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Skin Morphogenesis

Embryonic Chicken Skin Explant Cultures

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1. Introduction

Chicken skin development is an excellent model to study the mechanisms of morphogenesis. It has a long experimental history and has been well characterized phenotypically. Chicken skin offers distinct patterns, large numbers of different cutaneous appendages, availability of in vitro culture systems, accessibility to microsurgery *in ovo*, and the existence of skin mutants. All these factors make feather an ideal model for the investigation of epithelial-mesenchymal interactions and the regeneration of epithelial appendages ***(1–3)***.

In this chapter, we describe the model of feather explant cultures (*see* **Fig. 1**). Skin explant cultures were originally started by Wolff and Haffen ***(4)***. Sengel ***(3)*** has continued to develop the explant culture procedure and has stated that explants from E6.5 embryos still need fetal calf serum (FCS), but after E6.75 serum-free culture is possible. When epithelia and mesenchyma are separated, the organization of both components are lost. However, if the epithelium and mesenchyme are recombined shortly after separation, feather germs regenerate ***(5,6)***. The location of new buds are in accord to that of the mesenchyme. If the epithelium is rotated relative to the mesenchyme in the recombinants, the anterior-posterior orientation of the feather buds will follow that of the epithelium. This provides a unique opportunity to study the dependence of molecular expression on E-M contact and the sequence of molecular expression required for skin appendage formation. The following is the culture procedure we use routinely in our laboratory.

2. Materials

1. Eggs: Pathogen-free fertilized chicken eggs are from SPAFAS Connecticut Hatchery (Preston, CT). Non-pathogen-free eggs can be acquired from a local farm.
2. Media.
   a. Hanks's buffered saline solution (HBSS) (Gibco-BRL, Gaithersburg, MD).
   b. Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL supplemented with 2% FCS and gentamicin (1:1000).

From: *Methods in Molecular Biology, Vol. 136: Developmental Biology Protocols, Vol. II*
Edited by: R. S. Tuan and C. W. Lo © Humana Press Inc., Totowa, NJ

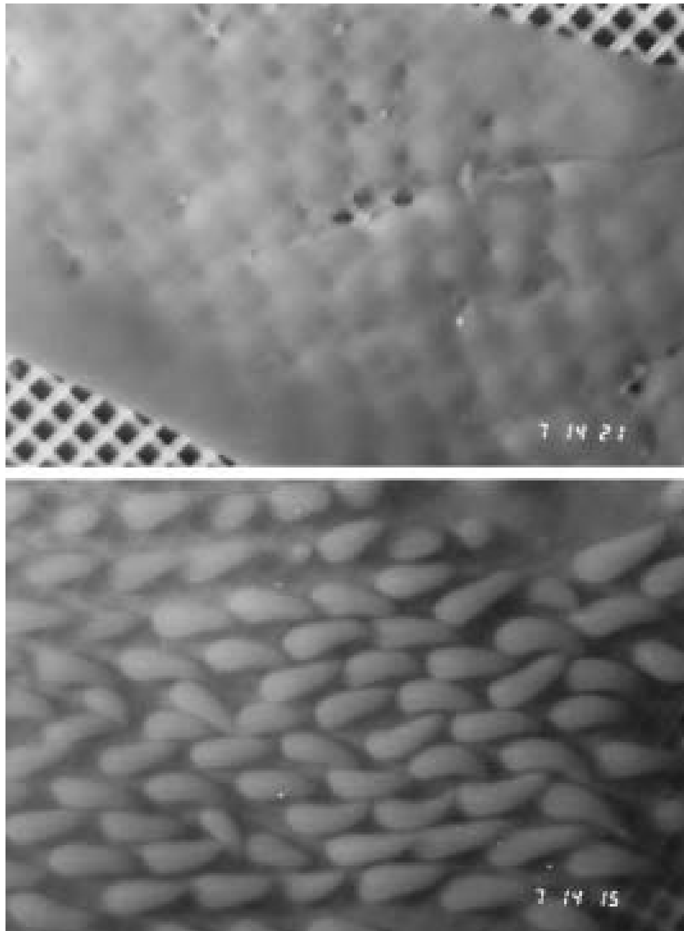

Fig. 1. An example of feather buds formation from cultured explants. A piece of dorsal skin from stage 31 chicken embryo was cultured for 4 d. Note the hexagonally arranged feather buds emerging from the homogeneous skin explant. Also note that most of the buds elongate toward the posterior end (caudal end of the dorsal skin, toward right edge of the panel).

c. Calcium-magnesium free saline (CMFS 10X)
   NaCl (1.37 *M*) 80 g
   KCl (0.04 *M*) 3 g
   NaH_2PO_4 (0.004 *M*) 0.5 g
   KH_2PO_4 (2 *M*) 0.25 g
   $NaHCO_3$ (0.12 *M*) 10 g
   Glucose (0.1 *M*) 20 g in 1000 mL distilled water, pH 7.3

3. Tissue culture supply: Dishes and insert are from Falcon (Los Angeles) (#3090, pore size 0.4 µm). Filters are from Millipore (Bedford, MA) (#110608, pore size 0.6 µm).
4. Tools
   Spatula
   Regular forceps
   Fine-point forceps
   Watchmaker's forceps (#5, titanium)

3. Methods

3.1. Skin Explant Culture

1. Eggs are incubated in a humidified incubator at 38°C and staged according to Hamburger and Hamilton ***(7)***. For feather development, stage 28–34 chicken embryo dorsal skins are most frequently used (*see* **Note 1**).
2. The outside of the egg is cleaned with alcohol. The shell is cracked with regular forceps. Gently remove the shell with sterile regular forceps. Avoid dropping shell debris into the egg.
3. Use another set of sterile forceps and spatula to get into the egg and remove the chicken embryo. Transfer the embryo to a 60-mm dish with HBSS. Rinse the embryo and transfer the embryo to different dishes until HBSS is clear.
4. Place embryo under the dissection microscope with the head toward the left (assuming you are right handed). With the left hand, use sterile fine-point forceps to hold the embryo at the neck. With the right hand, use scalpels to make two longitudinal cuts along the flank region parallel to the midline.
5. With the right hand switched to a watchmaker's forceps, grab the skin and gently rip it off toward the back. When the skin is near the tail, make a transverse cut anterior to the tail. In case there are muscles attached to the bottom, trim them off. Using transillumination, dermal condensations are visible. Record how many rows of dermal condensations have already formed (*see* **Note 2–4**).
6. Place tissue culture insert in the 60-mm dish. Add 2 mL/well of DMEM supplemented with 2% FCS, gentamicin (1:1000).

7a. Use a smoothed-edge spatula to transfer the skin to the insert. Spread the skin explant flat gently. Make sure that the bottom side is down. Mark the cephalic end of the dorsal skin so that you know the orientation later.

7b. If the filter method is used ***(8)***, spread the skin flat on the filter. Then lift the filter and transfer it to a dish with the same culture medium. Let the explant/filter float on top of the air-liquid surface. Alternatively, the explant filter can be placed on top of a metal meshwork that is supported on an organ culture dish (Costar, Cambridge, MA, #32360 (*see* **Note 5**).

8. For insert cultures, media should be placed in both the outside well and the inner chamber. In the inner chamber, a thin layer of media is left just to cover the explant so that the explant is moist and close to the air-liquid interface.
9. The skin explant cultures are incubated at 37°C in 5% carbon dioxide and 95% air. The medium is changed every 2 d.

3.2. Epithelial-Mesenchymal Recombination

1. Following the dissection of the skin, place the skin in twofold concentrated calcium-magnesium free saline with 0.25% EDTA. The dish is left on ice for 10 min. Some regions should have shown signs of separation of epithelium and mesenchyme. If not, leave it longer, or transfer to room temperature. Then from the separated region, use two watchmaker's forceps to grab the epithelium and mesenchyme and peel them off gently.
2. The epithelium is transparent. Leave it in the medium but remember which side is up and which end is cephalic. The mesenchyme is thicker and can be transferred to the insert as described. Also remember which end is cephalic.
3. Use a spatula to pick up the epithelium with the upper side facing up. Slide the epithelium, in desired orientation, on top of the denuded mesenchyme on the culture insert.
4. Press the transplanted epithelium down gently while the media is being withdrawn until a thin layer covers the explant. We use surface tension to hold them together. Leave the recombined explant semidry for 2 h in the incubator (*see* **Note 6**).

5. Gently add more media from the side. For recombinants, 10% FCS is usually required for better growth.

3.3. Perturbing Functions with Beads Coated with Growth Factors or Drugs

Localized delivery of growth factors or drugs ***(9–12)*** can be delivered by Affi-Gel Blue beads (Bio-Rad, Hercules, CA, 100–250 μm in diameter) or heparin-acrylic beads (200–250 μm diameter, Sigma Chemical, Co., St. Louis, MO).

1. Beads are washed three times in sterile phosphate-buffered saline (PBS) by short centrifugation.
2. Approximately 20–100 beads are added to 5 μL aliquots of growth factor solution.

3a. For the Affi-Gel Blue beads, incubate at 37°C for 1 h.

3b. For the heparin-acrylic beads, incubate at room temperature for 2 h.

4. Treated beads are picked up with the watchmaker's forceps and placed on top of the skin explants or sandwiched between the epithelium and mesenchyme.
5. If treated beads are not immediately used, they can be stored at 4°C for up to 1 wk.

3.4. Reconstitution of Skin Appendages with Dissociated Mesenchymal Cells and Intact Epithelium

The epithelial-mesenchymal recombination reset the development of epithelium, but not the mesenchyme. Previous placode epithelium disappears and the state, but the locations of new feather buds are in accord to that of the previous mesenchyma ***(5,6)***. To reset mesenchyme to the initial state, we have developed a new procedure to dissociate mesenchymal cells, reaggregate them, and allow them to reform feather buds with a new periodic pattern ***(13)***. This model allows us to study the most initial events that occur in the dermal mesenchyme before periodic patterning.

1. Stage 29–35 dorsal skins containing the spinal tract were incubated at 4°C in 2% trypsin for 15–20 min and then washed in medium containing 10% fetal calf serum.
2. Under a dissection microscope, the epithelium and the mesenchyme were separated using watchmaker's forceps.
3. The epithelia were trimmed to be of equal same size using transparent graph paper beneath the disk as a guide. The epithelia were left in the media in ice.
4. The mesenchyme was pooled (usually from five pieces of skin) and gently triturated into single cells by drawing them through fine pore pipets with decreasing diameters. Cells were filtered through Nitex netting when necessary. The viability of cells and completion of dissociation were checked microscopically with trypan blue inclusion.
5. The dissociated cells were counted, repelleted by mild centrifugation (6500 rpm for 4 min) and allowed to reaggregate at specific cell densities for 1 h at 37°C on culture insert dishes (Falcon). The number of explant cultures to set up is equivalent to the original number of pieces of skin used.
6. The epithelium was then placed on top of the mesenchyme and the reconstituted explants were cultured on tissue culture inserts (Falcon, LA).
7. At designated times, explants were observed, photographed or fixed. Feather buds usually re-form in about 24 h.

3.5. Morphological and Molecular Analysis

1. At day 2, many dermal condensations have formed. At day 4, many feather buds have formed (see **Fig. 1**). Photographs can be taken daily from live insert cultures.
2. The contoured buds can be traced and analyzed by image analysis software, where the average size of the buds can be determined. If the variation is big, expressing them as a

histogram can be more informative. The density of the buds can be calculated by dividing the number of buds by the total area of the explant. The total interbud space can be calculated by subtracting the total bud area from the total explant area, which can be expressed as percentage of the explant. The regularity of the feather pattern can be judged by connecting the center of each bud to form a grid pattern. Orientation can be determined by measuring the angle between the anterior-posterior axis of a certain bud and the original midline of the dorsal skin.

3. Whole-mount *in situ* hybridization can be carried out on the explants. Wash the explant three times with DEPC (diethyl/pyrocarbonate)/PBS (1:1000), then process the explants for *in situ* hybridization as usual ***(14)***.
4. For sectioning, wash explants with DEPC/PBS, after which the explants are fixed. After fixation, the explant can be removed gently from the insert and put in a histology cassette. During processing, keep the explant as flat as possible. The sections can be stained following sectioning for *in situ* hybridization or immunochemistry ***(14)***.

4. Notes

1. Shipped eggs are equivalent to day 1 incubation. When these eggs are stored at 4–10°C, they do not develop. To have embryos of certain ages at desired dates, these eggs can be stored in lower temperature and transferred to the incubator within a 1-wk period without too much drop in viability. Once development starts at 38°C, trying to stop it by lowing the temperature will be detrimental.
2. There are many tracts on chicken skin ***(2,3)***. The major tracts we have studied are spinal, femoral, humeral, and caudal. Different tracts develop at different times. Within the tract, there is also a temporal sequence of development. For the spinal tract, the feather buds start from the primary row over the midline of the body and then spread bilaterally toward the flank region. For the femoral tract, the feather buds start as a primary row at the junction between the leg and trunk and then spread medially and upward toward the body midline. It should be kept in mind that there are feather buds of different stages over the same piece of skin explant. This is important, as the tested reagents may have different effects on buds of different stages or from different tracts.
3. Although the Hamburger-Hamilton staging ***(7)*** indicated in both embryonic days and stages, stages are more accurate because the days can be ±1 d. For feather study, this is not even sufficient, as the development progresses faster than stages between stages 28–35. Therefore it is useful to indicate the number of rows of formed dermal condensations. The explant can then be named "stage X dorsal skin explants with Y rows of dermal condensations."
4. There are two ways to visualize the feather buds in the whole-mount view ***(1)***. Transillumination with a dissection microscope is useful for visualizing dermal condensations and distinguishing bud and interbud spaces ***(2)***. Oblique epi-illumination using fiberoptic light is useful in observing the surface morphology and three-dimensional view of the skin appendages.
5. One useful point for using the insert culture over the filter culture is that the live cultures can be photographed daily.
6. Recombination can be carried out between different stages of skin explants, different types of skin appendage, or even appendages from different classes of animals ***(15,16)***.

Acknowledgment

The author thanks Dr. Ting-Xin Jiang and Dr. Han-Sung Jung for help in setting up these cultures. This work was supported by grants from the National Institutes of Health AR42177 and the National Science Foundation IBN 9808874.

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