

Development of a Nude Mouse Model for the Study of Antlerogenesis—Mechanism of Tissue Interactions and Ossification Pathway

CHUNYI LI^{1*}, XIUHUA GAO², FUHE YANG², SHIRLEY K. MARTIN¹, STEPHEN R. HAINES³, XUMING DENG³, JOHN SCHOFIELD⁴, AND JO-ANN L. STANTON⁴

¹AgResearch Invermay Agricultural Centre, Mosgiel, New Zealand

²Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China

³Jilin University, Changchun, Jilin Province, People's Republic of China

⁴University of Otago, Dunedin, New Zealand

ABSTRACT In a previous study (Li et al., 2001. *J Exp Zool* 290:18–30) a nude mouse model was established to investigate deer antler development. In that study we found nude mice could support the singularly implanted antlerogenic periosteum (AP) to form pedicle-like, but not antler-like, bony protuberances. To further develop the model and at the same time to use the updated model for the investigation of antler formation, three experiments were carried out in this study. The results showed that (1) antler-like protuberances were successfully induced on the nude mouse heads via subcutaneous co-transplantation of AP and deer skin, and subsequent exposure through wounding of the deer xenografts; (2) deer skin epidermis and its attached half thickness of dermis were sufficient to interact with the AP, and the interactions were capable of transforming adult scalp skin into velvet; (3) the putative initial inductive molecules were primarily derived from the AP cellular layer, rather than fibrous layer; (4) initiation of the ossification center in the avascular cartilage of each mouse “antler” took place via metaplasia, rather than classical endochondral ossification. Further research is required to identify means for effective stimulation of calcification of the “mouse antlers” in order to create the opportunity to investigate antler regeneration using the nude mouse model. Overall, the nude mouse model, once further developed, has the potential to become a powerful tool to study underlying mechanism of antlerogenesis and organogenesis/regeneration in general. *J. Exp. Zool. (Mol. Dev. Evol.)* 310B, 2008. © 2008 Wiley-Liss, Inc.

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Antlers are cephalic appendages that develop in deer's postnatal life from the apices of permanent bony protuberances, known as pedicles. Deer start to form pedicles as they approach puberty (Li et al., 2003), and when they reach a species-specific body weight (Suttie and Kay, '82). Antlers then spontaneously transform from the distal ends of the pedicles when the pedicles acquire a species-specific height [5–6 cm in red deer; (Li, '97)]. Pedicles and antlers are male secondary sexual characters and, as such, their growth is under the control of androgen hormones. Initiation of pedicle growth is caused by increasing and subsequently elevated plasma testosterone (T) levels, whereas first antler transformation from a pedicle takes place when T levels are decreasing (Suttie et al.,

'91; Li et al., 2003). Antler growth occurs in the period when T levels are barely detectable. Antler calcification and velvet skin shedding are the consequence of high plasma levels of T, and antler casting and subsequent regeneration are linked to very low or undetectable levels of T (Bubenik, '82; Suttie et al., '95).

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*Correspondence to: Chunyi Li, AgResearch Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand.

E-mail: chunyi.li@agresearch.co.nz

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The potential to form a pedicle and an antler is exclusively held in the antlerogenic periosteum (AP) that overlies the frontal crest of a prepubertal deer. Removal of AP from the future antler growth region abrogates pedicle and antler development, and transplantation of the AP elsewhere on the deer body induces an ectopic antler to grow (Hartwig and Schrudde, '74; Goss and Powel, '85; Li and Suttie, 2001). Histological studies revealed that AP consists of two layers: an inner cellular layer that firmly abuts on the underlying bone tissue, and an outer fibrous layer that loosely attaches to the overlying skin via subcutaneous connective tissue (Li and Suttie, '94; Li and Suttie, 2000). Upon initiation of pedicle growth, cells of the AP start to actively proliferate and differentiate, first into osteoblasts to form bone via intramembranous ossification, then into a mixture of osteoblasts and chondroblasts to form osseocartilage through transitional ossification when the pedicle is over 1 cm high. Finally, the cells differentiate to chondroblasts in the later stage of pedicle and entire antler formation to form vascularized cartilage that is subsequently replaced by bone via modified endochondral ossification (Li and Suttie, '94). Therefore, pedicle and antler tissue are essentially the derivatives of the AP.

When carrying out AP transplantation experiments, Goss ('87) noticed that ectopic antlers could only be induced to form when the grafted AP became closely adhered to the overlying skin. This led Goss ('90) to suggest that this close association may be indispensable to facilitate transfer of molecules involved in the interactions between these two tissue components. Results from the detailed histological analysis of the process by Li and Suttie (2000) support this hypothesis. That study showed that transformation from a pedicle to an antler did not start until the interposed subcutaneous connective tissue between AP and the overlying skin was compressed from a very loose, thick layer to a thin, dense strip. However, experimental evidence was lacking as to whether the interactions required for transformation were achieved through diffusible molecules or direct physical contact.

To gain further insights into the nature of the tissue interactions that prevail in first antler generation, recently Li et al. (2008) carried out an experiment in which a piece of either impermeable or permeable membrane (0.45 μ m pore size) was physically interposed between AP and the overlying skin before pedicle initiation. It was

found that the impermeable membrane permanently inhibited antler formation, whereas the permeable membrane only slowed pedicle and antler initiation, but did not prevent these processes. These results clearly demonstrate that the indispensable tissue interactions required for antler formation are realized through diffusible molecules, rather than direct physical contacts. However, that study neither gave an indication of the origin of the putative inductive molecules (i.e. whether from the cellular, the fibrous or both layers of AP) nor of the tissue types (subcutaneous connective tissue, and skin dermis or epidermis) that are essential for responding to the induction.

Realizing some disadvantages of using deer themselves for antler research, such as high costs and significant handling issues, Li et al. (2001) established a nude mouse model to study antlerogenesis. Nude mice, which are congenitally athymic animals, have been widely used for biomedical research owing to their ability to accept xenografts (Demarchez et al., '92; Debiec et al., '94; Scott et al., '95). In their study, Li et al. (2001) convincingly demonstrated that nude mice can sufficiently support the AP to form pedicle-like bony protuberances when subcutaneously grafted onto their foreheads, but that their skin is not competent to interact with the grafted AP and hence fails to participate in antler formation. Therefore, further development of the nude mouse model, i.e. induction of antler-like structure formation, is essential if the model was to be used for the study of antlerogenesis. In addition, Li et al. (2001) also found that during the latter stages of formation of the pedicle-shaped protuberances was achieved via formation of avascular cartilage, rather than vascularized cartilage as is in the case during pedicle and antler formation in deer. However, the mechanism by which this avascular cartilage was replaced by bone during the final stages of protuberance formation was not elucidated.

The aim of this study was to (1) create antler-like protuberances on the heads of nude mice through co-transplantation of AP and deer skin; (2) identify the tissue layers/types that are essential for the establishment of the tissue interactions leading to antler formation, by tissue layer deletion and/or inversion before transplantation onto nude mice; (3) investigate the mechanism by which ossification of cartilage in pedicle/antler-like protuberances occurs and (4) determine whether mouse "antlers" could be induced to calcify through injection of exogenous sex hormones.

MATERIALS AND METHODS

Experimental animals and objectives

Animals

This study consisted of three experiments (Table 1). Red deer (*Cervus elaphus*) stag calves for Experiment I and II, and sika deer (*C. nippon*) stag calves for Experiment III, were selected before pedicle initiation to provide AP and scalp skin for transplantation. The deer were maintained either outside on pasture (red deer) or inside in an enclosure (sika deer) from birth to the time of tissue collection. The nude mice that were the recipients of the transplanted deer xenografts were kept in filtered cages in a pathogen-free environment. The sex, age and body weight of the selected mice were as shown in our previous report (Li et al., 2001) to be optimal for the growth of pedicle-like protuberances.

Objectives

Experiment I: To determine the best possible protocol for co-transplantation of AP and deer skin onto the foreheads of nude mice to create antler-like protuberances by: (1) "Two-step co-transplantation", first replacing a piece of mouse skin (4 mm × 5 mm) on the forehead region by the same size of deer skin, followed (after healing) by insertion of AP tissue directly under the deer skin; and (2) "one-step co-transplantation", subcutaneously transplanting deer skin presutured to AP tissue on the foreheads of the mice.

Experiment II: To investigate the effects of fusion between a pedicle/antler-like bony protuberance and the mouse skull, which was shown to greatly stimulate growth of a bony protuberance (Li et al., 2001) on the timing of the establishment of tissue interactions (as indicated by a change in skin type). At the same time to determine whether the antler-like bony protuberances could be induced to fully calcify, and then shed "velvet skin" to expose "hard antlers" through administration and subsequent withdrawal of exogenous sex hormones to mimic the T cycle in deer.

Experiment III: To explore whether diffusible signal molecules are derived from the AP cellular layer or fibrous layer. Although AP fibrous layer is naturally adjacent to skin, there is reason to believe that it is the cellular layer that secretes these putative molecules (Goss, '95; Li et al., 2008). The ideal way to test this would be to transplant one layer (cellular or fibrous) at a time together with deer skin on the nude mouse

forehead to determine which layer can induce somatic skin to transform into velvet skin. However, practically this was not feasible as AP tissue is too thin (approximately 2 mm in thickness for sika deer) to be accurately separated into these two layers. Therefore, we took an alternative approach comparing the rate of transformation of skin type from scalp to velvet between inverted (AP cellular layer facing the skin) and noninverted (AP fibrous layer facing skin) deer transplants. If the skin transformation were more rapid in the noninverted transplants, it would suggest that the signal molecules are primarily derived from the fibrous layer; otherwise they are more likely to be secreted by the cellular layer. The disadvantage of this alternative approach was that we could not exclude the possibility of some degree of participation by both of the layers.

Tissue collection and transplantation

Methods in common among these experiments are described together to improve clarity and brevity.

Tissue collection

The detailed procedures for collecting AP and scalp skin have been reported elsewhere (Li and Suttie, '94). Briefly, an incision was made in the deer scalp skin medial to the frontal crest. After lifting up the scalp skin, an incision was made on the periosteum along the longitudinal axis of each crest. Six pieces of AP (each around 4 mm × 5 mm) were collected from each frontal crest (Fig. 1A). Pieces of shaved deer scalp skin (4 mm × 5 mm) were also taken from directly above the sampled AP. Samples of each tissue type were placed in 20 mL DMEM medium containing antibiotics (500 U/mL Penicillin and 500 µg/mL Streptomycin) in a 50 mL Falcon centrifuge tube before being transferred to a pathogen-free room for tissue processing and transplantation surgery. Before transplantation, partial dermis adjacent to periosteum and the attached subcutaneous loose connective tissue (SLCT) were removed from each piece of deer skin using a scalpel under a dissecting microscope. Hence only the epidermis and attached partial dermis were used for transplantation.

Transplantation

Transplantation experiments were approved by the Animal Ethics Committee, Medical School of Otago University, New Zealand.

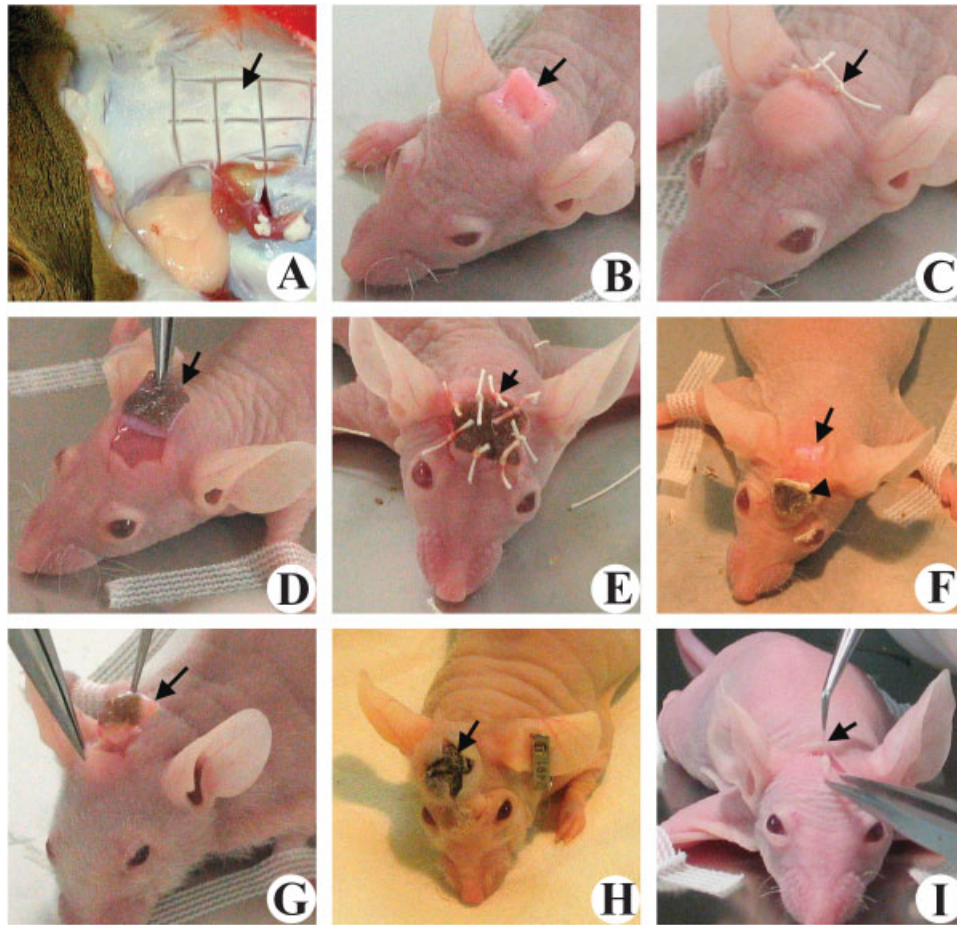


Fig. 1. Deer tissue sampling and transplantation surgery. (A) Antlerogenic periosteum (AP) sampling. Six pieces of AP (each around 4 mm × 5 mm) were delineated using a scalpel and collected by peeling off from each frontal crest (arrow). (B) A piece of AP (arrow) was inserted anteriorly through an incision in the mouse skin and placed at the mouse forehead region. (C) The skin incision was closed with silk suture (arrow) after AP was inserted subcutaneously. (D) A 4 mm × 5 mm piece of deer skin (arrow) was placed to cover the wound after removing an area of skin of the same size from the mouse forehead region. (E) Deer skin was sutured to the surrounding mouse skin (arrow). (F) A piece of AP (arrow) was inserted anteriorly through an incision in the mouse skin and placed right underneath the pregrafted deer skin (16 days after deer skin transplantation). Notice that the deer skin appeared dry and hard (arrow head), although it was still viable. (G) AP+deer skin (arrow) was inserted anteriorly through an incision in the mouse skin and placed at the mouse forehead region. (H) Excision of apical mouse skin overlying the deer xenograft to expose deer skin by first cutting a cross (arrow) using a scalpel and then trimming away the apical mouse skin to the shoulder level of each protuberance using a pair of scissors. (I) Mouse periosteum and loose connective tissue were scratched and at least partially removed (arrow) before deer tissue insertion in Experiments II and III.

Before surgery, each nude mouse was anesthetized by intraperitoneal (i.p.) injection of Ketamine (75 mg/kg equivalent to 200 μ L/mouse, Parnell Laboratories New Zealand Limited, Auckland) and Domitor (1 mg/kg equivalent to 250 μ L/mouse, Parnell Laboratories New Zealand Limited). After completion of the surgery, Antisedan was used i.p. at the dose of 1 mg/kg (approximately 250 μ L/mouse, Parnell Laboratories) to reverse the anesthesia. The mice were monitored until they fully recovered from anesthesia

after each surgery, and then observed weekly and photographed when necessary.

The design of each experiment is described below, and is also summarized in Table 1. In each experiment a group of mice in which AP tissue alone was used for transplantation served as a generic control, in addition to the experiment-specific controls. During surgery, a 4–5 mm long incision was made coronally on the scalp at the shortest distance between the ears of each nude mouse, and the skin was lifted to form a pocket

TABLE 1. *Experimental design*

Group	At transplantation				Days after transplantation		
	Date	Deer (age ¹)	Number of mice (age ²)	Scratch mouse periosteum ³	Exposure of deer skin	Wounding	Tissue sampling
<i>Experiment I</i>							
AP	31 Jul	Red (8)	5 (4)	–	–	56	112
D skin replacing M skin	15 Jul	Red (8)	5 (4)	–	–	–	128
D skin under M skin	15 Jul	Red (8)	5 (4)	–	56	–	128
AP under D skin	31 Jul	Red (8)	10 (4)	–	–	–	112
AP+D skin	2 Jul	Red (8)	10 (4)	–	69	72	141
<i>Experiment II</i>							
AP	15 Jun	Red (7)	4 (3)	+	–	30	174
D skin under M skin	15 Jun	Red (7)	3 (3)	–	21	–	174
AP+D skin	15 Jun	Red (7)	28 (3)	+	21	30	174
<i>Experiment III</i>							
AP	9 Apr	Sika (9)	12 (7)	+	–	–	–
AP+D skin	9 Apr	Sika (9)	12 (7)	+	11	24	69
Inverted-AP+D skin ⁴	9 Apr	Sika (9)	12 (7)	+	11	24	69

AP = antlerogenic periosteum; D = deer; M = mouse.

¹Age in months.

²Age in weeks.

³Scratch mouse periosteum at the implantation site to promote fusion between AP and the mouse skull.

⁴Inverted co-transplantation of AP and deer skin (i.e. with the AP cellular layer facing the skin, rather than in the normal orientation with the AP fibrous layer facing the skin).

anterior to the incision. AP was then inserted into the pocket (Fig. 1B) and the wound was closed with silk sutures (Fig. 1C).

Experiment I. For the two-step co-transplantation, a 4 mm × 5 mm piece of skin was removed from the forehead region of each mouse using a scalpel, and was immediately replaced by a similar sized piece of deer skin (Fig. 1D). This was then sutured to the surrounding mouse skin with silk threads (Fig. 1E). Once the grafted deer skin had properly healed and fused with nude mouse skin (7–10 days), a piece of AP was inserted underneath the deer skin after the procedure described above (Fig. 1F). For the one-step co-transplantation, AP and deer skin were first sutured together with the fibrous layer facing the deer skin, before being inserted under the mouse skin after the same procedure as for the generic control group (Fig. 1G). An attempt was made to position each deer xenograft into a skin pocket, so that the cellular layer was resting directly on the mouse skull. Once a pedicle-like protuberance was formed (after about 3 weeks), the mouse skin on the apex of each protuberance was excised to expose the transplanted deer tissue (Fig. 1H). Two experiment-specific control groups were included in Experiment I: (1) two-step-style transplantation of deer skin without AP (mouse

skin substitution) and (2) one-step-style subcutaneous transplantation of deer skin only (subcutaneous transplantation) (Table 1).

Experiment II. In this experiment, fusion between the grafted deer tissue and the underlying mouse skull was facilitated by scraping away the periosteum overlying each mouse skull at the transplantation site (Fig. 1I). Removal of the mouse periosteum at the transplantation site has been shown to be a very effective way to promote fusion between the deer and mouse tissue (Li et al., 2001). Transplantation was carried out after the same procedure as for the two-step co-transplantation group in Experiment I. The apex of each resultant antler-like protuberance was wounded by puncture with a needle in order to promote “antler” tissue formation, a method that was found to be effective for the induction of antler formation in deer (Jaczewski, '82).

To try and induce full calcification of the protuberances on the animals co-transplanted with AP and deer skin, 27 of the 28 mice in Experiment II were randomly allocated into seven groups at the time protuberances had visibly stopped growing (29 September–16 October). Mice in Group 1 were each injected with 30 µL saline as a control treatment. Mice in Groups 2, 3 and 4 were injected with 1.5, 2.5 and 5 mg T

(Durateston, Intervet Australia, Melbourne) in 30, 50 and 100 μ L vehicle, respectively. Mice in Groups 5, 6 and 7 were injected with 7.5, 11.25 and 22.5 ng estradiol (Estral, Stockguard, New Zealand, Hamilton) in 10, 25 and 30 μ L vehicle, respectively. Three weeks after sex hormone injection, blood was sampled via the saphenous vein from both the control and the highest T dose (5 mg/mouse) groups. T levels were measured by radioimmunoassay by Canterbury Health Laboratories.

Experiment III. Thirty six nude mice were randomly allocated into three groups. Group 1 served as the generic control, and mice in that group were treated as previously described. In Group 2, AP and deer skin were sutured together with the cellular layer facing the skin (inverted co-transplantation) before being grafted into the mouse skin pocket after the same procedure as in Experiment II. In Group 3, AP and deer skin were sutured together with the fibrous layer facing the skin (noninverted co-transplantation) before transplantation. Unfortunately, most mice in this experiment became infected owing to a Specific Pathogen Free barrier failure and only two mice survived from each of Groups 2 and 3. Consequently, data from only four mice were recorded in this study. These mice were sacrificed 69 days after transplantation surgery to avoid the risk of further infection.

Tissue sampling and histology

Tissue sampling

At the termination of each experiment, the protuberances formed on heads of mice were sampled for histological examination using the procedure reported by Li et al. (2001). To facilitate discussion, the final shape of each protuberance was given a descriptive name based on its dimensions as described in Li et al. (2001). A protuberance with height equivalent to or less than half its width was called “dish shaped”, height similar to its width was called “dome shaped” and height greater than the width was called “pedicle shaped”. A “pedicle-shaped” protuberance, if apically covered with velvet-like skin, was further termed “antler-like”.

Histology

The collected protuberances were preserved in 10% buffered formalin immediately after removal. The tissue samples were then decalcified and divided sagittally into two even parts after

the procedure reported by Li et al. (2001). The trimmed tissue blocks were dehydrated, embedded in paraplast wax, sectioned at 5 μ m (five consecutive sections) and counterstained with hematoxylin (H)/eosin (E) and alcian blue.

RESULTS

Morphology

The morphological results from each of the three experiments are shown in Table 2.

Experiment I

Three of the five AP grafts (generic control) formed pedicle-shaped (Fig. 2A) and two formed dome-shaped protuberances. However, all ten animals that received deer skin either through two-step-style or one-step-style transplantation only formed dish-shaped bumps, although the deer skin transplanted in the two-step-style had become hard and dry at the time of AP tissue transplantation (Fig. 1F), while that transplanted in the one-step-style was perfectly viable and produced long hairs (Fig. 2B).

In the one-step co-transplantation group, nine out of ten deer grafts formed pedicle-shaped protuberances. The apex of each protuberance was black (Fig. 2C), which was caused by the pigmented deer skin being visible through the transparent nude mouse skin. At the time of excising the apical mouse skin, all the exposed deer skin had not only survived but had also produced long hairs (Fig. 2D). Four out of nine deer skin transplants in this group were transformed from scalp into velvet type (Fig. 2E and F) after apical wounding.

In the two-step co-transplantation group, seven out of ten deer skin transplants survived, but their areas were substantially reduced by ingrowth of mouse skin surrounding the transplantation sites. All these deer skin transplants were pushed sideways by the upwardly growing protuberances, some anteriorly (Fig. 2G) and some posteriorly (Fig. 2H). The remaining three grafted deer skin transplants in this group became desiccated, but remained in place (Fig. 2I).

Experiment II

All 28 deer xenografts in the AP+deer skin group formed pedicle-shaped protuberances that were black at the apices (Fig. 3A). The exposed deer skin was fully fused with the surrounding mouse skin after the removal of the apical mouse

TABLE 2. Morphological and histological features of the protuberances formed from deer xenografts

Group	Shape ¹ (Di/Do/Pe/An)	Fused/ not fused ^{1,2}	Days to change in skin type ¹ (after: Tr/SE/Wo)	Number having skin with velvet features ²
<i>Experiment I</i>				
AP	0/ 2 / 3 / 0	1 / 4	–	0
D skin replacing M skin	5/ 0 / 0 / 0	0 / 5	–	0
D skin under M skin	5/ 0 / 0 / 0	0 / 5	–	0
AP under D skin	0/ 6 / 1 / 3	3 / 7	–	0
AP+D skin	0/ 1 / 5 / 4	5 / 5	93/24/21	4
<i>Experiment II</i>				
AP	0/ 1 / 3 / 0	3 / 1	–	0
D skin under M skin	3/ 0 / 0 / 0	–	–	–
AP+D skin	0/ 5 / 10 / 13	24 / 4	43/22/13	13
<i>Experiment III</i>				
AP	No data ³	No data	No data	No data
AP+D skin	0/ 0 / 2 / 0	1 / 1	–	0
Inverted-AP+D skin ⁴	0/ 0 / 2 / 0	0 / 2	–	2

AP = antlerogenic periosteum; D = deer; M = mouse; Di = dish-shaped; Do = dome-shaped; Pe = pedicle-shaped; An = antler-like; Tr = transplantation; SE = skin exposure; Wo = wounding.

¹By morphology.

²By histology.

³No data were available for mice with the AP grafts, as they all died prematurely owing to infection.

⁴Inverted co-transplantation of AP and deer skin (i.e. with the AP cellular layer facing the skin, rather than in the normal orientation with the AP fibrous layer facing the skin).

skin, and had typical scalp skin hairs (Fig. 3B). Thirteen out of the 24 AP+deer skin grafts that fused with the underlying mouse skull formed antler-like protuberances, which were apically covered with velvet-like skin (shiny surface and sparsely populated hairs) (Fig. 3C and D). Apical wounding to induce antler growth, however, in most cases in this experiment seriously damaged the growth center of each protuberance as well as its apical deer-derived skin (Fig. 3E and F). The AP-alone grafts in the generic control group formed pedicle-shaped protuberances similar to those in Figure 2A.

None of the injected doses of T or estradiol caused any visible changes in morphology of these protuberances, including those that were capped with velvet-like skin. Analysis of the plasma of the group administered the highest dose of T showed that there was no significant increase in mean circulating T level (7.6 nmol/L) compared with controls (7.4 nmol/L plasma) detectable 3 weeks after steroid treatments.

Experiment III

The xenografts from the four surviving mice formed pedicle-shaped protuberances 45 days after the deer skin was exposed by excising apical mouse skin (Fig. 4A and C). However, no morphological change in skin type from scalp to velvet was

visibly obvious in these mice at the time of tissue sampling (Fig. 4B and D), 69 days after transplantation surgery.

Histology

The histological results from each of the three experiments are shown in Table 2.

Experiment I

The gross histological structures of all the fused protuberances (Fig. 5A and H) were similar and could be divided into four portions. Distal-proximally, these portions were apical perichondrium, cartilaginous tissue, osseocartilaginous tissue (mixture of bone and cartilage) and osseous tissue. In all cases, the cartilaginous tissue formed in the protuberances was avascular. The osseocartilaginous and osseous portions were of a well-organized structure. Remodelling could be seen within the osseous portion. In unfused protuberances, the histological structures were not consistent, although they all contained components of cartilage and bone (Fig. 5B, C and F).

In the two-step co-transplantation group, none of the growth centers, where cartilage tissue resides in each protuberance, was associated with deer skin, nor had AP-derived tissue and the enveloping mouse skin formed close contact

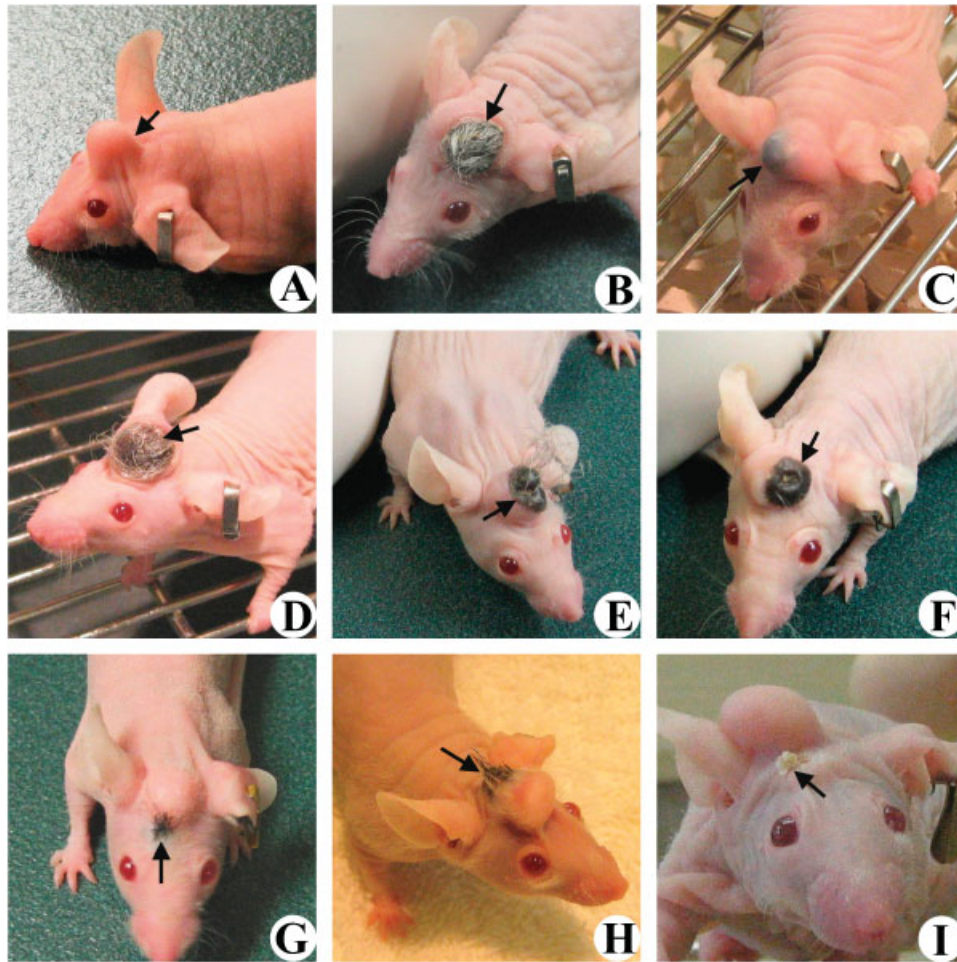


Fig. 2. Morphology of the protuberances in Experiment I. (A) Pedicle-shaped protuberance (arrow) formed from the subcutaneously transplanted AP in the generic control group. (B) Deer scalp skin that was subcutaneously implanted and subsequently exposed by excising the overlying mouse skin. Notice that the skin was perfectly viable and looked no different to the original scalp skin (arrow). (C) Pedicle-shaped protuberance with the dark-coloured apex (arrow), which was owing to the pigmented deer skin being visible through the transparent nude mouse skin. (D) Freshly exposed deer scalp skin that was overlying an upwardly growing bony protuberance. Notice that the deer skin had typical deer skin hairs (arrow). (E) and (F) Antler-like protuberances capped with velvet-like skin. Notice that the velvet-like skin was shiny and had very sparsely populated hairs (arrows). (G) Surviving deer skin (arrow) that had been pushed anteriorly by the upwardly growing protuberance (on a mouse from the group in which AP was transplanted beneath the pregrafted deer skin). (H) Surviving deer skin (arrow) that had been pushed posteriorly, from the same group as in Figure 2G. (I) Grafted deer skin (arrow) that had desiccated but still remained in place in front of the protuberance, also from the same group as in Figure 2G.

(Fig. 5A and B). In contrast, in the one-step co-transplantation group, four out of ten AP-derived tissues not only had apical deer skin (Fig. 5C and F), but had also become closely adhered to it (Fig. 5E). The closely adhered deer skin clearly possessed velvet skin features at the time of tissue sampling, as demonstrated by the presence of thickened epidermis and multi-lobed sebaceous glands, and absence of sweat glands and arrector pili muscle (Fig. 5D and G). Close adhesion between the AP-derived tissue and the enveloping

mouse skin was not observed in this experiment (Fig. 5A, B and H).

An interesting feature noticed in the core cartilage region of each protuberance was that one or more ossification centers were initiated seemingly through an unusual mechanism in which cartilaginous tissue was directly converted into bone (Fig. 5C, H and I), rather than through classical chondroclasia (bone replacement achieved via cartilage destruction), as was the case in the rest of the protuberance.

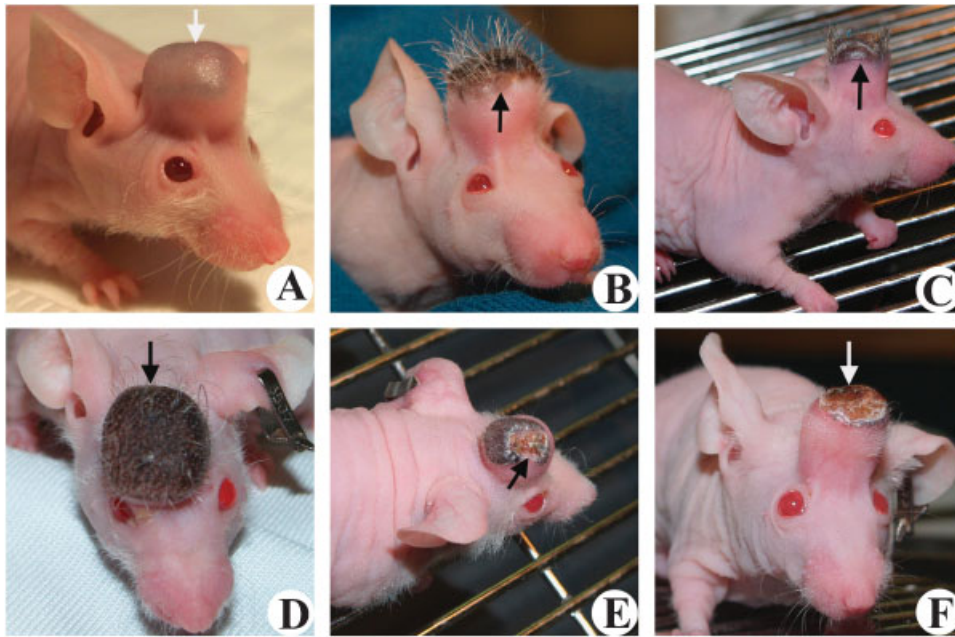


Fig. 3. Morphology of the protuberances in Experiment II. (A) Pedicle-shaped protuberance with dark-coloured apex (arrow), which resembles the one in Figure 2C. (B) Pedicle-shaped protuberance capped with deer scalp skin. Notice that deer skin had nicely healed with the surrounding mouse skin (arrow). (C) Antler-like protuberance capped with velvet-like skin, which resembles those in Figures 2E and F but with a much taller shaft. (D) Top view of an antler-like protuberance before mechanical wounding. Notice that deer scalp skin was beginning to transform to velvet-like skin, which was shiny and had sparsely populated hairs, even without mechanical wounding. (E) and (F) Antler-like protuberances from the wounding group. Notice that wounding had seriously damaged the apical velvet-like skin (arrows).

Experiment II

The gross histological structures of all the fused protuberances (24 out of 28) were comparable to counterparts in Experiment I (Fig. 5A and H). No clear correlation was detected between bone structure (compact, intermediate or cancellous) and abundance of cartilage in each protuberance. In some protuberances having shafts comprising cancellous bone, the apical growth centers were entirely occupied with cartilage tissue (Fig. 6A), whereas others had only negligible amounts of cartilage in their apical growth centers (Fig. 6B). In contrast, some protuberances in which the apical growth centers were predominantly filled with cartilage had shafts that entirely consisted of compact bone (Fig. 6C). Further, there was no clear association identified between sex hormone administration and the bone structure or cartilage content of each protuberance (Table 3).

Mechanical wounding on the apex of some antler-like protuberances caused either partial (Fig. 6D) or total (Fig. 6E) damage to the growth center and apical deer skin, and hence probably disrupted further growth of these protuberances.



Fig. 4. Morphology of the protuberances in Experiment III. (A) and (C) Pedicle-shaped protuberances with dark-coloured apices (arrows), which resemble the one in Figure 2C. A, from the noninverted co-transplantation group; and C, from the inverted co-transplantation group. (B) and (D) Pedicle-shaped protuberances capped with deer skin. Notice that the apical deer skin had grown long hairs (arrows). B, from the noninverted co-transplantation group and D, from the inverted co-transplantation group.

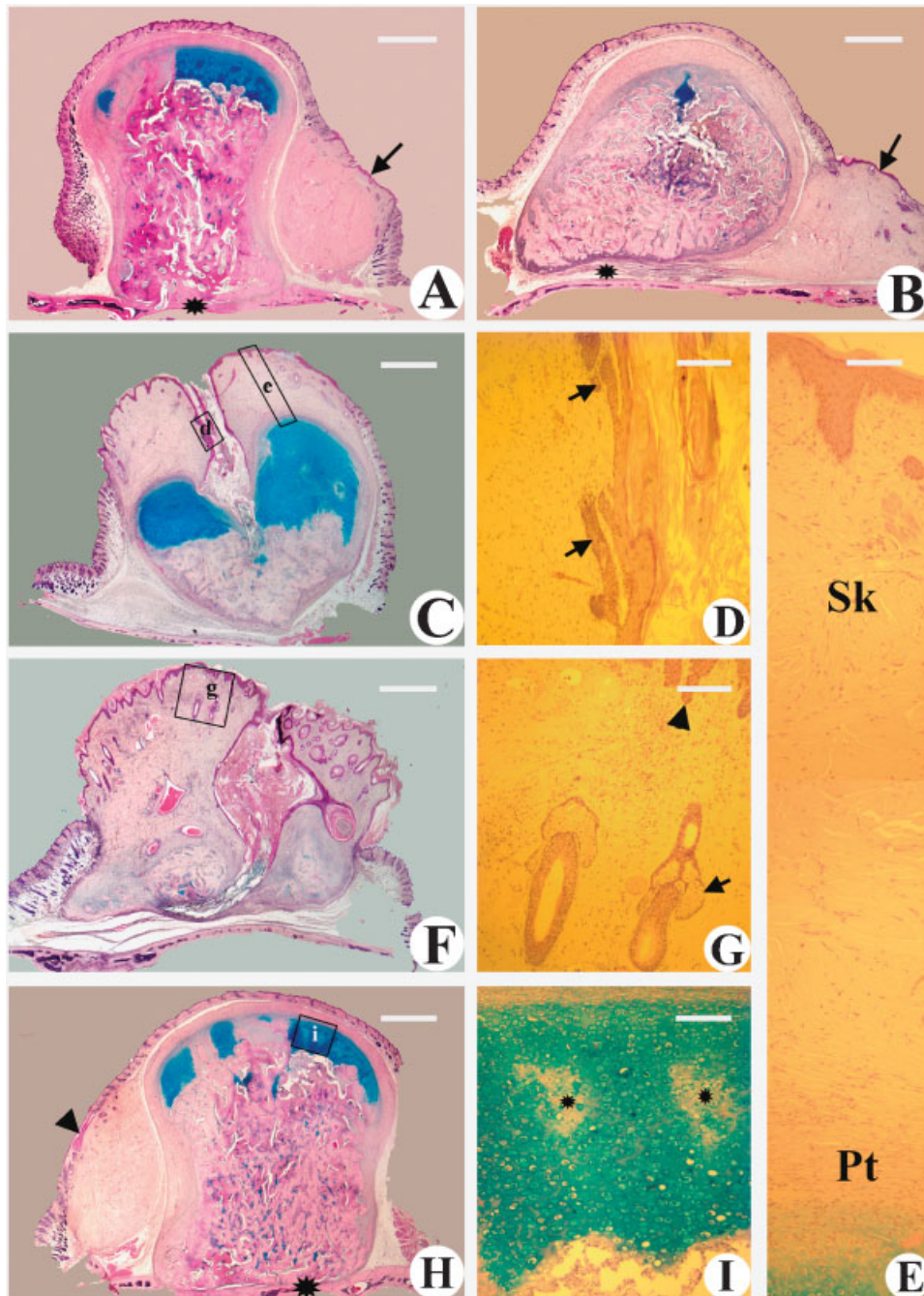
The histological structure of deer skin from the skin-only control group maintained its original features, such as thin epidermis, small mono-lobed

sebaceous glands and presence of sweat glands and arrector pili muscle (Fig. 6F and G).

Without exception, close adhesion between the AP-derived tissue and the deer skin (Fig. 7A and B) occurred in all the antler-like protuberances (13 out of 28). Fused deer skin acquired convincing velvet skin features: presence of thickened epidermis, large bi- or multi-lobed sebaceous glands and absence of sweat glands and arrector pili muscle (Fig. 7B and C). Close adhesion between

the AP-derived tissue and the enveloping mouse skin was not observed in this experiment and, except for mechanical stretch, the original features of the mouse skin were maintained (Fig. 6C).

Histological examination confirmed the finding in Experiment I that the ossification center in the core region of cartilage, which was located in the growth center of each protuberance, was initiated and expanded through direct cartilage conversion



(Fig. 7D–G), a phenomenon known as metaplasia. These protuberances were more advanced than those from Experiment I in terms of the degree of formation of the ossification centers, and they therefore further offered the opportunity to observe the formation and remodelling of bone trabeculae in these centers. Bone erosion in these centers was clearly discernable at this stage, although typical multinucleated osteoclasts were not obvious (Fig. 7G). Therefore, the bone tissue that formed through metaplasia was seemingly remodelled via osteoclasia. In contrast to the metaplastic bone formation in the cartilage core, at the osseocartilaginous interface in the shaft of each pedicle-shaped protuberance, the distal cartilage was replaced by the proximal bone tissue (lined with active osteoblasts) through the means of cartilage destruction (Fig. 7D and H), a process known as chondroclasia, although chondroclast-like cells could not be clearly observed at lower magnification. Interestingly, the chondroclasia zone in this case was much narrower and less impressive than is observed in true antler tissue formation (Banks and Newbrey, '82).

Experiment III

Close adhesion between the AP-derived tissue and the overlying deer skin (Fig. 8A and D) was observed in all four surviving mice from Groups 2 and 3 (2/group). However, the degree to which deer skin transformed to velvet clearly set apart

these two groups: inverted and noninverted co-transplantation. In the former, the deer skin in the vicinity of the mechanical injury site had acquired velvet skin features, with thickened epidermis and de novo hair follicle formation (Fig. 8B). However, in the latter the deer skin either retained the original scalp features (Fig. 8E), or had somewhat thickened epidermis, but still possessed sweat glands and arrector pili muscle (Fig. 8F). An interesting phenomenon was noticed between the cancellous bone formed from the AP cellular layer and deer skin in the inverted co-transplantation group. In these protuberances, a layer that resembled the original AP cellular layer had formed (Fig. 8C).

DISCUSSION

This study extends our previous work on the development of a nude mouse model for the investigation of antlerogenesis (Li et al., 2001). In this study, we successfully created for the first time antler-like protuberances that were capped with velvet-like skin via subcutaneous co-transplantation of the AP and deer skin and subsequent exposure by removal of covering mouse skin. Using this model we made the following new findings: (1) Antler-like head pieces can be induced on the nude mice via subcutaneous co-transplantation of AP and deer skin, and subsequent exposure through wounding. (2) Deer skin epidermis and its attached partial (as opposed

◀ Fig. 5. Histology of the protuberances in Experiment I. All sections were cut sagittally through the central region of each protuberance. Hematoxylin and eosin/alcian blue counterstaining. (A) Section of a pedicle-shaped protuberance from the group in which AP was transplanted beneath the pregrafted deer skin. Notice that the protuberance was fused to the mouse skull (asterisk), and deer skin was located at the base of the protuberance (arrow). Bar = 1.5 mm. (B) Section of a dome-shaped protuberance from the same group as in Figure 5A. Notice that the protuberance was not fused to the mouse skull (asterisk), and deer skin was also located at the base of the protuberance (arrow). Bar = 1.22 mm. (C) Section of a dome-shaped and unfused protuberance from the AP+deer skin group. Notice that mechanical wounding had damaged the growth center, and that deer skin had closely adhered to the underlying intact AP-derived cartilage and showed velvet skin features, such as thickened epidermis. Bar = 1.33 mm. (D) Higher magnification of the area labelled “d” in Figure 5C to show that the downwardly growing epidermis had acquired some velvet-like features, such as de novo hair follicle formation (arrows). Bar = 163 μ m. (E) Higher magnification of the area (montage) labelled “e” in Figure 5C to show that the deer skin (Sk) and AP-derived tissue (Pt) had closely adhered together. Bar = 111 μ m. (F) Section of a dome-shaped and unfused protuberance from the AP+deer skin group. Notice that mechanical wounding had seriously damaged the growth center, which had probably disrupted further growth of the protuberance. Interestingly, deer skin had nicely adhered to the underlying intact AP-derived osseocartilage and showed velvet skin features (see the next image in this figure). Bar = 1.06 mm. (G) Higher magnification of the area labelled “g” in Figure 5F to show the velvet-like skin features of the adhered deer skin. These features include de novo formation of hair follicles (arrowhead), multi-lobed sebaceous glands (arrow) and absence of sweat glands. Bar = 194 μ m. (H) Section of a pedicle-shaped and fused protuberance from the group in which AP was transplanted beneath the pregrafted deer skin. Notice that two ossification centers started to initiate in the core region of cartilage (labelled “i”) that was located in the tip of the protuberance. The deer skin was located at the base of the protuberance (arrowhead). Bar = 1.44 mm. (I) Higher magnification of the area labelled “i” in Figure 5H to show the two newly initiated ossification centers (asterisks). Notice that these ossification centers were initiated through an unusual mechanism of metaplasia (direct conversion from cartilage to bone), rather than a classical pathway of chondroclasia (cartilage is first destroyed before being replaced by bone). Bar = 179 μ m.

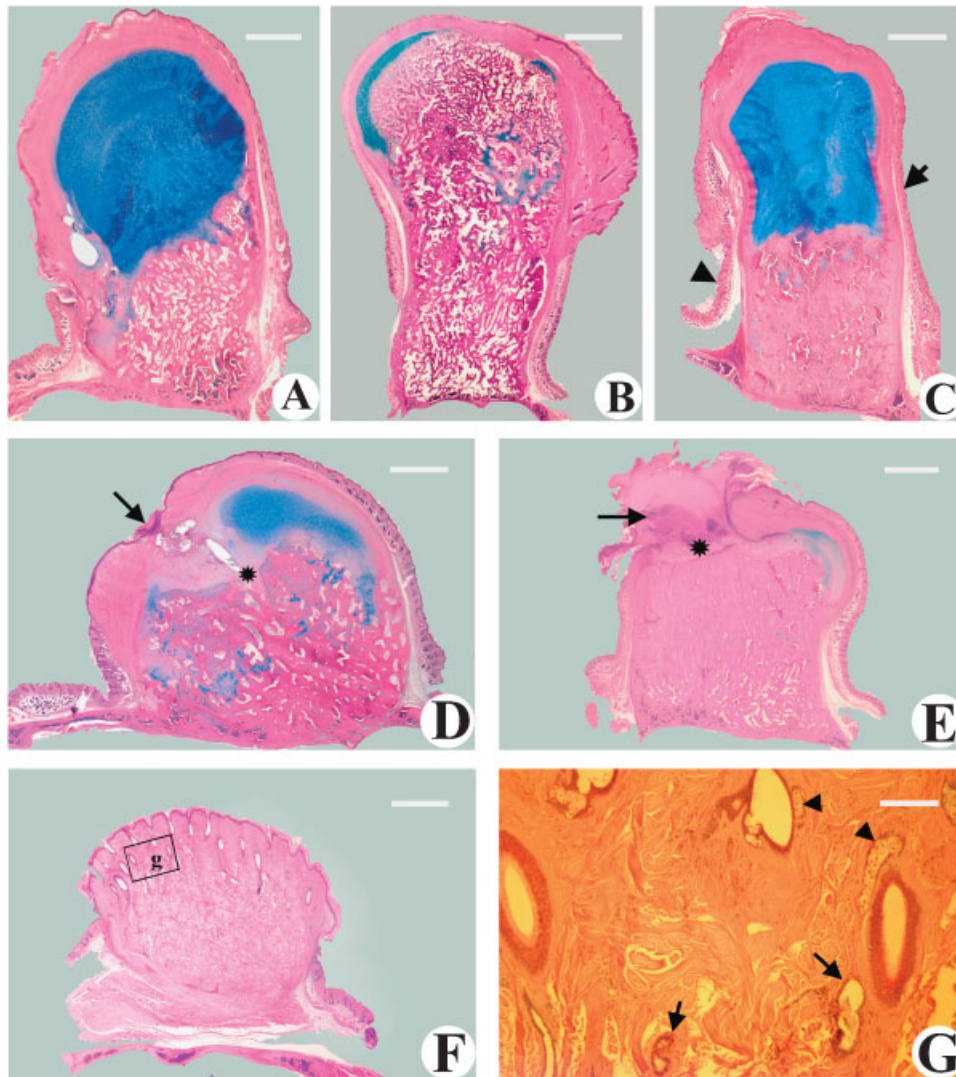


Fig. 6. Histology of the protuberances in Experiment II (1). All sections were cut sagittally through the central region of each protuberance. Hematoxylin and eosin/alcian blue counterstaining. (A–C) Three fused antler-like protuberances. Notice that there was no correlation between the bone structure (compact, cancellous or intermediate form) and abundance of cartilage mass: in A, the distal half of protuberance was entirely occupied with cartilage, and the proximal half comprises cancellous bone; in B, a negligible amount of cartilage was found in the apical growth center and the rest of the protuberance was made up of cancellous bone; whereas in C, the distal half was filled with cartilage but the proximal half was compact bone. Bar = 2.33, 2.80 and 1.80 mm in A, B and C, respectively. (D–E) Two fused dome-shaped protuberances. Notice that mechanical wounding (arrows) caused either partial (in D) or total (in E) damage to the growth center and apical deer skin. Transformation from deer skin toward velvet skin was not observed in these cases. Bar = 1.60 and 2.22 mm in D and E, respectively. (F) Section from the deer skin alone control transplantation group. Notice that at the time of tissue sampling, deer skin still kept its original scalp skin features, such as thin and undulated epidermis (also see Figure 6G). Bar = 1.40 mm. (G) Higher magnification of the area labelled “g” in Figure 6F to show that the singular transplanted deer skin did not transform to velvet-like skin, and still kept its original scalp skin features, such as small and mono-lobed sebaceous glands (arrowheads) and presence of sweat glands (arrows). Bar = 144 μ m.

to full thickness) dermis are sufficient to establish interactions with the AP-derived tissue capable of transforming adult scalp skin to velvet. (3) The putative initial inductive molecules may be derived from the distant AP cellular layer, rather than the AP fibrous layer naturally adjacent to skin. (4) Initiation of ossification in the core

avascular cartilage region in each mouse “antler” takes place via unusual metaplastic osteogenesis, rather than classical endochondral ossification. (5) Fusion between the mouse skull and the grafted AP tissue substantially shortens the time required for transformation from scalp skin to antler velvet. Overall, the nude mouse body can

TABLE 3. Bone structure and cartilage content of 27 protuberances exposed to different sex hormone treatments

Group	Dose of sex hormone (amount/volume)	Bone structure			Cartilage content		
		Compact	Intermediate	Cancellous	High	Medium	Low
<i>No sex hormone (saline control)</i>							
1	–/30 μL		2	1	1	1	1
<i>Testosterone</i>							
2	1.5 mg/30 μl	2	2	2	2	2	2
3	2.5 mg/50 μL	2	1		2		1
4	5.0 mg/100 μl	2		1	1		2
<i>Estradiol</i>							
5	7.5 ng/10 μL		3	3	2	1	3
6	11.3 ng/15 μl	2		1			3
7	22.5 ng/30 μL	1	1	1	3		

provide a milieu sufficient to sustain the heterotypic tissue interactions of xenografts. Because organogenesis, including deer antler formation and regeneration, relies on heterotypic tissue interactions, the nude mouse system can be developed as a novel model for the study of these biological processes.

It is open to speculation why injection of exogenous sex hormones did not induce the mouse “antlers” to fully calcify. It is possible that the injected exogenous hormones were converted into inactive metabolites given that 3 weeks after the injections we failed to detect elevation of these hormones. Alternative approaches are required to overcome this problem if the mechanism of antler regeneration is to be studied using the nude mouse model.

Key tissue types for interactions and the origin of the initial inductive signal

Organ formation (Gilbert, 2003) and regeneration (Stocum, 2006) rely on heterotypic tissue interactions. Tissue interactions involved in antler development are unique in that they take place in postnatal life between two interactive tissue types (periosteum and epidermis of the overlying skin) separated by distances that, on a histological scale, are exceedingly long (over 1 mm), and yet these interactions are potent enough to transform the adult deer scalp skin into a unique pelage, known as antler velvet (Li and Suttie, 2000; Li et al., 2008). In our previous studies we demonstrated that both antler generation (Li et al., 2008) and regeneration (Li et al., 2007) only require transient tissue interactions, achieved via diffusible molecules. However, those studies neither established whether all tissue types (subcutaneous

connective tissue and dermis) interposed between AP (inducer) and epidermis of the overlying skin (responder) are necessary for the establishment of these interactions, nor the precise tissue origin (AP fibrous or cellular layer) of the initial inductive signals. In this study, we used our nude mouse model to explore these questions. The results showed that without SLCT and its attached partial dermis, AP-derived tissue transformed deer scalp skin into antler velvet at both morphological and histological levels. Therefore, only AP, skin epidermis and its adhered partial dermis are required for the establishment of the inductive interactions. Interestingly, deer scalp skin in the inverted co-transplantation group transformed to velvet-like skin at the histological level earlier than in the noninverted co-transplantation group. These results have recently been confirmed by us in a deer model, with the transplantation of AP tissue in the inverted orientation (cellular layer facing the skin) having initiated transformation of overlying skin from scalp to velvet at least a month earlier than transplantation in the noninverted orientation (Unpublished data). The implication is that the initial inductive signals are derived from the distant AP cellular layer rather than the fibrous layer naturally adjacent to the skin.

Incorporating the results from this study, we have further refined our working hypothesis (Li et al., 2008) for this novel type of tissue interaction. This is illustrated graphically in Figure 9. The cellular layer cells of AP release the instructive diffusible molecules, which traverse the periosteal fibrous layer, compressed SLCT layer and its associated partial dermis to act in a long-distance-paracrine manner on the dermal cells roughly at the level where dermal papilla

cells reside (Fig. 9A1). The altered dermal cells exert their influence via paracrine and juxtacrine (Rendl et al., 2008) mechanisms on the overlying epidermis, which then transforms into antler velvet (Fig. 9A2). In turn, the transformed epidermal cells send instructive feedback signals in a similar paracrine and juxtacrine manner, to the dermis (Fig. 9B1), which then relays the signal back to the periosteal cells to initiate first antler formation (Fig. 9B2).

Nature and fate of the preosseous tissue/ avascular cartilage

The history of antler research is riddled with controversy over the presence of cartilage. What makes one immediately think that antler preosseous tissue is not true cartilage is the vascular nature of the tissue, as typical hyaline cartilage is

a relatively avascular tissue. Although the cartilaginous nature of these mature cell types has been confirmed by ultrastructural evidence (Banks and Neal, '70; Banks, '73; Banks, '74; Banks and Newbrey, '82) and histochemical studies (Frasier and Banks, '73), the vascular system of the tissue has always been a topic of debate. Thus far, the preosseous vascularized tissue has been described as cartilaginous (Lojda, '56), "chondroid" (Wislocki et al., '47; Beresford, '80) and "fibrocellular" (Kuhlman et al., '63). However, Banks and Newbrey ('82) considered the tissue as true cartilage and inferred that the rich vascularization may be a reflection of the high metabolic demands of fast growth.

Interestingly, in this study AP (antler stem cells), once grafted to nude mice, formed avascular tissue. The appearance of chondrocyte-like cells residing in cartilage-like lacunae and the presence of a high abundance of proteoglycans (shown by deep alcian blue staining) indicated the cartilaginous nature of the avascular tissue. Therefore, we propose that the vasculature of antler cartilage is not constitutive, but rather is caused by extrinsic factors, such as the metabolic demands suggested by Banks and Newbrey ('82). If that is the case, then the degree of vasculature

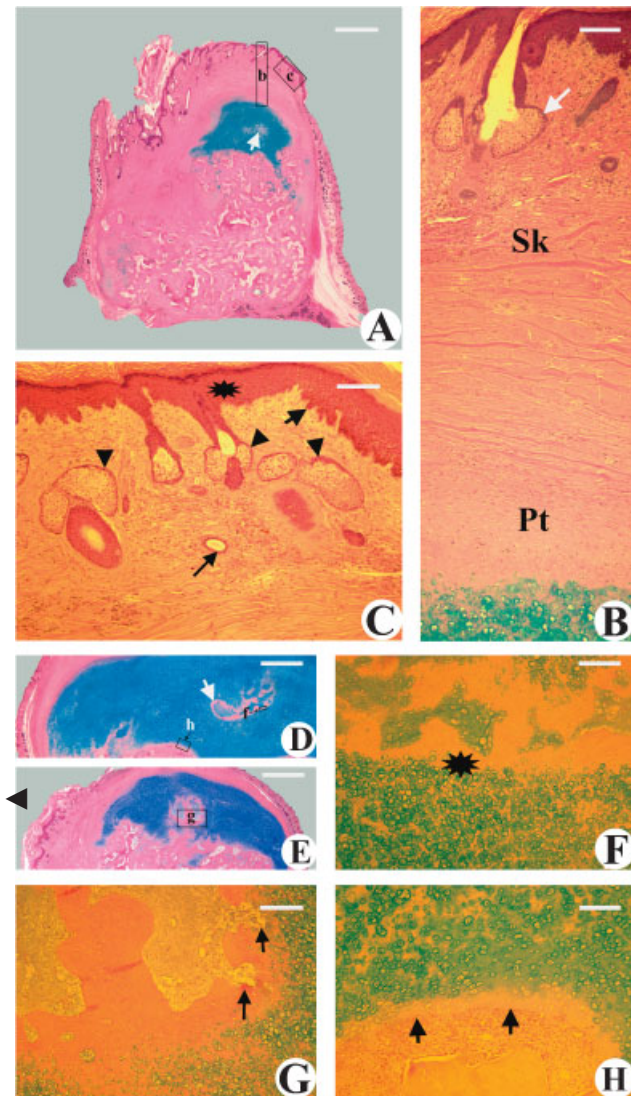


Fig. 7. Histology of the protuberances in Experiment II (2). All sections were cut sagittally through the central region of each protuberance. Hematoxylin and eosin/alcian blue counterstaining. (A) Section of a fused and antler-like protuberance to show the velvet-like skin features of its capped deer skin (seen more clearly at higher magnification in the next two images), and an early ossification center in the core region of the cartilage (arrow). Bar = 1.69 mm. (B) Higher magnification of the area labelled "b" in Figure 7A. Notice that deer skin (Sk) was nicely adhered to the underlying AP-derived tissue (Pt), and had multi-lobed sebaceous glands (arrow). Bar = 130 μ m. (C) Higher magnification of the area labelled "c" in Figure 7A to show the typical features of velvet skin: thickened epidermis (asterisk), multi-lobed sebaceous glands (arrowheads), de novo formation of hair follicles (short arrow) and absence of sweat glands. Long arrow points to a blood vessel. Bar = 124 μ m. (D) Region from the distal portion of an antler-like protuberance to show a newly initiated ossification center (arrow) in the core region of cartilage tissue. Bar = 0.90 mm. (E) Region from the distal portion of another antler-like protuberance to show the remodelling process in an ossification center (labelled "g") that was formed via metaplasia. Bar = 1.66 mm. (F) Higher magnification of the area labelled "f" in Figure 7D. Notice that the ossification center was initiated and expanded via metaplasia (asterisk), rather than chondroclasia. Bar = 154 μ m. (G) Higher magnification of the area labelled "g" in Figure 7E. Notice that the ossification center was remodelled via osteoclasia (arrows). Bar = 176 μ m. (H) Higher magnification of the area labelled "h" in Figure 7D. Notice that the distal cartilage was replaced via chondroclasia (arrows). Bar = 136 μ m.

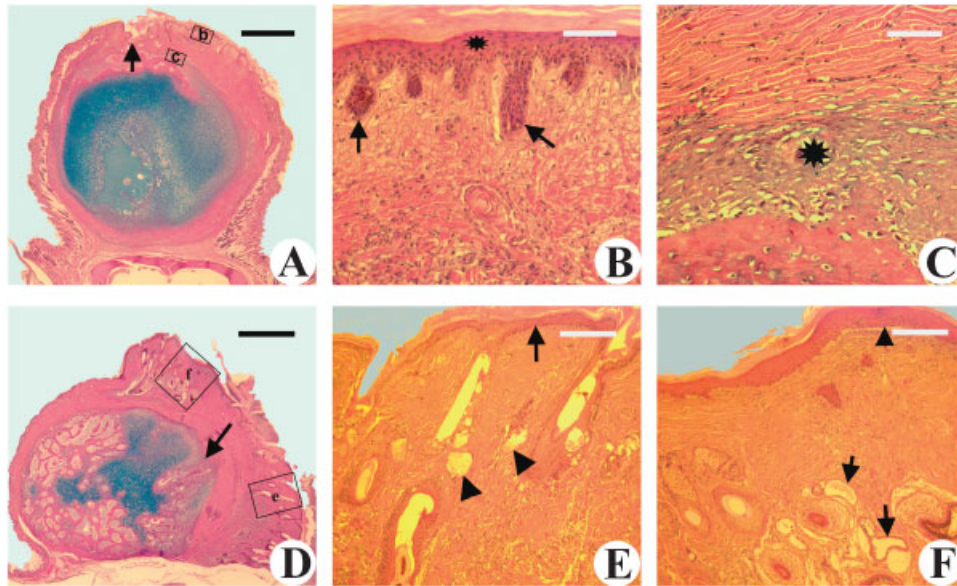


Fig. 8. Histology of the protuberances in Experiment III. All sections were cut sagittally through the center region of each protuberance. Hematoxylin and eosin/alcian blue counterstaining. (A) Section of an unfused and dome-shaped protuberance from the inverted co-transplantation group to show that the apical deer skin had closely adhered to the cancellous bone formed from the cellular layer, and the wounding mark (arrow). Notice that deer skin in the vicinity of wounding site acquired some velvet skin features (see next image). Bar = 1.11 mm. (B) Higher magnification of the area labelled “b” in Figure 8A to show the detailed features of velvet-like deer skin: the thickened epidermis (asterisk) and de novo hair follicle formation (arrows). Bar = 89 μ m. (C) Higher magnification of the area labelled “c” in Figure 8A to show a layer (asterisk), reminiscent of AP cellular layer, interposed between the AP-derived cancellous bone and the overlying deer skin. Bar = 87 μ m. (D) Section of an unfused and dome-shaped protuberance from the noninverted co-transplantation group to show that the apical deer skin closely adhered to the AP-derived tissue, and the wounding mark (arrow). Bar = 1.11 mm. (E) Higher magnification of the area labelled “e” in Figure 8D. Notice that the deer skin still kept its original scalp skin features: thin epidermis (arrow) and small sebaceous glands (arrowheads). Bar = 51 μ m. (F) Higher magnification of the area labelled “f” in Figure 8D. Notice that the deer skin in this region was at a very early stage of transformation toward velvet skin as its epidermis (arrowhead) had become thicker and its sebaceous glands (arrowhead) larger than those in Figure 8E, but it still possessed sweat glands (arrows). Bar = 167 μ m.

of antler cartilage must be proportional to the intensity of metabolic demands from the most vascular in the fastest growing antlers, such as elk antler with 2.75 cm/day (Goss, '70), to the least vascular in the slowest growing antler, such as the nude mouse “antlers” (this study).

Equally as controversial as the nature of the vascularized preosseous tissue in antler is the fate of this tissue type, which has also been intensively debated in the last century. Some investigators (Macewen, '20; Wislocki et al., '47; Goss, '70) concluded that cartilage formed during antler growth is directly converted into bone (i.e. metaplastic osteogenesis), whereas others (Davis, '62; Banks and Newbrey, '82) reported that antler development occurs by classical endochondral ossification that includes cartilage development, calcification, removal and replacement by bone. The authors from the latter group convincingly demonstrated that parallel arrays of smooth cartilaginous trabeculae in antlers are converted to rough, irregular columns by the

activity of chondroclasts, a phenomenon that has also been observed more recently in some studies (Kierdorf et al., '94; Li and Suttie, '94; Kierdorf et al., 2003; Li et al., 2005). Banks ('74) initially thought that subtle differences in the antler ossification process among deer species may have been responsible for the interpretive differences reported in the literature, but later hypothesized (Banks and Newbrey, '82) that subtle differences between calcified cartilage and woven bone may instead have been the basis of the various interpretations.

Interestingly, the results from this study clearly showed that both metaplastic osteogenesis and endochondral ossification took place (co-existence) in the cartilage tissue of a mouse “antler”. The former occurred in the central region of cartilage via direct conversion from cartilage to bone (Fig. 7D and F), whereas the latter occurred proximodistally at the interface of bone and cartilage tissue via chondroclasia (Fig 7H). To enable the latter process to take place, cartilage-

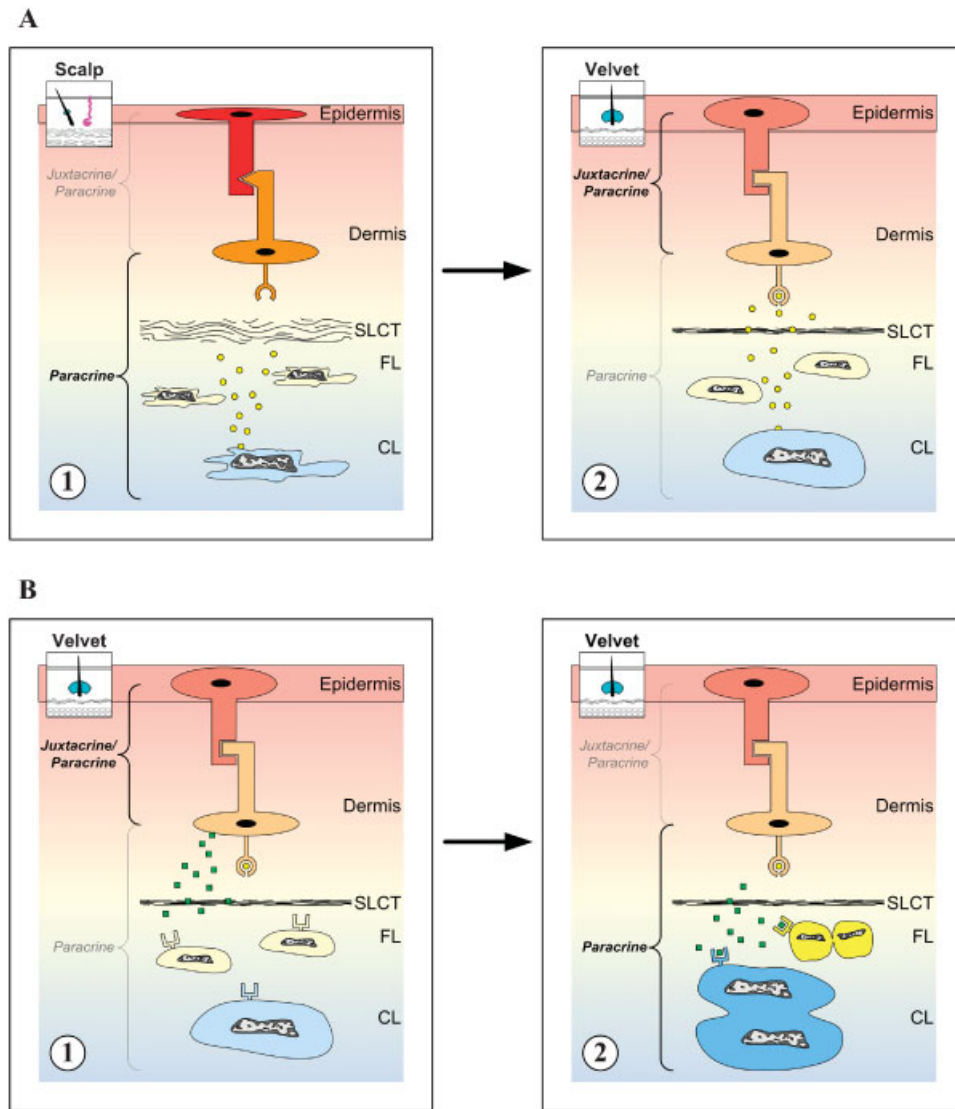


Fig. 9. Schematic representation of the long distance paracrine interactions between antlerogenic periosteum and the overlying skin during antlerogenesis. **(A)** Induction process. (1) Antlerogenesis is initiated by the secretion of putative signal molecules (small circles) by cells of the cellular layer (CL) of antlerogenic periosteum. These molecules diffuse through the fibrous layer (FL), compressed subcutaneous loose connective tissue (SLCT) and its attached partial dermis to reach and act on the dermal cells at the level of dermal papilla cells. (2) The altered dermal cells exert their influence via both paracrine and juxtacrine mechanisms on the overlying epidermis (angled hair shaft, mono-lobed sebaceous glands, presence of sebaceous glands), which is then transformed into antler velvet (displaying the presence of vertical hair shaft, large multi-lobed sebaceous glands, the absence of sweat glands and a thickened epidermis). **(B)** Feedback process. (1) Feedback is received by the altered dermis from the thickened (induced) epidermis via both paracrine and juxtacrine mechanisms. Subsequently, the dermal cells secrete putative feedback molecules (small squares), which traverse its attached partial dermal tissue and the SLCT. (2) These molecules directly act on the periosteal cells. It is these feedback signals that stimulate periosteal cells to rapidly proliferate and differentiate and thus grow an antler.

resorbing cells must be preformed. Formation of these cells requires the availability of precursor cells of macrophage and osteoblasts. It is known that nude mouse body can provide functional macrophages (Cheers and Waller, '75) and the grafted AP is the perfect source of preosteoblasts for the purpose (De Grooth et al., '94). Hence, it is conceivable that chondroclasia can take place

in an immune-compromised animal, the nude mouse.

Based on these findings, we would like to offer an alternative explanation for the existing interpretive differences regarding the mechanism leading to antler ossification. Normally, both processes of endochondral ossification and metaplastic osteogenesis could occur in the cartilage

tissue of a growing antler. Which process dominates the tissue remodelling depends on the status of antler growth. For antlers that grow slowly (owing to malnourishment, small deer species or for any other reason), a low level of vasculature and thick cartilaginous columns would be expected. Chondrocytes can only be adequately nourished within a limited distance by the closest blood vessels, as cartilaginous cells obtain their nutrients solely through diffusion (Stockwell, '79). Hence ossification inevitably occurs in the central region of these thick cartilaginous columns. We recently demonstrated using HIF-1 α in situ hybridization that the environment in the central region of antler cartilage is hypoxic (unpublished data), which supports our hypothesis. Thus, in slow growing antlers metaplastic osteogenesis would dominate the antler ossification process, although thus far it is not known why ossification takes place through metaplasia rather than cartilage replacement. Therefore, investigators sampling tissue from these antlers would claim that antler cartilage was directly turned into bone. However, tissue samples derived from rapid growing antlers, with high levels of vasculature and thin cartilaginous columns, would be expected to display endochondral ossification, in which tissue remodelling occurs by destroying and removing chondrocytes along the surface of cartilaginous columns, and subsequently attracting osteoblast progenitor cells to form bone tissue. In this case, investigators would infer that antler cartilage was replaced through classical endochondral ossification. Although this hypothesis is certainly an interesting one that deserves further testing, thus far we have not been able to find the experimental data to substantiate it. Although the hypothesis remains to be proven, it illustrates how this nude mouse model of antlerogenesis is already helping to clarify some contentious issues in antler biology. Furthermore, as it is a potentially useful tool for investigating the underlying mechanism of metaplasia, and given that metaplastic osteogenesis has been implicated in osteoarthritis (Collins et al., '94), the nude mouse model could also prove to be of great value in the study of this debilitating disease.

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