and were arranged in hexagonal patterns similar to that of intact skin (Fig. 1F). At 5 days, these buds grew to form long feather buds (Fig. 1G). These buds expressed normal molecular markers such as SHH and continued to grow to form barb ridges and follicular structures (H.-S. J. et al., unpublished data). The formation was epithelium-dependent, since the mesenchymal

cells alone did not form any buds and eventually became chicken embryo fibroblasts (Fig. 1H).

All feather primordia appeared simultaneously and all mesenchymal cells had an equal probability to become primordia or interprimordia in this reconstituted explant

Surprisingly, all feather primordia appeared simultaneously in the reconstituted explant cultures. This led us to question whether the sequential propagation from the primary row, as

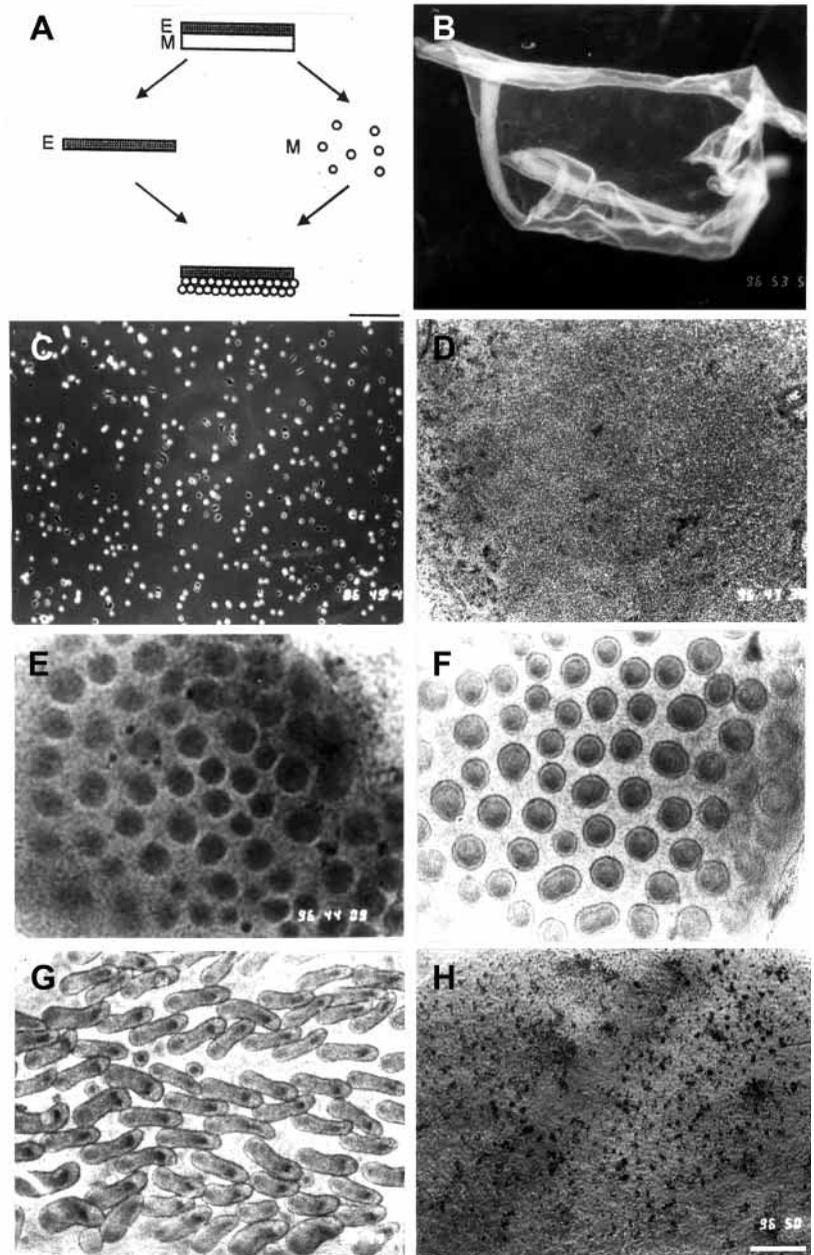


Fig. 1. Reconstitution of the feather spacing pattern from dissociated mesenchymal cells. (A) Schematic diagram depicting reconstituted feather buds with intact epithelium (E) and dissociated mesenchymal (M) cells. (B) Intact epithelium. (C) Dissociated mesenchymal cells. Reconstituted explants shown (D) immediately, (E) 1 day, (F) 2 days and (G) 5 days after reconstitution. Note, feather bud orientation was determined by the epithelium. (H) Mesenchyme cultured without epithelium. Scale bar, 500 μ m.

reported widely in the literature (reviewed in Sengel, 1975, 1976), is essential. We followed the reappearance of feather primordia in normal explants and reconstituted explants (Fig. 2). At the time of explant preparation, the primary row (positioned in the middle of the panel and across the explant) had already formed. In the control explant, some initial dermal condensing processes have taken place with visible fibrous lattices (Stuart and Moscona, 1967). Subsequent lateral rows of buds were added sequentially at a rate of about 6 hours per row (Fig. 2A). In the reconstituted explants, no dermal condensations were observed before 24 hours (Fig. 2A'). Then all of the feather primordia appeared simultaneously in the reconstituted explant, while those in control explants only had clearly formed three rows of dermal condensations (Fig. 2B,B'). At 42 hours, well-formed buds appeared all over the reconstituted explant. In regular explants, only 5 rows of buds were well formed and new buds were still forming in the lateral explant (Fig. 2C,C'). This indicates that a periodic pattern can form without sequential propagation from the primary row and suggests that all mesenchymal cells were reset to an equivalent state in this experimental procedure.

We further tested whether all cells were reset to an equivalent state without memory of previous cues. If cells becoming dermal condensations are predetermined and retain 'memory' (whether it is in the form of differential cell adhesiveness or determined 'cellular fate'), following mesenchymal dissociation and reaggregation, the original dermal condensation cells should sort themselves out to form new feather primordia. However, if all cells are reset and a new order has to be established, then each cell has an equal opportunity to be part of a new dermal condensation. We microinjected DiI into dermal condensations before dissociation (Fig. 3A). The labeled mesenchyme was then dissociated into single cells, plated at high density and recombined with non-labeled intact epithelium. After 3 days, new feather buds reappeared and the labeled cells were randomly distributed within and outside of the buds (Fig. 3A'). In another experiment, DiI was microinjected into the epidermal placode prior to mesenchymal dissociation (Fig. 3B) and then the intact epithelium was recombined with non-labeled reaggregated mesenchyme. Evenly spaced feather buds reformed without reference to the previous epidermal placodes in 2 days (Fig. 3B'). Therefore after dissociation and separation, all epithelial and mesenchymal cells appear to be reset and have an equal chance to contribute to feather primordia.

To test further whether cells in the primary row were predetermined or predisposed to become feather primordia, DiI was injected into the mesenchyme of feather buds in the primary rows (Fig. 3C). After mesenchymal dissociation, reaggregation and reconstitution with intact nonlabeled epithelium, the labeled cells became randomly distributed in new buds and interbud domains after 2 days in culture (Fig. 3C'). Finally, we prepared reconstituted explants from regions of lateral skin, not including the primary row. Again, feather buds formed in these primary row-deficient reconstituted explants (data not shown). Thus while dissociation disrupted existing cell contacts and extracellular matrix, the cells have an

intrinsic property to reform periodic patterns when reaggregated. We then asked what factors might affect this intrinsic property.

The size, but not the number, of feather primordia remained constant when different numbers of mesenchymal cells were used in the reconstituted explant

In vivo, mesenchymal cells accumulate in the developing dermis through random cell proliferation (Noveen et al., 1995a) and migration from the dermatome, somatopleurae or neural crest (Sengel, 1976). When does the cell density reach the threshold required to initiate periodic patterning? Once formed, how are the size, number and spacing determined? Would varying the number of available mesenchymal cells (placed subjacent to a constant-sized sheet of epithelium) alter the size or the number of primordia (Fig. 4A)? The reconstituted explant cultures enable us to address some of these issues. If all cells had an equal chance of initiating or

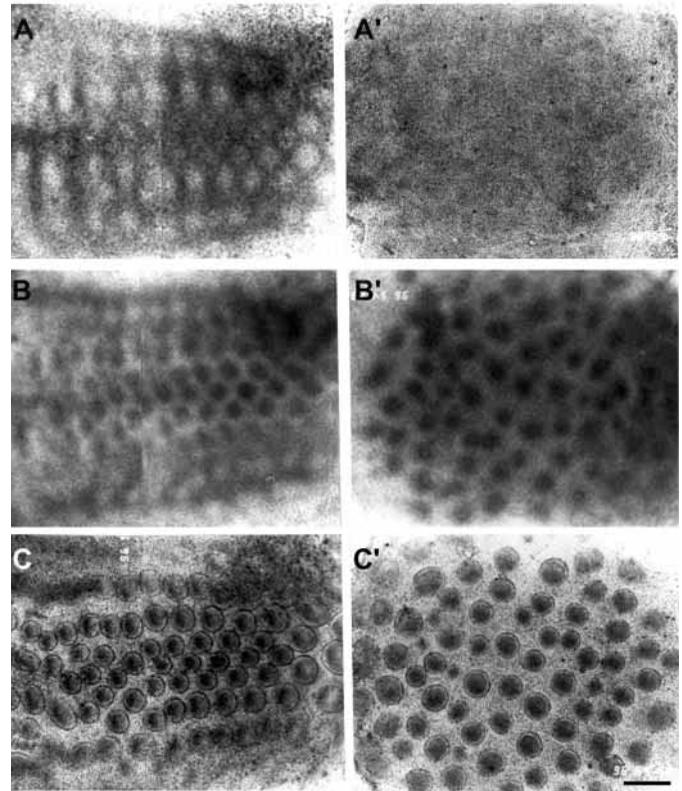


Fig. 2. Feather buds form sequentially in intact skin explants but simultaneously in reconstituted explants. Reappearance of feather buds in regular and reconstituted explants. The midline is horizontally placed from the 9 o'clock (anterior) to 3 o'clock (posterior) positions in the panel. (A-C) Regular skin explants and (A'-C') reconstituted explants shown at 18 hours (A,A'), 27 hours (B,B') and 42 hours (C,C'). The condensations formed simultaneously in the reconstituted explant but sequentially in the regular explants. Note, the feather bud density was higher in the midline region of regular explants than in reconstituted explants because the density in the reconstituted explant was averaged. The spacing in the reconstituted explants was wider but fairly evenly spaced (see below, Fig. 4). Anterior, left; posterior, right. Scale bar, 500 μ m.

being recruited into primordia, then the size of the primordia should remain constant, reflecting an intrinsic property of the microenvironment and the number of formed primordia would be subject to change. If a select predetermined subpopulation of cells was destined to initiate primordia formation, the number of primordia would be maintained, and the size of dermal condensations would vary, reflecting the availability of recruitable mesenchymal cells (smaller dermal condensations when fewer cells are provided).

The results showed that the effects of cell density fell into three phases (Fig. 4B,C). At low cell density, no buds formed. Once the threshold level was achieved (about 0.2×10^7 cells/ml cell suspension), dermal condensations began to appear. When feather primordia appeared, the average size was similar, in the range of 200–300 μm in diameter. Within the range of $0.2\text{--}3 \times 10^7$ cells/ml, the numbers of feather primordia gradually increased. In these explants, activation centers initially occurred randomly. The availability of more mesenchymal cells increased the number of surviving condensations. Since the size of the total explant and the size of individual primordia were constant, the increased buds had to form at the expense of the interbud space. At lower density, the few feather buds that formed appeared to be randomly distributed in the explants. As the bud density increased, they started to be more closely arranged. However, a minimal spacing was always maintained between primordia (this can also be seen clearly in Fig. 6C,D for an *in vivo* example). The presence of lateral inhibition zones around the centers of activation led to the appearance of even spacing and suggests underlying interactions. When the highest packing was achieved, the buds appeared hexagonally arranged – but this is not because of predetermined patterning codes. After the bud number reached a plateau, the extra mesenchymal cells piled up to form ‘thicker’ dermal condensations, which grew faster.

Therefore, mesenchymal cells derived from a specific body region are capable of selforganization to form feather primordia of a certain size. We have previously proposed that periodic feather patterning is based on the relative strengths of activator and inhibitor pathways (Jung et al., 1998). Here we further postulate that this ‘intrinsic property’ is based on the different number of morphogen ligands and their receptors on the cells (Jung et al., 1998).

Alteration of mesenchymal cell properties with RCAS transduced BMP receptor and Noggin can change the size of the primordia

We and others previously have used beads and viral gene delivery to enhance or suppress bud formation in cultured explants or in embryos (Noveen et al., 1995b; Noveen et al., 1996; Widelitz et al., 1996; Song et al., 1996; Morgan et al., 1998; Jung et al., 1998; Noramly and Morgan, 1998). Activators promote feather formation (FGFs), while inhibitors suppress feather formation (BMPs). To our surprise, both activators and inhibitors are expressed from within the forming feather buds (Jung et al., 1998). This led us to propose that feather bud size is modulated by relative ratios of activators and inhibitors as shown schematically from both lateral and top views (Fig. 5A, left and right columns, respectively).

In our previous work, perturbations were carried out in explants that already had passed the initial primordium-forming stage, so we obtained fused feather bud domains or inhibited feather buds. The reconstitution assay provides a unique opportunity to test this hypothesis from the initial stage of feather primordia formation. If our hypothesis is true, increasing the sensitivity to the endogenous inhibitors should decrease the size of the feather buds. We therefore ectopically expressed BMPR 1a from RCAS in reconstituted explants. Average feather bud size was decreased by around 40% (Fig. 5B,C). Along this line, reducing the available BMP in the system should reduce the strength of inhibitor activity and increase the bud size. We then expressed exogenous Noggin, an antagonist of BMPs, using the RCAS virus. Indeed Noggin expression increased the size of feather buds by about two-fold on average. This observation is different from the effect of Noggin *in vivo*, which caused the formation of mostly fused buds (Noramly and Morgan, 1998) possibly due to the timing of exogenous gene function. RCAS-BMP2 and RCAS-BMPR 1b viruses also reduced the size of the buds, although not as dramatically (not shown). These data are consistent with the notion that the size of feather primordia are determined by competition between activators and inhibitors.

Progressively restrictive expression of NCAM in mesenchyme during periodic patterning

We then examined the cellular processes involved in the emergence of patterned primordia. Immunostaining of NCAM was used to visualize the presence of numerous microaggregates (diameter ranging over 25–50 μm containing 5–20 cells) as early as 4 hours of culture (Fig. 6A). The microaggregates were randomly distributed in the explant. We reported earlier that NCAM was present at a basal level in the dense dermis prior to dermal condensation formation (Chuong and Edelman, 1985). Antibodies to NCAM perturb the formation of feather buds (Jiang and Chuong, 1992). Here we observed that at 4–8 hours NCAM was expressed at a basal level in most cells, varying within a moderate range. The heterogeneous staining representing microaggregates was discerned in high-power view (Fig. 6A, right column). Smaller, highly NCAM-positive cell clusters were observed first, followed by larger but fewer consolidated dermal condensations. In earlier stages, this microheterogeneity can be appreciated with the aid of pseudocolor images (Fig. 6A right column, 2nd panel). Cells in clusters expressed higher levels of NCAM, and those in between clusters expressed low levels of NCAM (Fig. 6A, right column, 3rd and 4th panel, arrow 1–3).

When feather primordia emerged, the periodic arrangement of NCAM expression levels became larger in amplitude: NCAM became more enriched in the dermal condensations but disappeared from the interbud regions. At 18 hours, the periodic patterning of NCAM became more clear and the blurred border sharpened by 24 hours. The microaggregates were in an unstable and reversible state. Since the number of initial microaggregates far outnumbered the number of dermal condensations, logically, only some of the microaggregates survived and became independent dermal condensations. Some microaggregates

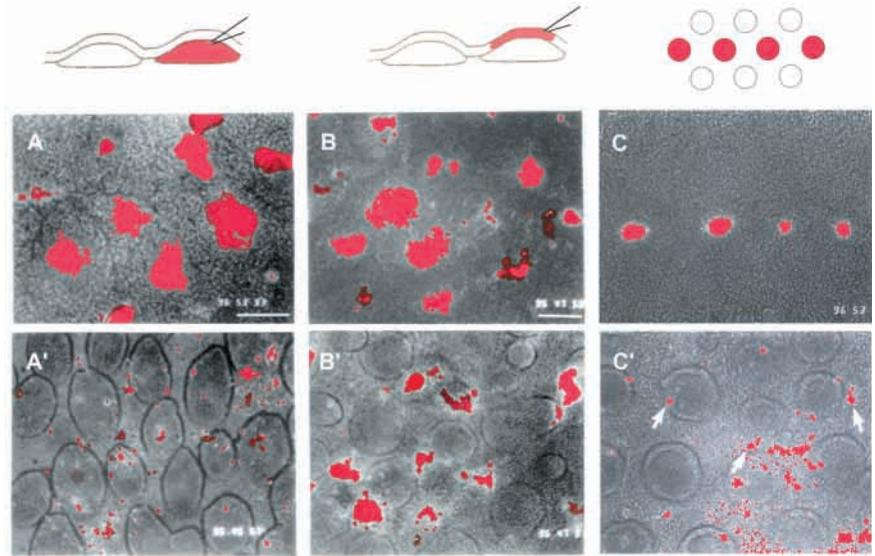


Fig. 3. Reconstituted feather buds form without reference to the original epidermal placodes, dermal condensations or primary row. DiI (labeled in red) was injected into the dermal condensations (A), epidermal placodes (B) or primary row mesenchyme (C). Diagrams show the method of injection. Cells were then dissociated, reassociated and combined with cells from non-labelled mesenchyme (A) or epithelium (B,C). (A',B',C') Subsequent distribution of DiI-labeled cells. Because of the nature of the assay, the photographs depict related but not identical fields. Scale bar, 200 μm .

dissociated into single interprimordial cells, while some merged to form larger, more stable aggregates, suggesting a competitive mechanism. A high-power view showed that the difference of NCAM staining was due to different amounts of NCAM on

different cells (compare arrow 1-3), not due to different cell densities (Fig. 6A, right lower panels).

Epithelial-mesenchymal interactions have been demonstrated to be essential for skin appendage formation. Reconstituted

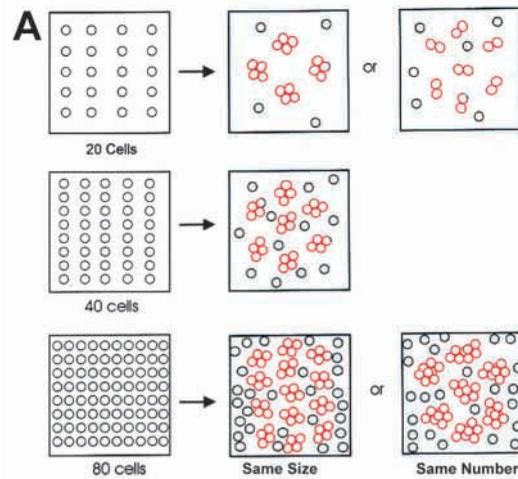
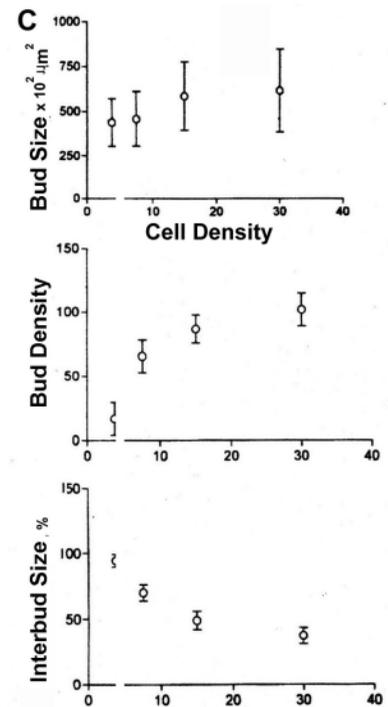
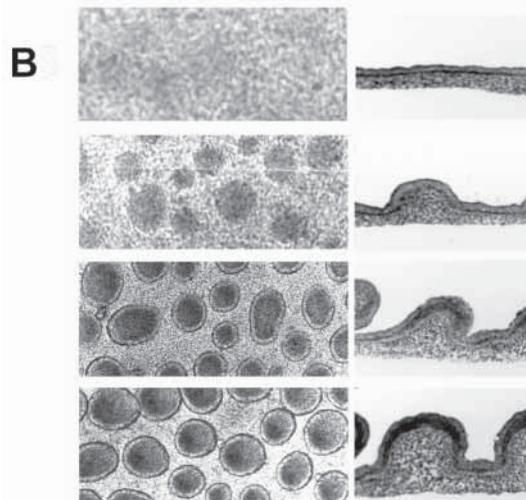


Fig. 4. Altered feather bud number and interbud spacing produced by changing the number of mesenchymal cells in the explant. (A) Schematic diagram of possible changes in periodic spacing when cell density or growth factor concentrations are altered. Independent (black circles) or aggregate-forming (red circles) cells. (B) More available cells (increasing cell numbers from top to bottom from 0.19, 0.75, 1.5 to 3×10^7 cells) increased bud density, but not bud size after 1 day in culture (Top view, left column; cross section, right column). Buds first formed randomly and sparsely and appeared evenly spaced. At maximum bud density, primordia were hexagonally arranged. (C) Bud size and number and explant size were measured using the Mocha image analysis program (see Materials and Methods). Bud size was relatively constant. The increased bud density was generated at the expense of the interbud spacing, although a minimum distance between feather primordia was maintained (C, lower panel). Size bar, 200 μm .



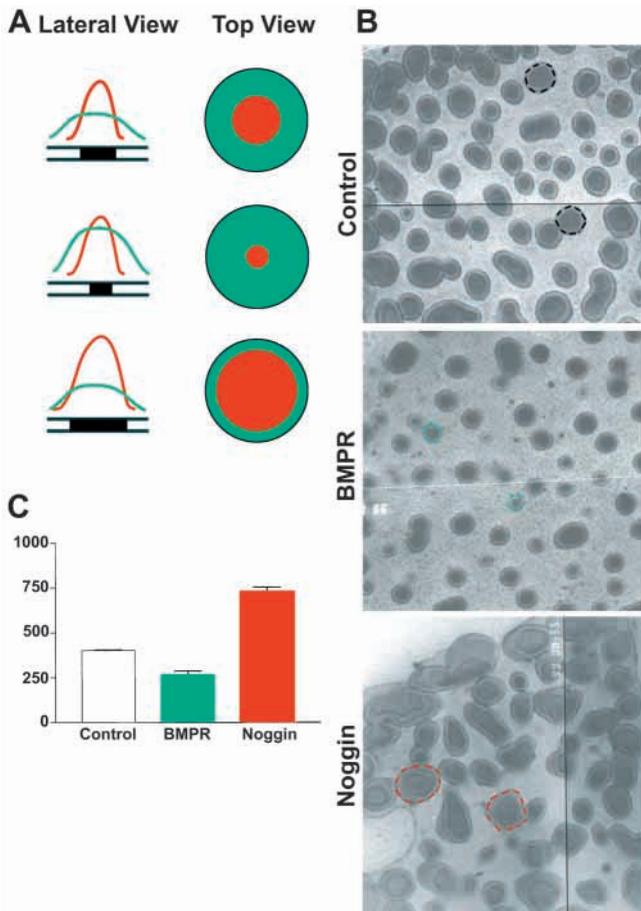


Fig. 5. Alteration of the size of feather primordia by RCAS-transduced BMP receptor and Noggin in feather mesenchymal cells. (A) Schematic diagram of the effect of activators (FGFs, SHH; red) and inhibitors (BMPs; green) on feather formation from the lateral and top view. The effective ratio of activators versus inhibitors can regulate the size of the feather buds. (B) Reconstituted skin explants prepared with 2×10^7 mesenchymal cells were transduced with RCAS (Control), RCAS-BMPR 1a (BMPR) or RCAS-Noggin (Noggin) and cultured for 3 days. The borders of some of the feather buds are outlined (dotted lines). (C) Data from three independent experiments were plotted. The data demonstrate that expression of BMPR reduces the average size of feather buds, while expression of Noggin increases the average size of feather buds.

explants without epithelium still can form microaggregates, however, they fail to progress into dermal condensations, suggesting that the molecular cascades driving the consolidation process require interactions with epithelia. Explants without epithelium are similar after 24 hours to explants with epithelium within 10 hours (Fig. 6A, right column, 1st panel). Cell proliferation does not seem to play a role in dermal condensation since the process occurs within the first 18 hours of culture (Wessels, 1965; Noveen et al., 1995a); rather it is likely due to cell rearrangements driven by cell adhesion. The basal expression of NCAM, together with other adhesion molecules, endow mesenchymal cells with adhesiveness and the intrinsic tendency to form microaggregates through random collisions when high cell density is achieved. The result of the evolution

of the microaggregates depends on the signaling regulatory loops present in the local environment.

How do these results correlate with those *in vivo*? The temporal sequences of signaling molecule expression parallel that found *in vivo* (Noveen et al., 1995a, 1996; Chuong et al., 1996; Widelitz et al., 1996, 1997; Song et al., 1996; Morgan et al., 1998; Jung et al., 1998; Noramly and Morgan, 1998). Previously we examined NCAM expression in feathers (Chuong and Edelman, 1985); our new studies use whole-mount staining at much earlier stages (Fig. 6B). NCAM first appeared in the midline stripe, and then moved bilaterally in an approximately 500 μm wide stripe (the feather-forming morphogenetic stripe) flanking both sides of the expanding spinal tract. Individual feather primordia then fragmented out from the stripes. The feather border sharpened as the primordia matured toward the midline. These data suggest that a similar periodic arrangement of NCAM expression takes place *in vivo*. It appears that the increased cell density in the morphogenetic stripes can trigger the periodic patterning process and that our reconstitution assay recapitulates the events taking place in the morphogenetic stripe.

Progressively restrictive expression of β -catenin in epithelium during periodic patterning

NCAM is mainly expressed in the mesenchyme. L-CAM (or E-cadherin) is expressed uniformly in the epithelia (Chuong and Edelman, 1985), but β -catenin transcripts (Lu et al., 1997) are expressed homogeneously in the feather tract field and then progressively become restricted into individual primordia. Prior to feather formation, moderate expression was first expressed as a stripe from the caudal end of the spinal tract along the midline. β -catenin expression extended along the midline in a pattern that preceded, but later matched, the morphologically detectable feather primordia. This is best seen in H&H stage 29 embryos (Fig. 6C). From the thoracic and cervical tracts, expression of two primary rows were observed. Gradually, β -catenin mRNA expression became enriched within the placodes of the few feather buds that had formed in the posterior regions. Expression was reduced in the interbud regions immediately surrounding those buds, thus forming a halo. Paralleling this expression pattern, the feather bud boundary was initially blurred, but sharpened over time. As the spinal tract expanded bilaterally, moderate basal levels of β -catenin staining extended laterally beyond the zone where feathers had formed. Thus the moderate and homogeneous β -catenin expression levels were converted into a periodically arranged pattern expressing elevated β -catenin levels in primordia and reduced β -catenin levels in the interprimordial space.

How does the widespread tract distribution pattern become limited to a discrete primordia expression pattern? The transitional process is observed in the spinal tract but is best seen in the femoral tract (Fig. 6D,D'). In the femoral tract, β -catenin was first expressed as a continuous patch prior to feather formation. When feather primordia appeared from the posterior lateral corner of the femoral tract and sequentially formed anteriorly, feather primordia emerged with increased β -catenin expression. A concentric halo appeared outside of the formed primordia. The next primordium emerged anterior to

the first one. As development proceeded, a halo also formed outside the second primordium. The distance between the two primordia gradually increased and more mature feather buds grew further apart. During these dynamic periodic patterning processes, the size remained constant at around $150 \pm 11 \mu\text{m}$ in diameter, and the width of the halo (ring) was $50 \pm 6 \mu\text{m}$. It should be noted that this is the size of feather primordia in the femoral tract. In different regions such as feathers in the head above the beak, the diameter of the primordia can be as small as $50 \pm 4 \mu\text{m}$ (not shown). We consider these to be region-dependent characteristics (see Fig. 5 and associated text). Thus the moderate levels of homogeneous β -catenin expression appear to define the epidermal region of feather tract field that is competent to respond to periodic patterning signals from the mesenchyme.

DISCUSSION

An experimental system to study periodic pattern formation from the beginning

The exquisite arrangement of feather primordia is a striking phenomenon that has intrigued many scientists. Because feather primordia appear sequentially *in vivo*, this patterning led scientists to propose models in which new feather primordia form through sequential propagation (Sengel, 1975, 1976). In some cases, pre-existing primordia were proposed to work as a template for the next emerging primordia. This approach has an intrinsic problem, since, logically, one would ask what set up the initial pattern? If it is positional information (Wolpert, 1971), then what set up the difference in positional value? If it is differential adhesiveness (Steinberg and Takeichi, 1994) or mechanical force (Oster et al., 1983), then what set up the initial difference of adhesion? Could it be the molecular zip code or specific promoters as is found in *Drosophila* segmentation (Lewis, 1978; Struhl et al., 1992)?

To address this, it is very important to have an experimental system in which all cells have the same probability of becoming primordia or interprimordia, and in which the size and number of feather primordia are not irreversibly predetermined. We used a reconstitution model in which the dissociated mesenchymal cells indeed have the ability to selforganize into periodically arranged primordia that grow into normal feather buds (Fig. 1). In the early phase (about 24 hours) of this formation, the fates are still reversible. DiI labeling was used to show that there were no predetermined differences and no cellular or molecular 'memory' following mesenchymal dissociation and reaggregation (Fig. 3). Furthermore, periodic feather primordia appeared simultaneously (Fig. 2), suggesting that neither the primary row nor sequential propagation were essential for periodic patterning. This finding uncoupled periodic patterning from sequential propagation. These data suggest that there are no predetermined molecular addresses, and the periodic patterning process of feather morphogenesis is likely a physical-chemical consequence based on the properties of the cells.

If the above argument is correct and all cells have an equivalent opportunity to form feathered or non-feathered

regions, we would expect that the earliest expressed molecules should be all over the feather field, reflecting their equivalency, and then gradually become restricted to regions where the feather primordia will form. We followed the temporal pattern of molecular expression from our work and the literature (reviewed in Widelitz et al., 1997; Widelitz and Chuong, 1998). The results suggest that there are two modes of molecular expression (Fig. 7, top row). The earliest expressed genes followed a 'restrictive mode', with early ubiquitous and moderate expression levels all over the feather field. This pattern gradually became restricted to the primordia regions, with expression levels intensified in the primordia but suppressed in the interprimordial regions. *Ncam*, β -catenin (Fig. 6), *FGF4* and *Wnt-7a* (Widelitz et al., 1999), and may be *Bmp2* (Noramly and Morgan, 1998) and *Delta* (Crowe et al., 1998; Viallet et al., 1998) follow this mode. Other genes follow a 'de novo mode', starting later and directly in the patterned primordia. These include *Shh* (Ting-Berret and Chuong, 1996; Morgan et al., 1998), *Gli3* (Chuong et al., 1999), *Msx1*, *Msx2* (Noveen et al., 1995a), genes encoding tenascin (*TnC*) (Jiang and Chuong, 1992) and *myc* (Desbiens et al., 1991), and other growth and differentiation related genes. This is also consistent with the recent findings that skin from *Shh* knockout mice can form periodically arranged hair germs (St-Jacques et al., 1998; Chiang et al., 1999).

We recently proposed a staging system for skin appendage formation composed of induction, morphogenesis, differentiation and cycling phases (Wu-Kuo and Chuong, 1999). The induction phase marks the change from one homogeneous state into two states: primordia or interprimordia states. The morphogenesis phase marks further regional specification of the primordial domain with the formation of the AP axis and PD axis. We think that the molecules with a restrictive mode of molecular expression correspond to those involved in the inductive phase of feather formation. Molecules with a de novo mode of expression correspond to those involved in the morphogenesis phase for further growth or determination of subdomains of feather primordia.

A selforganizing periodic patterning model involving reaction-diffusion and competitive equilibrium

To focus on the induction phase, we tried to understand the formation of feather primordia from the beginning. The above data suggest that patterning is flexible, relying on self-organizing properties of cells similar to those proposed by Newman (Newman and Frisch, 1979; Newman and Comper, 1990) rather than strict molecular addresses. With the new data presented here and those in the literature, we propose that the following events occur in the feather primordium induction phase that also lead to periodic patterning (Fig. 7, mid-rows). (1) Cells become competent to form feather primordia and form the feather field. At this initial stage, they have equivalent probabilities of becoming primordia or interprimordia (Figs 2, 3). They have comparable medium levels of cell adhesion molecules and ligands/receptors for secreted morphogens (Fig. 6 and Jung et al., 1998). (2) When a threshold of cell density is reached, the random collision of these cells, which are adhesive, leads to the random formation of many unstable microaggregates (Fig. 6). (3) The formation

of microaggregates leads to increased concentrations of adhesion molecules (e.g., NCAM, Fig. 6) and activators in short-range selfenhancing loops. In this autocrine-like fashion, some microaggregates increase in size or merge by collision. (4) When the size of aggregates reaches a certain threshold, a long-range lateral inhibition pathway (possibly based on the BMP pathway) is triggered to suppress the neighboring regions from becoming feather primordia, and thus make the successful aggregates more stable. (5) Periodic patterns form in the whole reconstituted system. We favor the presence of a reaction-diffusion mechanism (Nagorcka and Mooney, 1985; Jung et al., 1998) because of the expression pattern of activators and inhibitors in the primordia, their effect on feather formation and the regulatory relationship among activators/inhibitors. (6) The survival of microaggregates is based on competition. Some microaggregates consolidate to become bigger and eventually stable dermal condensations, while others disappear. Thus the initial random fluctuation is transformed into a wave-like periodic pattern and cell fates are determined. (7) Consolidated dermal condensations send a message to the epithelium. When competent, the epithelium responds by forming epidermal placodes above the condensations (Fig. 6D,D').

The locations of surviving dermal condensations are randomly and therefore evenly spaced. The hexagonal pattern is the result of highest packing efficiency. Among all the available models, this model is most consistent with all available experimental and published data, and can explain several classical phenomena. For example, when identically sized skin explants from the same region but varying ages were cultured, they generated the same size, not same number, of buds (Linsenmayer, 1972). This is because there is no predetermined pattern and the formation of feather primordia simply depends on the number of available mesenchymal cells. When a skin explant was stretched, the number of buds increased (Davidson, 1983a,b). Stretching led to a larger area of competent epithelium yet there was no change in mesenchymal cell number, so the experimental condition created a decrease in the number of mesenchymal cells per unit area. Since the number of mesenchymal cells was sufficient to be above the threshold, they could accommodate the larger epithelial surface area. Davidson also showed that an incision on the lateral region does not stop the 'propagation' of buds. This can be easily comprehended now since our results suggest that a local intrinsic property is sufficient to initiate feather formation. Similarly, patchy suppression of feather bud formation in the skin did not suppress the formation of buds lateral to the inhibited area (Noramly and Morgan, 1998). It also has been shown that when a primary row is damaged and the mesenchyme has to reorganize, a new primary row is established at the site of the previous morphogenetic stripe (Novel, 1973). This may simply be explained by the higher cell density in the 'morphogenetic stripe'. Finally, ectopic tracts can be induced when an inert object is inserted in early skin or an extra limb bud is initiated in the flank regions (Sengel, 1976). These can also be explained by the accumulation of cells as a response to the foreign body.

While this paper was in revision, some related papers came out. According to our model, Noggin and Follistatin, both BMP antagonists, should be activators for feather formation (Jung et al., 1998) and enhance the levels of NCAM and/or members of the β -catenin pathway. Noggin knockout mice showed reduced NCAM and *Lef-1* expression in the skin while suppressing hair induction (Botchkarev et al., 1999). Ectopic Noggin added to skin cultures upregulates *Lef-1* expression and stimulates hair induction. In contrast, BMP4 is shown to reduce NCAM expression in mouse skin explant cultures. In chicken, local application of Follistatin-coated beads to the skin explant can induce ectopic feather buds (Patel et al., 1999). Enhanced β -catenin activity mediated by RCAS also induces new feather buds (Noramly et al., 1999). These results are consistent with the model presented here (Figs 6C,D, 7).

The size, number, spacing and in vivo propagation of feather primordia

In periodic pattern formation, periodic elements can form with different size, number and spacing. A good model should be plastic and able to respond to variables to alter the size, number or spacing. The difference of feather size is most obvious and is based on the combination of initial different sizes of feather primordia and different growth rates (our unpublished data). Our reconstituted system also provides a unique opportunity for us to analyze the molecular factors that can modulate the number, size and spacing of individual elements. We showed that the size of feather primordia remains constant when the number of available mesenchymal cells (per unit area of epithelium) varies. On the contrary, the number of feather primordia formed (also per unit area of epithelium), within a range, is the function of the number of available mesenchymal cells. The increasing cell density (per unit area of overlaying epithelium, not per unit volume of dermal region) implies the formation of more microaggregates. The size of microaggregates (3-5 cells) is a constant based on cell adhesion molecules such as NCAM. The size of stable dermal condensations, or aggregates of microaggregates, is determined by the relative strength of activators and inhibitors and is fixed for mesenchymal cells derived from a specific body region (see below). Thus the increase of cell numbers is translated into the survival of more dermal condensations supported by the epithelium. In other words, the diameter of the formed primordia can be viewed as the wavelength of the equilibrated pattern. In this case, patterning is the formation and maintenance of a minimum distance between repetitive neighboring elements. The number of peaks (feather primordia) and valleys (lateral inhibitory zone surrounding the feather primordia) that can form is constrained by the dimensions of the system, in this case, the size of the epithelium.

Since different sizes of feather primordia do form, how is this achieved? We were able to demonstrate that alterations of BMP pathway activity can change the size of individual primordia. This was demonstrated with RCAS-BMP receptor or RCAS-Noggin transduced mesenchymal cells that formed smaller and larger feather primordia, respectively, in reconstituted explant cultures. This is consistent with our earlier hypothesis that the size of feather primordia is

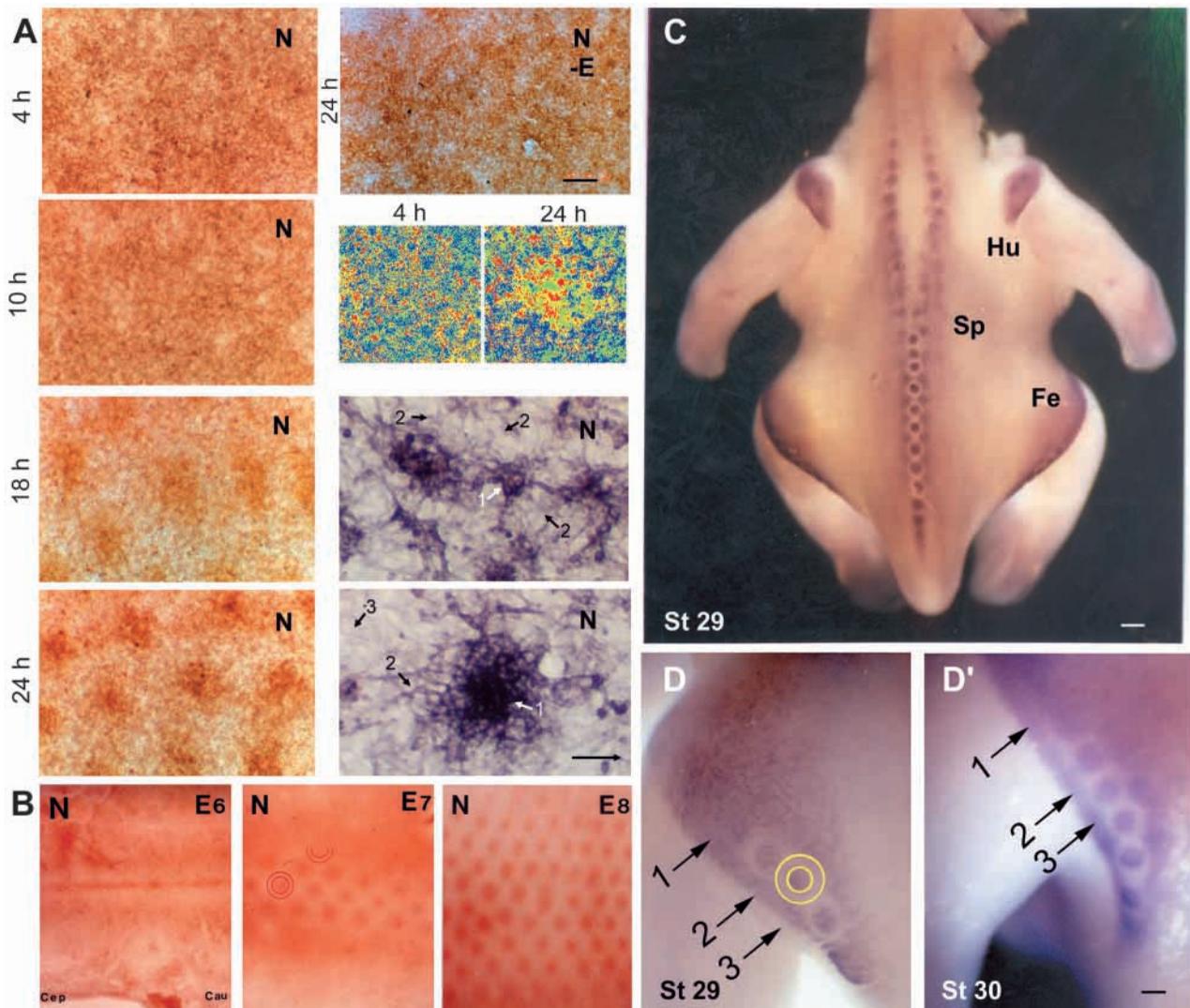


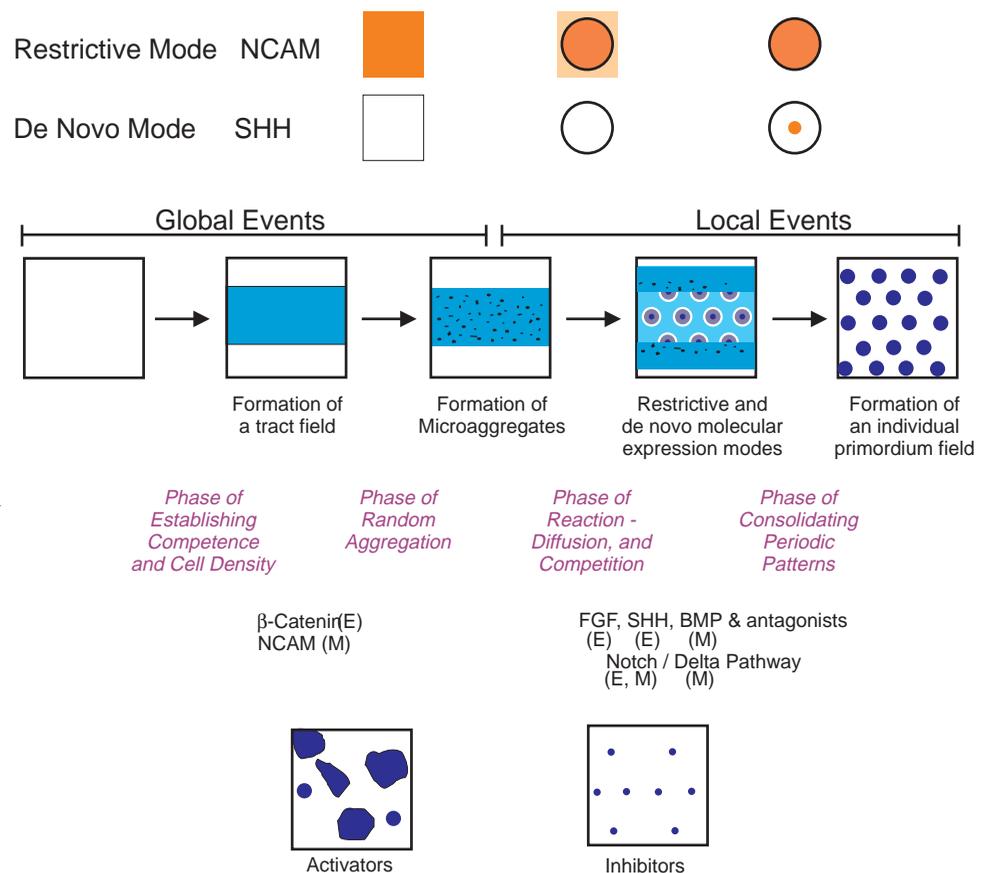
Fig. 6. Cell adhesion molecules as mediators of periodic patterning. (A) Immunostaining of NCAM in the reconstituted explant or dissociated mesenchymal cells (N-E, right column) after 4, 10, 18 and 24 hours in culture. Size bar, 200 μ m. Pseudocolor and higher power views (A, right column). NCAM (N) was first expressed all over the explants at a medium level. At 18 hours, NCAM started to be seen to be expressed intensively within aggregates (arrow 1), at basal levels surrounding aggregates (arrow 2) and not expressed in unaggregated cells (arrow 3). Size bar, 500 μ m. (B) Expression of NCAM in vivo at E6-8. Note the alteration from absence to homogenous staining to individual primordia (double red circle) expression pattern. Development starts from the midline of the spinal tract. (C,D) Whole-mount in situ hybridization of β -catenin expression in the dorsal (C) or femoral (D,D') tracts at H&H stage 29. Yellow circles show the primordium and the outside halo. The arrows point to three sequentially developing feather buds (number 1 is the youngest). Development starts from the posterior-lateral corner of the femoral tract. Size bars, (C) 500 μ m; (D,D') 250 μ m.

determined by the relative strength of activators and inhibitors (Jiang et al., 1998). In the earlier cases, FGF1, FGF2, FGF4, BMP2 and BMP4 ligands were used to demonstrate the principles while we also proposed that BMP antagonists could work as activators and FGF antagonists could work as inhibitors. Here we showed that Noggin, a BMP antagonist (Re'em Kalba et al., 1995), can indeed increase the average size of reconstituted feather primordia. Consistent with this, the in vivo delivery of Noggin caused fusion of feather buds (Noramly and Morgan, 1998). Finally, increasing the number of BMP receptors on mesenchymal cells can have similar, and even more apparent, effects than the delivery of BMP2 and BMP4, suggesting that there may

be ligand excess in this explant system. Put together, these results suggest that the size of feather primordia indeed reflects the balanced activities of activators and inhibitors, or the concentration of morphogen ligands in the system and the number of receptors on the cells (Fig. 7, lower row). In vivo, different-sized feather buds form on different body regions. It should be noted that the initial feather primordia size, as stained by β -catenin, is the same as the final feather bud size (Fig. 6D,D'). They do not initiate as smaller sized buds and grow into their final size. This further conforms that epithelial placodes reflect the equilibrated states of consolidated dermal condensations.

In vivo, feather buds indeed form sequentially away from

Fig. 7. Global and local events combine to generate periodic feather patterns. Molecules with a restrictive mode of expression start earlier and initially are distributed all over the feather field, but then become restricted to regions forming the feather primordia. Molecules with a de novo mode of expression appear later and directly in the primordia. The periodic patterning of feather buds from homogeneous skin (1st panel) requires a competent feather tract (blue color, 2nd panel). Adhesion of cells, contacting through random collisions leads to the formation of microaggregates (dark-blue dots, 3rd panel). These cells produce and respond to activators as well as inhibitors through a mechanism involving reaction-diffusion (Jung et al., 1998). The microcellular aggregates are consolidated into dermal condensations (purple color) with a lateral inhibitory zone (4th panel). In vivo, this occurs from the midline and the remaining primordium-forming regions propagate bilaterally as morphogenetic stripes. In the in vitro reconstitution explant, all buds form simultaneously since cells are evenly mixed before plating. Eventually, an equilibrium is achieved and successful feather primordia are evenly spaced (5th panel). In each panel, the midline is horizontally placed in the middle of the square. The expression patterns of some molecules regulating feather morphogenesis are shown. The final pattern reflects an equilibrium state, not the result of a pre-assigned pattern. While activators (e.g., FGF, BMP antagonists such as Noggin and Follistatin, PKA) and inhibitors (e.g. BMPs, FGF antagonists, PKC, Delta) are involved in normal development, perturbing their concentrations caused alterations in the number, size and spacing of primordia in different ways.



the primary row. How do we explain this? We think that it may reflect a developmental wave spreading from the midline (primary row of the spinal tract) toward the lateral edge of the body. A zone about 500 μm wide, named the morphogenetic stripe, can be observed lateral to the last row of formed buds and is the birth site of new feather primordia. Molecules expressed within this stripe, such as NCAM, show a homogeneous, non-periodic staining pattern (Fig. 6B). We think the reason that feather primordia emerge from this zone can be as simple as the homogeneous high cell density in this zone (demonstrated by DAPI staining, not shown). Indeed, we consider that our reconstituted model is the equivalent of the morphogenetic stripe, and feather primordia in vivo appear to propagate because the morphogenetic stripe moves across the body region. The increase of cell density in the morphogenetic stripe is consistent with our experimental data that a threshold of cell numbers is required for feather primordia to form.

What drives the propagation of feather formation in vivo? This is the next question to be explored and the question may become what initiates the feather field. In normal development, the global propagation and local events combine to generate the exquisite temporal and spatial feather patterns. In this paper, based on our new data and those in the literature, we propose a feather-patterning model that can account for the

local events (Fig. 7 mid-row). It provides good explanations for many intriguing and seemingly inconsistent phenomena of feather formation in the literature. It also provides an explanation for both inductive and patterning events. While more signaling mechanisms remain to be worked out, the main framework of the model is likely to be sustained. However, it is likely that multiple mechanisms are in operation to achieve periodic patterning of one organ and that different organs may use different mechanisms. It is also possible to generate spatial periodicity through temporal control such as those observed in somite formation (Palmeirim et al., 1997; Cooke, 1998).

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