

Stem Cells Responsible for Deer Antler Regeneration Are Unable to Recapitulate the Process of First Antler Development—Revealed Through Intradermal and Subcutaneous Tissue Transplantation



CHUNYI LI^{1*}, FUHE YANG^{2,3}, STEPHEN HAINES¹,
HAIPING ZHAO^{2,3}, WENYING WANG¹, XIUMEI XING^{2,3},
HONGMEI SUN^{2,3}, WENHUI CHU^{2,3}, XIAOPING LU^{2,3},
LINLING LIU^{2,3}, AND CHRIS MCMAHON⁴

¹AgResearch Invermay Agricultural Centre, Mosgiel, New Zealand

²Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, Jilin, P. R. China

³State Key Laboratory for Molecular Biology of Special Economical Animals, P. R. China

⁴Ruakura Agricultural Centre, Hamilton, New Zealand

ABSTRACT

Antlers offer a unique model for the study of whether regeneration recapitulates development in a mammalian organ. Research, to date, supports the full recapitulation in antler, but a recent report that subcutaneously transplanted (ST) pedicle periosteum (PP) failed to induce that ectopic antler formation could argue against recapitulation, as antlerogenic periosteum (AP) can readily do so. However, it was not clear in that study whether the result was caused by inability of the PP to interact with the skin or owing to failure to create the required close contact to it. This study was designed to clarify this uncertainty by adopting intradermal transplantation (IT) to achieve the required close contact without the need for significant mass expansion. The results showed that IT of 1/8 of the original AP mass or more was sufficient for antler induction, whereas ST of 1/4–AP or less could not do so within 2 years. The minimum amount of AP required for antler induction using the IT approach was somewhere between 1/8 and 1/12–AP (<30 mg). The results further demonstrated that IT of 62–84 mg PP failed to induce ectopic antler formation, even if the PP had fused with the surrounding skin. Because this mass of PP was 2–3 times the minimum amount of AP required for antler induction, we conclude that PP does not recapitulate AP in induction of ectopic antler development. It is likely that PP has been restricted for antler regeneration and lost the potential to initiate antler development. *J. Exp. Zool. (Mol. Dev. Evol.)* 314B, 2010. © 2010 Wiley-Liss, Inc.

How to cite this article: Li C, Yang F, Haines S, Zhao H, Wang W, Xing X, Sun H, Chu W, Lu X, Liu L, McMahon C. 2010. Stem cells responsible for deer antler regeneration are unable to recapitulate the process of first antler development—revealed through intradermal and subcutaneous tissue transplantation. *J. Exp. Zool. (Mol. Dev. Evol.)* 314B:[page range].

J. Exp. Zool. (Mol. Dev. Evol.) 314B, 2010

Establishing whether regeneration of an organ recapitulates the process of its development can provide valuable insights into growth for regenerative medicine (Stocum, 2006). However, thus far, only few studies have addressed regeneration of mammalian organs (Dor and Stanger, 2007). This situation has undoubtedly

Grant Sponsors: Chinese Natural Science Foundation; New Zealand Foundation for Research, Science and Technology.

*Correspondence to: Chunyi Li, AgResearch Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand. E-mail: chunyi.li@agresearch.co.nz

Received 30 March 2010; Revised 30 April 2010; Accepted 9 May 2010

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/jez.b.21361

been caused by the lack of availability of models of mammalian organ regeneration, owing to the fact that during evolution most mammalian organs have lost the ability to grow back when damaged or lost as a result of disease, accident, or amputation (Carlson, 2007). For the few exceptions that do regenerate to some extent, it is debatable whether they truly reflect full (molecular, cellular, and tissue levels) or only partial recapitulation of development. For example, adult mammalian liver is unique in that it can restore itself at the tissue and functional levels after injury (Michalopoulos and DeFrances, '97). However, this restoration has been described as hepatocyte hyperplasia rather than true regeneration, as at a transcriptional level this regeneration does not call up development (Otu et al., 2007). Most conclusions drawn thus far about mammalian organ recapitulation (Simon et al., '97; Vlaskalin et al., 2004) have been based on an extrapolation of regeneration studies in newts, the lower vertebrates that have great ability to completely grow back severed or damaged limbs (Gardiner and Bryant, '96). Suitable mammalian models of organ regeneration are lacking at present, but are highly desired if successful strategies are to be devised for the restoration of damaged organs or limbs. One plausible mammalian model is the use of deer antlers.

Deer antlers are the only mammalian organs that, once lost, can fully regenerate (Goss, '83). Therefore, they offer a unique opportunity to learn whether mammalian epimorphic regeneration, a phenomenon of *de novo* formation of appendages distal to the level of amputation, recapitulates development. Advantages of the antler model include the fact that they are external organs, thus facilitating access and observation, and both development and regeneration naturally occur in the same animals during postnatal life, which allows more comprehensive comparisons to be made. Uniquely, antler development and hence regeneration can be artificially induced elsewhere on the deer body by a simple transplantation of antlerogenic periosteum (AP), the bone membrane that overlies the frontal crests of pre-pubertal deer (Hartwig and Schrudde, '74). Therefore, not only associative but also functional recapitulation can be evaluated using the deer antler model.

Experimental data, reported to date, support the concept that antler regeneration fully recapitulates development. Gene expression studies carried out using regenerating antlers demonstrate that the molecular mechanisms of development are recapitulated (Faucheux et al., 2004; Mount et al., 2006). At the cellular level, antler development (Hartwig and Schrudde, '74; Goss and Powel, '85; Li and Suttie, 2001) and regeneration (Li et al., 2007a) rely on distinctive populations of cells, which respectively reside in AP and in the periosteum (PP) that envelops the pedicle (i.e. the permanent bony protuberance from which an antler drops off and subsequently regenerates). The cells from both AP and PP have been demonstrated to be adult stem cells for their self-renewal, expression of stem cell markers, and multipotency (Rolf et al., 2008; Li et al., 2009), suggesting cellular

recapitulation. Both antler development and regeneration are triggered by the transient interactions between the skin and underlying periosteal tissue—AP in the case of development (Li et al., 2008) and PP for regeneration (Li et al., 2007b). AP and PP first causes the transformation of the deer scalp skin into specialized pelage called antler velvet and, in turn, feedback from the antler velvet induces the periosteal cells to proliferate and differentiate to build up antler tissue. This then undergoes three ossification stages (Li and Suttie, '94; Li et al., 2005)—intramembranous, transitional, and finally modified endochondral ossifications—indicating a full spatiotemporal recapitulation at a tissue level.

While carrying out a tissue deletion and transplantation experiment in order to identify the stem cells responsible for antler regeneration, Li et al. (2007a) observed that subcutaneously transplanted PP failed to induce ectopic antler development, whereas the grafted AP can readily do so (Hartwig and Schrudde, '74; Goss, '90). This result raises the possibility that this may not be the case of recapitulation. However, it is not clear whether this exception was caused by the inability of the PP to interact with the overlying skin or owing to a failure to create the required close contact between the two tissue types. In that study, the grafted PP did not grow and expand from its original mass to the extent that could create significant mechanical tension to the overlying skin, the manner in which close contact between AP-derived tissue and overlying skin is achieved during natural antler development (Li and Suttie, 2000). It is known that an initial minimum tissue mass (at least half the original AP) is required for the grafted AP to expand enough to create a significant mechanical pressure on the overlying skin, which results in their close contact (Goss and Powel, '85). It is this close physical contact that is prerequisite for the establishment of the interactions between these two tissue types, which in turn trigger antler development (Goss, '90; Li and Suttie, 2000; Li et al., 2008). It was clearly shown that grafted PP did not have the ability to significantly expand its mass through growth (Li et al., 2007a). Therefore, to confirm whether or not PP recapitulates ectopic antler induction, it was necessary to find alternative means of creating the requisite close contact with skin without subjecting PP tissue to substantial expansion. We hypothesized that this could be achieved by intradermal transplantation (IT) of the PP tissue, rather than subcutaneous transplantation (ST), as used earlier, i.e. by delivery of PP tissue directly into the skin. We further hypothesized that, because of the close association created immediately between the grafted tissue and the skin, less initial tissue mass would need to be transplanted by this method than by ST in order to induce development of an antler.

The aims of this study were to determine whether (1) IT was a valid approach for antler induction; (2) IT approach could substantially reduce the minimum AP mass required in ST for induction of antler formation, and (3) PP could induce antler development through the IT approach.

MATERIALS AND METHODS

Experimental Animals and Design

Animals

This study consisted of two separate experiments, involving transplantation of AP tissue in the first and PP tissue in the second (Table 1). In the first, performed in the Northern hemisphere (China), nine 10-month-old male Sika deer (*Cervus nippon*) calves were selected for AP transplantation at the time of pedicle initiation. For the second experiment, performed in the Southern hemisphere (New Zealand), three 2-year-old red deer (*C. elaphus*) stags were selected for PP transplantation just before antler regeneration. The animals were maintained either outside on pasture for red deer (routine farming scheme in New Zealand) or inside in an enclosure for sika deer (routine farming scheme in China), during the experimental period. The reason to change species from Chinese sika deer to New Zealand red deer was to enable us to commence the experiment 6 months early and progress the overall study more rapidly than if it were to be conducted within a single hemisphere. We believe this was justified because, to the best of our knowledge, there is essentially no difference between these two deer species in the ability to induce ectopic antler formation by transplanted AP (Li et al., 2001, 2008).

Design

AP transplantation experiment (Northern hemisphere). Nine sika deer were randomly allocated into three groups, each consisting of three deer. Two types of AP transplantation, IT and ST, were performed on the head of each deer with the same amount of AP tissue grafted at each of the two sites (Table 1). This amount was one-quarter, one-eighth, and one-twelfth of full-size AP tissue for deer in Group 1 ("1/4-AP" group), Group 2 ("1/8-AP" group), and Group 3 ("1/12-AP" group), respectively. AP transplantation took place during the period of normal pedicle initiation in order to maximize the possibility of ectopic antler induction.

The rationale for this design was as follows: It had been shown that subcutaneous transplantation of the total, one-half or one-quarter of the AP tissue from a single future antler growth region resulted within 2 years in 100, 20 and 0% ectopic antler induction, respectively (Goss and Powel, '85). Therefore, if induction of an ectopic antler succeeded following intradermal transplantation of one-quarter of the AP tissue mass, this would demonstrate that the requisite close association with skin could be achieved by this approach, without requiring substantial expansion of AP tissue through subsequent growth. Treatments that involved the use of smaller quantities of AP tissue were included in the experiment, in order to try and establish the minimum amounts of transplanted tissue required for antler induction using IT, as compared with the control ST method. The results would also give an indication of how much PP tissue should be grafted in the subsequent experiment, which would be performed in the Southern hemisphere.

PP transplantation experiment (Southern hemisphere). Three red deer stags were selected for PP transplantation and, as in the earlier experiment, both IT and ST were performed on the head of each deer. The age of the stags was chosen based on the fact that the pedicles of 2-year-old red deer stags are long enough (around 4.5 cm) to provide sufficient PP tissue for transplantation and their scalp skin thick enough (at least twice that of yearlings) to facilitate the cutting of intradermal pockets using a scalpel. The experiment was carried out in September, a month before antler regeneration (in the Southern hemisphere), to maximize the chance of ectopic antler induction.

The selection of the quantity of PP tissue for IT was based on the minimum amount of AP tissue found in the first experiment, to be sufficient for antler induction by that transplantation method. By delivering more than that minimum amount of tissue, we aimed to ensure that the quantity of tissue transplanted would not be limiting to the chances of successfully inducing antler growth. For each deer, we loaded as much PP tissue as the intradermal pocket could comfortably accommodate, and left-over PP tissue was used for the control ST treatment (Table 1).

Autologous transplantation (grafting back to the same animal) was used in both experiments, and all the transplantation surgery was carried out by one person (C Li).

AP/PP Collection and Transplantation

Tissue collection and transplantation experiments had full approval from the AgResearch Invermay Animal Ethics Committee in New Zealand. All surgeries in these experiments were conducted under general anesthesia using intravenous fentanyl citrate/azaperone/xylazine hydrochloride (Fentazin 5, Parnell Laboratories Ltd., Auckland, New Zealand) at a dose rate of 1.8 mL/100 kg live weight. The anesthesia was reversed with naloxone/yohimbine (Contran H, Parnell Laboratories Ltd., Auckland, New Zealand). Long-acting Penstrep LA (BOMAC Laboratories Ltd., Auckland, New Zealand) was injected subcutaneously following each surgery. Animals from each experiment were observed daily for 7 days after surgery, weekly thereafter, and photographed when necessary.

Collection

The detailed procedures for AP and PP collection have been reported elsewhere (Li and Suttie, '94; Li and Suttie, 2003; Li et al., 2007a). Frontal crest or pedicle on one side of each animal was randomly selected for AP or PP sampling, respectively. Each piece of sampled AP or PP was immediately placed in a 50 mL centrifuge tube containing 20 mL of sterile 2% penicillin/streptomycin DMEM medium (Invitrogen), and was then cut into small explants (each around 1 mm²) in a laminar flow hood. The small minced explants of each sampled AP piece were thoroughly washed in media and then divided based on the number of explants into one-quarter, one-eighth, or one-twelfth aliquots. These aliquots were immediately transplanted back to deer in the 1/4-AP, the 1/8-AP, and the 1/12-AP groups, respectively. In the

Table 1. Intradermal and subcutaneous transplantations of AP or PP.

Experiment (species, hemisphere)	Group	Tissue	Deer #	Sample site (pedicle height) (mm)	Date of surgery	Graft type	Mass of tissue (mg)	Time to appearance of antler (days)	At time of tissue sampling	
									Days after graft	Outgrowth size (H × W × L) (mm)
I (sika deer, Northern)	1	1/4 AP	1	R (13)	April 16	IT	60	118	150	19.0 × 13.4 × 14.7
						ST	60	None	150	8.9 × 24.9 × 15.4
			2	L (5)	April 11	IT	60	62	122	11 × 13.6 × 13.9
						ST	60	None	122	Palpable
			3	R (5)	April 11	IT	60	65	155 ¹	5 × 10 × 9.5
						ST	60	None	155 ¹	5 × 10 × 9.5
	2	1/8 AP	4	R (<5)	April 9	IT	30	58	124	14.5 × 12.7 × 14.2
						ST	30	None	124	Palpable
			5	R (8)	April 17	IT	30	57	144	6.8 × 8.9 × 10.0
						ST	30	None	144	Palpable
			6	R (13)	April 9	IT	30	114	435	7.7 × 15.7 × 26.1
						ST	30	None	435	6.6 × 15.2 × 19.0
II (red deer, Southern)	3	1/12 AP	7	R (<5)	April 9	IT/ST	20/20	None/none	455	None/none
			8	R (<5)	April 16	IT/ST	20/20	None/none	454	None/none
			9	R (8)	April 9	IT/ST	20/20	None/none	456	None/none
			10	L (45)	September 29	IT/ST	78/131	None/none	431	None/none
			11	R (45)	September 29	IT/ST	62/156	None/none	431	None/none
			12	L (50)	September 29	IT/ST	84/257	None/none	431	None/none
	1	PP								
	2	PP								
	3	PP								

AP, antlerogenic periosteum; PP, pedicle periosteum; IT, intradermal transplantation; ST, subcutaneous transplantation; R, right; L, left; H, height; W, width; L, length.

¹At final observation, as the tissue was not sampled.

case of minced PP, the required amount from each deer was subsampled after weighing for autologous IT, and the remainder was set aside for ST.

Both AP and PP were minced before transplantation, in order to homogenize each tissue type. This was considered necessary because it has been demonstrated that the antlerogenic potential

of the central portion of AP is greater than that of its outer regions (Goss and Powel, '85), as is also the case for PP from different sides (anterior, posterior, lateral, and medial) of a pedicle (Goss, '61, '95). Because mincing of AP before transplantation does not abrogate formation of well-organized antlers (Goss, '90), we felt confident this homogenization would allow us to

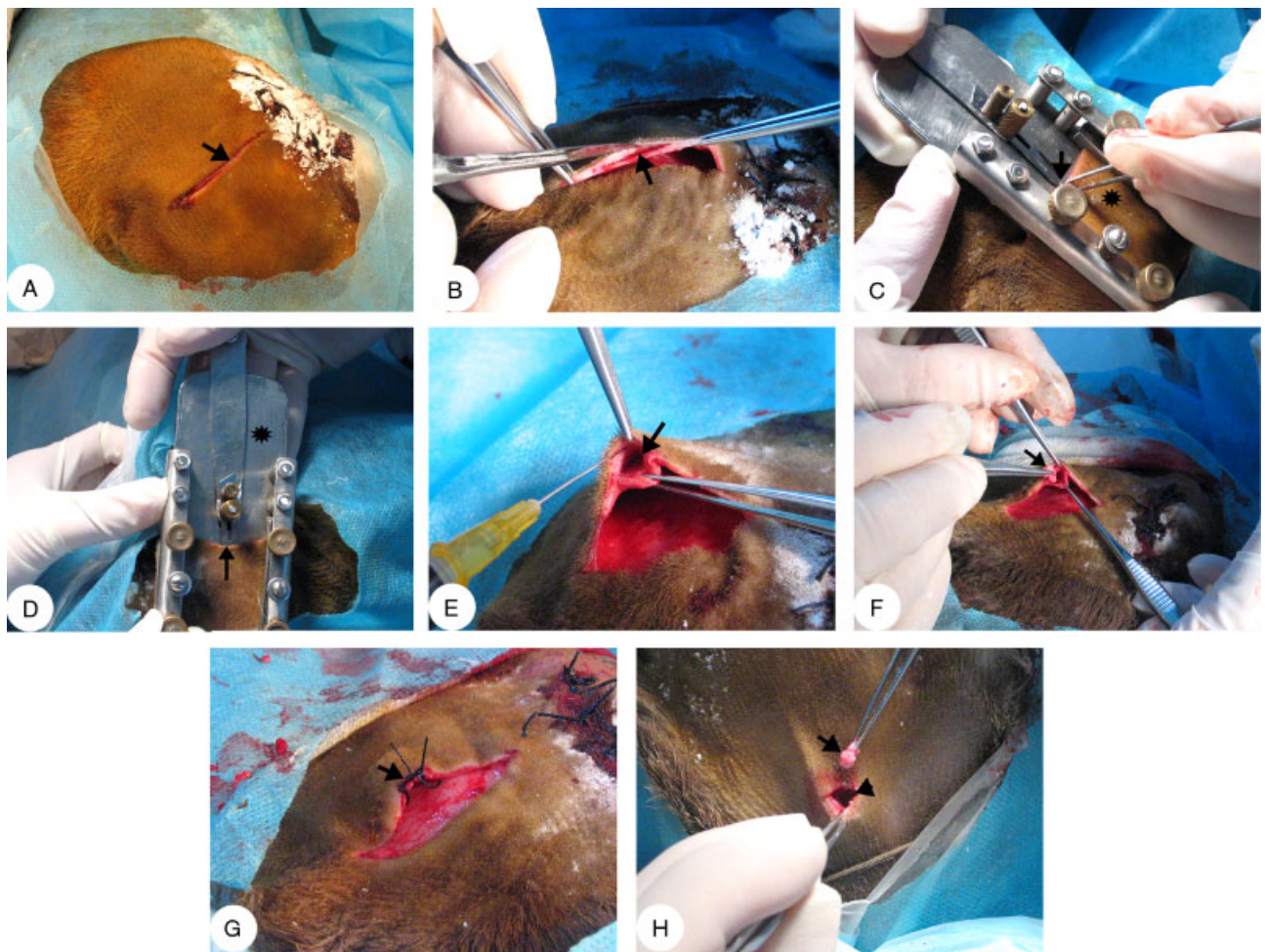


Figure 1. Antlerogenic periosteum (AP) transplantation surgery. (A) A 45 mm long skin incision (arrow) was positioned centrally and cut coronally along the line drawn between the anterior points of the two frontal crests using a scalpel. (B) A 20 mm long and 1–2 mm deep skin incision (arrow) was made on the cut surface of the skin at rostral side using a scalpel, in order to guide the purpose-built device-attached blade at the initial slicing stage. (C) The base of the device was inserted under the skin through the 45 mm long skin incision and the skin was then tightly clamped (asterisk) when the tip of the device-attached blade was properly inserted into the 1–2 mm deep skin incision (arrow) with the help of a pair of forceps. (D) The 1–2 mm deep skin incision was extended to form an intradermal pocket (10–15 mm in depth) by rotating the handle, to which the blade (arrow) was attached, of the device between 10 o'clock and 2 o'clock while the blade was moving forward inside the skin along the slot in the device base (asterisk). (E) An intradermal pocket (arrow) made using the device was opened using two pairs of fine forceps. (F) A pellet of the minced AP (arrow) was carefully loaded into an intradermal pocket. (G) A suture (arrow) was used to seal the opening of an AP-loaded intradermal pocket. (H) A 15 mm long incision was made coronally in the skin rostral to the IT site and a subcutaneous pocket was created anteriorly by blunt dissection via the incision. The pellet of minced AP (arrowhead) was then loaded into the pocket (arrowhead).

determine whether induction of ectopic antler formation would be more readily achieved by IT than by ST, when grafting small explants of the original pieces of tissue.

Transplantation

Each site of operation was thoroughly shaved and then sterilized with 70% alcohol and 1% iodine tincture. Aliquots of freshly minced AP or PP tissue were transplanted using either IT or ST approach. All skin incisions were closed using nonabsorbable suture (2-0 black braided silk, Ethicon, NJ).

AP. It was necessary to use a custom-built device to create an intradermal pocket in the scalp skin of the yearling sika stags because, at that age, the skin is very thin (1.5–2 mm). As a result, it would have been almost impossible to precisely cut an intradermal pocket without damaging hair follicles or puncturing the pocket simply by use of a handheld scalpel. The intact hair follicles are considered to be necessary because the interactions that lead to antler development require the presence of hair follicles (Goss, '87), and partial deer skin can successfully participate in antler formation so long as its hair follicle layer is still intact (Li et al., 2008). The device consisted of two main parts: a base with two parallel metal clamps located at both edges to hold the skin flat and tightly extended and a handle for

manipulation of an attached scalpel blade (Fig. 1C and D). The handle was attached to the base by a screw (3 mm diameter), which protruded through a centrally located slot (5 mm wide) in the base. This design facilitated free lateral movement of the scalpel blade, whereas its movement in the longitudinal direction was guided by the slot and its height above the base was fixed at a distance (1 mm) that ensured the cut was made below the level of hair bulbs.

For IT, a 45 mm long skin incision (Fig. 1A) was positioned centrally and cut coronally along the line drawn between the anterior points of the two frontal crests, where the skin was found to be the thickest (15–20 mm for calves) on a deer's head. To create an intradermal pocket for IT, a 20 mm wide and 1–2 mm deep incision (Fig. 1B) was made on the freshly cut surface from the first 45 mm long skin incision at the rostral side with a handheld scalpel, in order to provide a guide for the blade that was attached to the device. The base of the device was inserted under the skin through the 45 mm long skin incision and the skin was then tightly clamped when the tip of the device-attached blade was properly inserted into the 1–2 mm deep skin incision (Fig. 1C). The depth of the 1–2 mm deep incision was then extended to create an intradermal pocket by lateral movements of the handle between the 10 and 2 o'clock positions about the

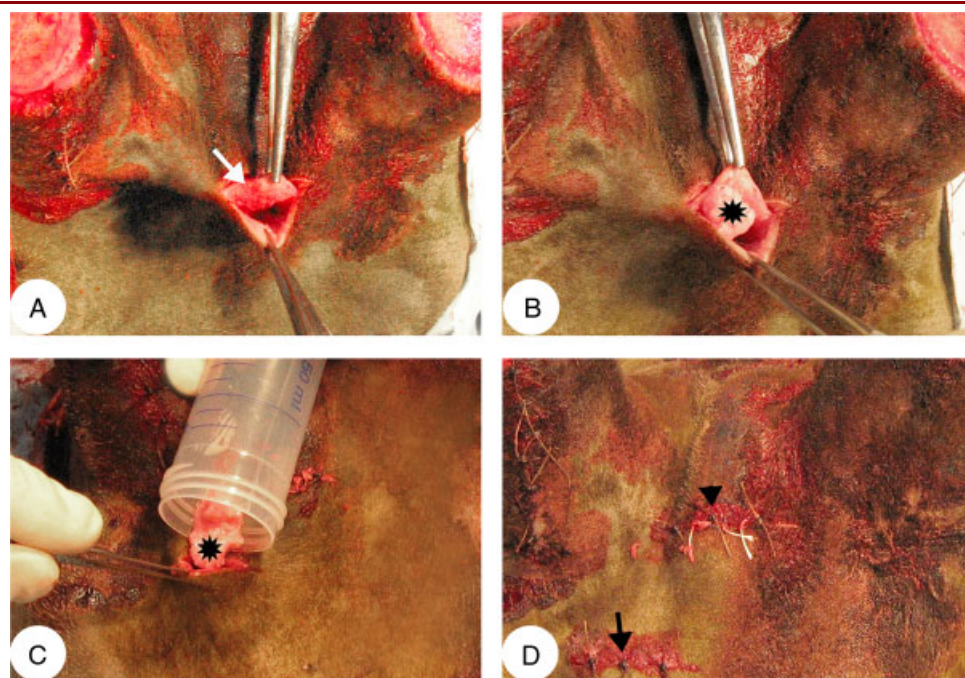


Figure 2. Pedicle periosteum (PP) transplantation surgery. (A) An intradermal pocket (arrow) made using a hand-held scalpel was opened using two pairs of fine forceps. (B) A pellet of the minced PP (asterisk) was carefully loaded into an intradermal pocket. (C) A pellet of minced PP (asterisk) was loaded into a subcutaneous pocket (arrow). (D) Both skin openings of the intradermal pocket (arrowhead) and the subcutaneous pocket (arrow) were sutured.

central pivot axis of the blade (Fig. 1D). By this means, an intradermal pocket, approximately 10–15 mm deep (Fig. 1E), was formed. The mouth of each intradermal pocket was opened using two pairs of fine forceps (Fig. 1E), to facilitate the loading of the pellet of minced AP into the pocket (Fig. 1F). A suture was then used to seal the opening of each intradermal pocket (Fig. 1G).

For ST, a 15 mm long incision in the skin was made coronally rostral to the IT site and a subcutaneous pocket was created anteriorly by blunt dissection. The pellet of minced AP was then loaded into each ST pocket (Fig. 1H).

PP. For IT, a 25 mm long skin incision was positioned centrally and cut coronally along the line drawn between the anterior

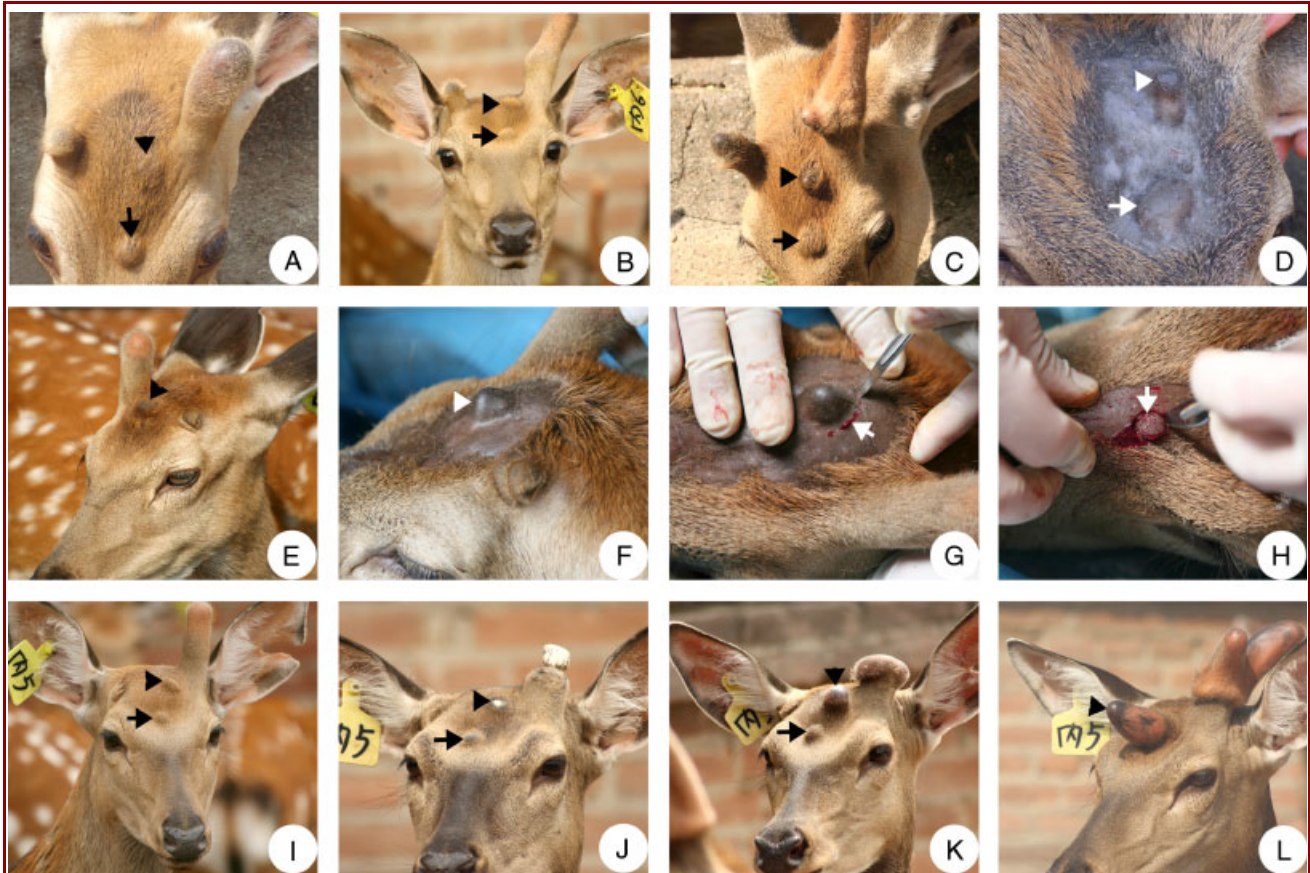
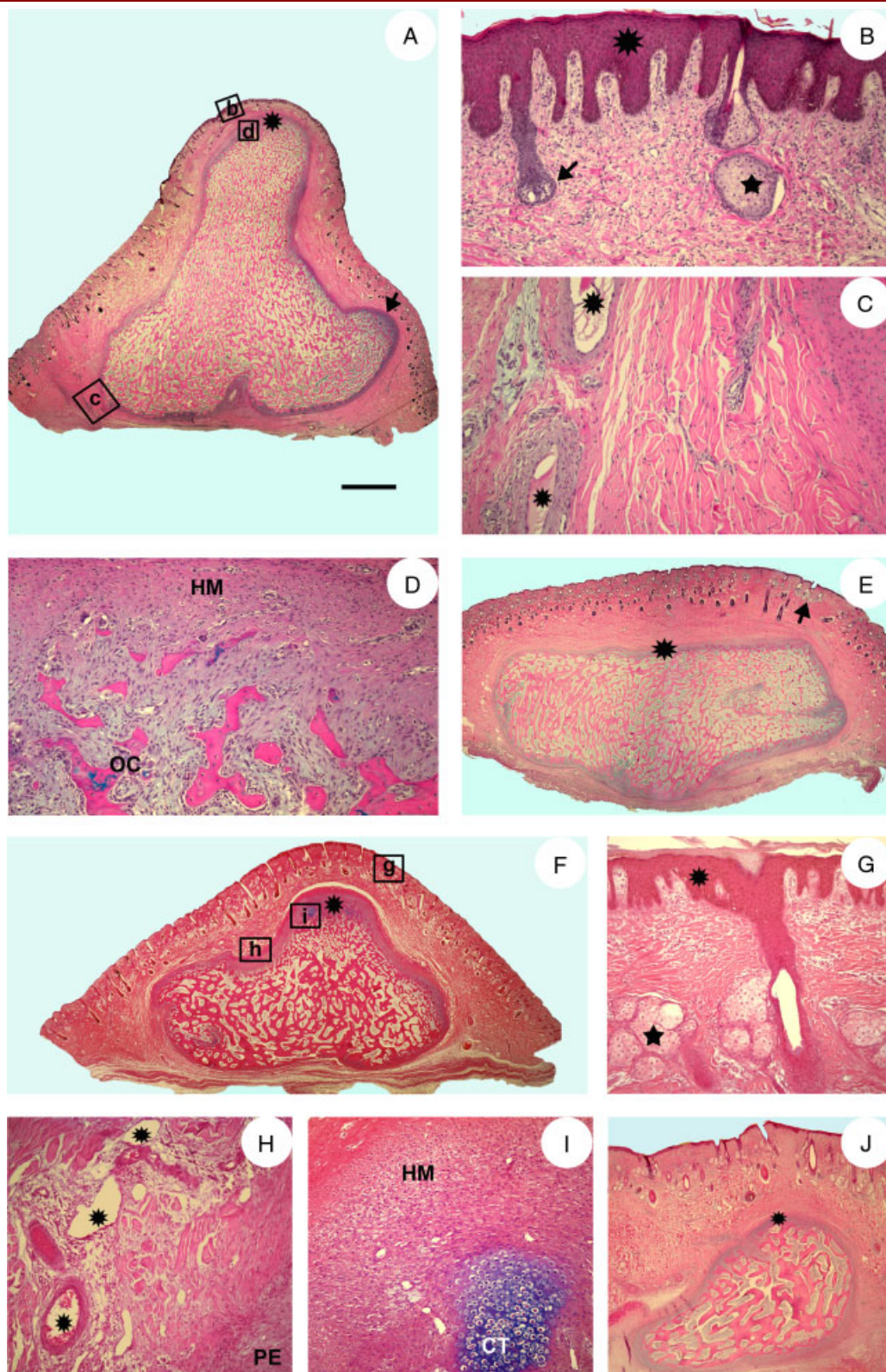


Figure 3. Morphology of the outgrowths formed in the 1/4-AP Group. (A–D) Deer 1. (A) Protuberance formed at the IT site (arrowhead) was smaller than the one at the ST site (arrow) 1 month after AP transplantation. (B) Protuberances from both IT (arrowhead) and ST (arrow) sites reached similar height around 2 months after AP transplantation. (C) Protuberance at the ST site (arrow) was subsequently surpassed by the IT-site one, which had clearly transformed into an antler (arrowhead) at the end of the first antler growth season. (D) Protuberances of both the IT and ST sites at the time of tissue sampling. Notice that the IT-site protuberance (arrowhead) was at least double the height of the ST-site protuberance (arrow, 19 mm vs. 8.9 mm). (E–H) Deer 2. (E) A protuberance (arrowhead) was formed at the IT site around a month after AP transplantation, whereas no outgrowth could be seen at the ST site. (F) The IT-site protuberance reached 11 mm high and had transformed into an antler (arrowhead, side view) 2 months after AP transplantation. (G) The IT-site protuberance was sampled by cutting the skin around its base using a scalpel (arrow). (H) A barely palpable bulge formed from the ST site was sampled by the same procedure as for the IT-site protuberance (arrow). (I–L) Deer 3. (I) Similar height protuberances were formed at the IT (arrowhead) and the ST (arrow) sites 50 days after AP transplantation. (J) Antler transformation from the IT-site protuberance was revealed by the end of the first antler growth season (5 months after AP transplantation) when its apical skin was peeled off to expose a bare bony tip (arrowhead), whereas no antler transformation took place from the ST-site protuberance (arrow). (K) Antler regeneration (arrowhead) at the IT site occurred after the miniature hard antler was cast. (L) A much larger antler regenerated from the protuberance at the IT site (arrowhead) during summer.



sides of the two pedicles. A handheld scalpel, rather than the custom-built device, was used in this group as the skin of a 2-year-old red deer is thick enough (3–4 mm) to be readily separated into two layers to make an intradermal pocket (Fig. 2A). The minced PP explants were carefully loaded into each pocket (Fig. 2B).

For ST, the procedure used in AP transplantation was repeated, and subsequently the minced PP was transferred into each pocket (Fig. 2C). Both skin incisions were sutured after the periosteum tissue loaded (Fig. 2D).

Transplantation Site Wounding and Tissue Sampling

Wounding. Because there was no sign of ectopic antler formation in the 1/12-AP group or in the PP group in the second year following the operation, wounding of both transplantation sites on each deer in these groups was performed early in the antler growth season (Fig. 7C, F, G and K, and Fig. 8B, F and J, respectively). A cross-shaped cut was made through the center of each IT or ST site using a scalpel. Each cut was deep enough (through the skin) to include the underlying AP- or PP-derived tissues (1–2 mm in depth), as it is known that deep mechanical wounding can facilitate antler formation and regeneration (Jaczewski, '82; Goss, '90).

Sampling. Tissue sampling was carried out simultaneously from both IT and ST sites of each deer, at the time when an outgrowth from one site had convincingly transformed into an antler. Without exception, all antler growth occurred at the IT site rather than the ST site. After thoroughly shaving each outgrowth (protuberance/bulge) and its surrounding area (Figs. 3D, F, 5D

and H), an incision was made through the full thickness of skin around the base of each protuberance using a scalpel (Figs. 3G, H and 5E). The antler outgrowth was removed from the underlying frontal bone and sagittally bisected into two equal parts (Fig. 5F).

Those deer that had not developed an outgrowth from either the IT or ST site by the end of the second antler growth season were slaughtered and tissues from these sites were sampled. The collected tissue samples were immediately placed in 10% formalin and processed for subsequent histological examination, as described by Li and Suttie ('94).

To facilitate description and discussion, a specific name was given to each of these outgrowths formed from IT or ST sites, based on their visibility: an outgrowth that could be clearly seen from a distance was called "protuberance", whereas one that could only be seen after hair removal was termed a "bulge."

The height and diameter of each sampled outgrowth were measured using a calliper (Fig. 5H, I)

RESULTS

AP Transplantation

Observation data were summarized in Table 1. None of the outgrowths from IT or ST sites were fused to the underlying frontal bone in this study.

1/4-AP Group

Morphology. All three IT sites in the group formed protuberances and these outgrowths were transformed into antlers (as indicated

Figure 4. Histology of the tissue samples collected from the 1/4-AP Group. Tissue sections were sagittally cut through the center of each outgrowth/transplantation site. Hematoxylin and eosin/Alcian blue counterstaining. The scale bar (10 mm) shown in (A) applies to all figures, but in each case represents a different length that is indicated below in the legend for each figure. (A–E) Deer 1. (A) A dominant growth center (asterisk) was formed from the fused AP explants at the IT site, and the AP-derived tissue had fused with the surrounding dermal tissue. The center was located on the tip of the protuberance and covered by typical velvet skin (for detailed structure, see (B–D)). Although a second growth center was formed (arrow), which was located at the lower right corner, the center was covered by typical scalp skin. Bar = 3.33 mm. (B) Higher magnification of the area labeled "b" in (A) to show that the apical skin had typical velvet features, including thick epidermis (asterisk), enlarged sebaceous glands (star), and de novo formation of hair follicles (arrow). Bar = 0.15 mm. (C) Higher magnification of the area labeled "c" in (A) to show some thick blood vessels (asterisks) adjacent to the AP-derived bony tissue. Bar = 0.16 mm. (D) Higher magnification of the area labeled "d" in (A) to show the growth center consisting of a hyperplastic mesenchymal layer (HM) and underlying osseocartilag tissue (OC). Bar = 0.16 mm. (E) AP-derived tissue had fused with the overlying dermis (asterisk) at the ST site. No dominant growth center was found from the fused AP pieces nor was antler velvet skin observed covering its apex. However, some enlarged sebaceous glands (arrow) at the upper right corner were detected, suggesting the initiation of velvet skin transformation might have begun. Bar = 2.83 mm. (F, J) Deer 2. (F) A dominant growth center (asterisk) was formed from the fused AP explants at the IT site. The center was located on the tip of the protuberance and covered by typical velvet skin (for detailed structure, see (G–I)). Bar = 2.46 mm. (G) Higher magnification of the area labeled "g" in (F) to show the apical skin that had acquired velvet skin features: thick epidermis (asterisk) and enlarged sebaceous glands (star). Bar = 0.18 mm. (H) Higher magnification of the area labeled "h" in (F) to show some of the thick blood vessels (asterisks) adjacent to the AP-derived bony tissue. Bar = 0.18 mm. (I) Higher magnification of the area labeled "i" in (F) to show the growth center consisting of a hyperplastic mesenchyme (HM) and underlying cartilage tissue (CT). Bar = 0.18 mm. (J) AP-derived tissue had fused with the overlying dermis (asterisk) at the ST site. No dominant growth center was formed from the fused AP pieces, nor was the antler velvet skin observed covering the outgrowth apex. Bar = 1.13 mm.

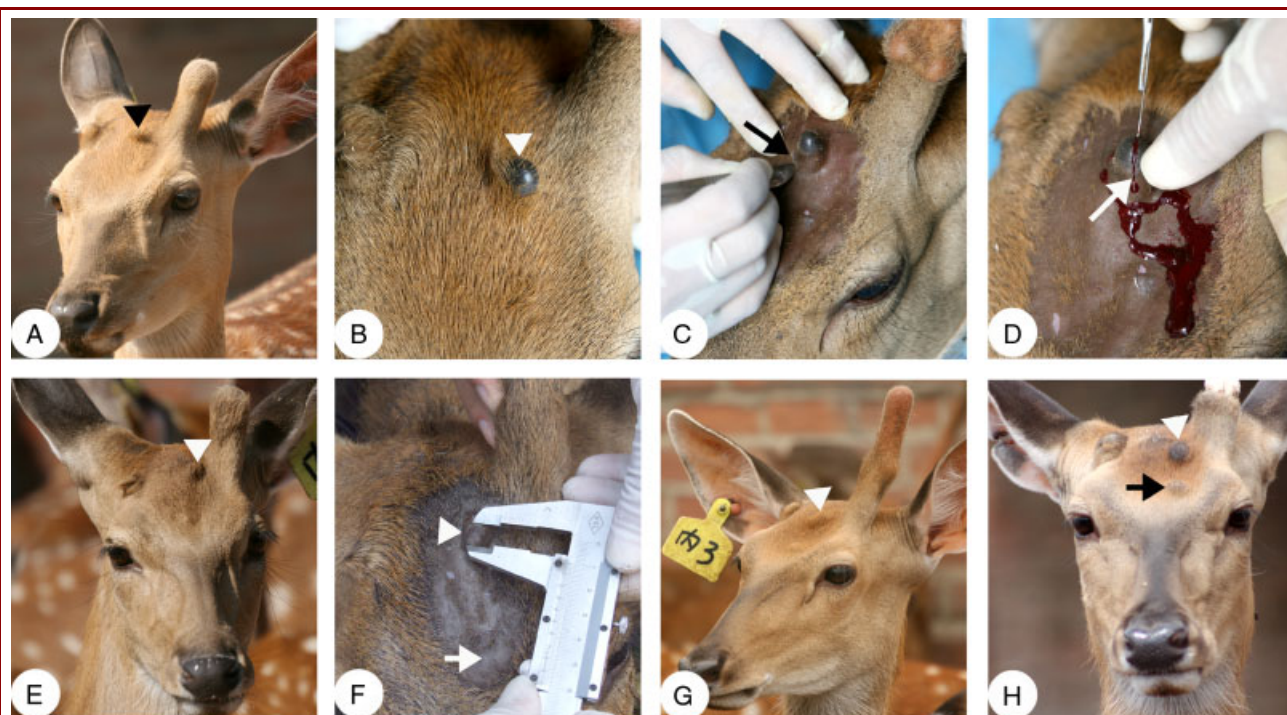


Figure 5. Morphology of the outgrowths formed in the 1/8-AP Group. (A–D) Deer 4. (A) A protuberance was formed at the IT site (arrowhead) but not at the ST site 30 days after AP transplantation. (B) Antler transformation (arrowhead) took place from the protuberance 60 days after AP transplantation and reached 14.5 mm high at the time of tissue sampling. Note that the apex of the protuberance was covered by shiny dark colored skin. Only a small bulge was found at the ST site. (C) The protuberance at the IT site was sampled by making an incision in the skin surrounding its base using a scalpel (arrow). Note that after shaving, it became clear that the protuberance was composed of a proximal pedicle part and a distal antler part. (D) The protuberance was sagittally cut into two equal parts by making a second incision (arrow). (E, F) Deer 5. (E) A small protuberance was formed at the IT site (arrowhead) but not at the ST site around a month after AP transplantation. (F) The protuberance reached 6.8 mm high 2 months after AP transplantation (at the time of tissue sampling) and transformed into an antler (arrowhead). Measurement was carried out using a calliper. The outgrowth was barely detectable at the ST site (arrow). (G, H) Deer 6. (G) A small protuberance was formed at the IT site (arrowhead) but not at the ST site in this deer at the time similar to that observed in Deer 5. (H) Antler transformation (arrowhead) took place almost 3 months after AP transplantation. At this stage, a small protuberance was also formed at the ST site (arrow).

by the sparse hairs on the shiny skin covering their apices) within 2–3 months after AP transplantation (Fig. 3 and Table 1). Two ST sites formed protuberances and one formed a palpable bulge, based on their morphology, but none of these structures was transformed into an antler (Fig. 3 and Table 1).

In Deer 1, the protuberance formed at the IT site was initially smaller than the one at the ST site (Fig. 3A), but it reached a similar size around 2 months after AP transplantation (Fig. 3B), became higher thereafter (Fig. 3C), and was double the height (19 mm vs. 8.9 mm) at the time of tissue sampling (5 months after transplantation; Fig. 3D). In Deer 2, a protuberance was formed at the IT site around a month after AP transplantation (Fig. 3E) and this reached 11 mm high at the time of tissue sampling (around 2 months; Fig. 3F and G). In contrast, only a barely palpable bulge (Fig. 3H) was formed at the ST site at the time of tissue sampling. In Deer 3, protuberances of similar sizes were formed at both IT

and ST sites 50 days after AP transplantation (Fig. 3I). Tissue sampling was not performed at that time, though, owing to uncertainty whether true antler tissue had differentiated. However, in the case of the protuberance at the IT site, this was subsequently confirmed at the end of the first antler growth season (5 months after AP transplantation) when it became totally calcified and its apical skin peeled off to expose a bare bony tip (Fig. 3J). In contrast, the protuberance at the ST site remained unchanged and antler transformation did not occur (Fig. 3J). During the following antler growth season (spring), the miniature hard antler at the IT site was cast and a much larger antler regenerated, and again no change was detected on the ST site (Fig. 3K and L).

Histology. Histological examination for Deer 1 and Deer 2 confirmed the morphological results that skin overlying the

protuberance from each IT site had transformed into antler velvet, whereas that from each ST site had not (Fig. 4).

In each IT-site protuberance, a dominant growth center located on the tip of the protuberance (Fig. 4A and F) was formed from the fused AP explants and covered by velvet skin (typified by thickened epidermis, enlarged sebaceous glands, and de novo formation of hair follicles; Fig. 4B and G). These growth centers each consisted of a hyperplastic mesenchymal layer and underlying cartilage tissue (Fig. 4D and I). In contrast, only original deer scalp skin was observed on the shaft of these protuberances (evidenced by thin epidermis, small sebaceous glands, arrector pili muscle, and sweat glands; Fig. 4A). There were numerous blood vessels that surrounded the AP-derived tissue (Fig. 4C and H). In each ST-site outgrowth, no single dominant growth center was encountered, nor was the antler velvet skin observed covering their apices (4E and J). All the minced AP explants had fused together in each outgrowth.

1/8-AP Group

Morphology. Morphological results from this group were comparable to those of the 1/4-AP group. All the outgrowths formed at the IT sites were protuberances and eventually transformed into antlers, whereas those at the ST sites were only bulges and none of them transformed into an antler (Fig. 5).

In Deer 4, a protuberance was formed at the IT site just 30 days after AP transplantation, but at that time no antler development could be observed at the ST site (Fig. 5A). Two months after AP transplantation, the protuberance at the IT site was transformed into a definitive antler covered with dark shiny skin that was sparsely populated with hair (Fig. 5B). Following hair removal at the time of tissue sampling (124 days after AP transplantation), the protuberance measured 14.5 mm in height and clearly consisted of two parts (proximal pedicle and distal antler), whereas only a small bulge was found at the ST site (Fig. 5C and D). In Deer 5 and 6, both IT-site protuberances were formed at similar times (40–50 days) after AP transplantation (Fig. 5E and G), although subsequent antler transformation took place at different times (after 57 and 114 days, respectively; Fig. 5F and H). A bulge was formed on each of the ST sites in Deer 5 (Fig. 5F) and Deer 6 (Fig. 5H).

Histology. Three compelling histological features that demonstrated for antler transformation had occurred in the 1/4-AP group were also clearly shown in the IT-site protuberances in this group: apical velvet skin, a dominant growth center and numerous surrounding blood vessels in the vicinity of the AP-derived tissue (Fig. 6A, G and J). The histological structures of the protuberance/bulges from the ST sites were comparable to those counterparts of the 1/4-AP group (data not shown).

In Deer 4, the IT-site protuberance had the greatest length to width ratio (4.5) of the bony core (Fig. 6A) and the thickest apical epidermal layer (Fig. 6D) in the whole study, and its growth

center could be clearly divided into hyperplastic mesenchyme (Fig. 6B), precartilage (Fig. 6A), osseocartilage (Fig. 6C), and cancellous bone (Fig. 6A). Interestingly, the lower parts of some hair follicles in the apical velvet skin were bent away from the growing direction of the AP-derived growth center (Fig. 6A and E), a phenomenon seemingly created by the growth force of the intradermal transplanted AP tissue directly underneath the hair follicles. On the proximal shaft of the protuberance, the covering skin remained as the original scalp skin in nature (Fig. 6A). A major blood vessel trunk was evident at the bottom corner of the protuberance (Fig. 6A and F). In the protuberance from Deer 5, the dominant growth center was less developed, but also consisted of hyperplastic mesenchymal, cartilage, and cancellous bone layers (Fig. 6I) and was covered by the typical velvet skin (Fig. 6H). Only a single growth center was detected in the protuberance of Deer 6 (Fig. 6J) and the center was covered by the typical velvet skin (Fig. 6K).

1/12-AP Group

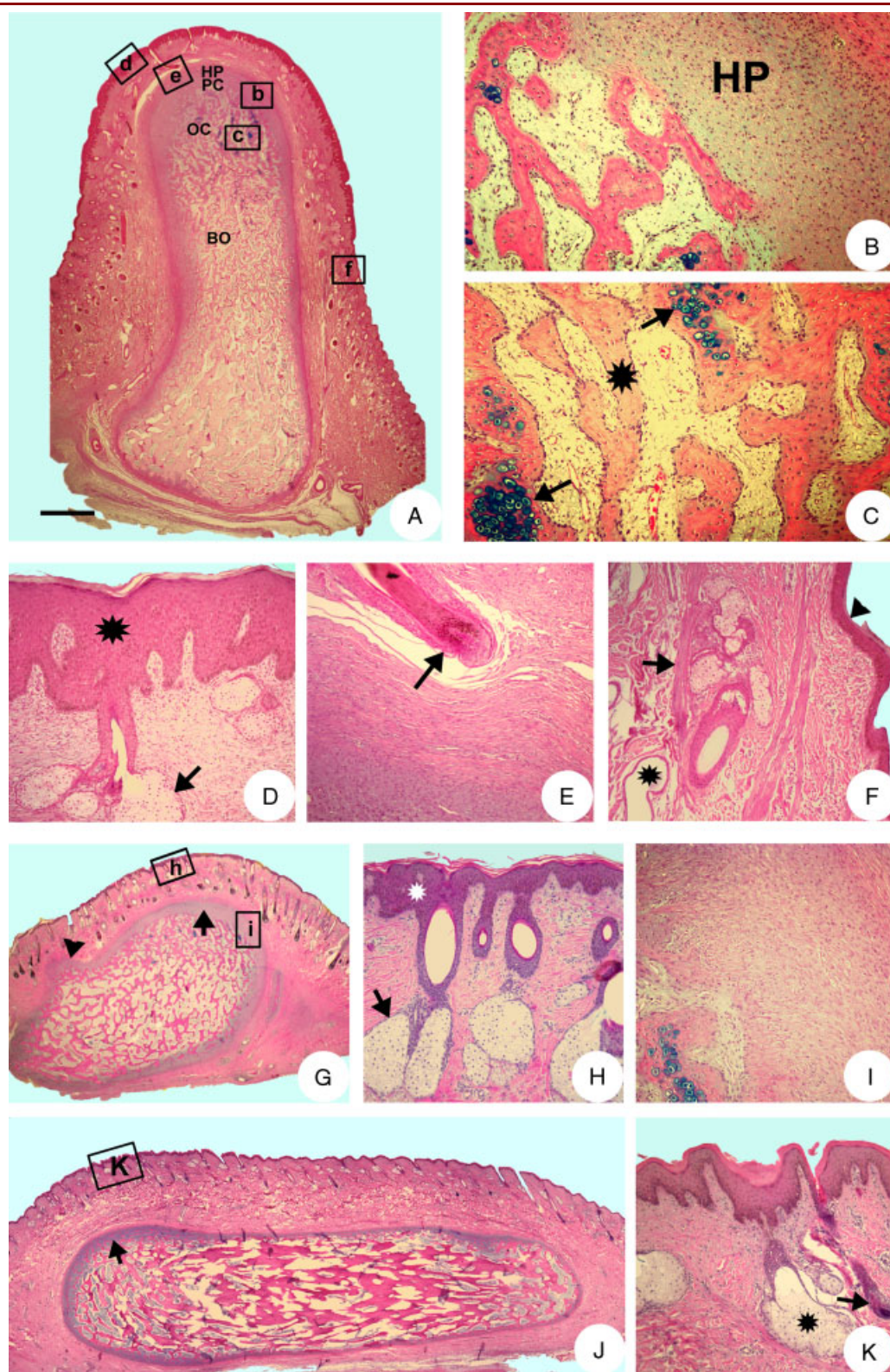
Morphology. In contrast to the 1/4- and 1/8-AP groups, no protuberances were formed at any of the IT or ST sites of deer in this group by the end of the first antler growth season. At the time of mechanical wounding in the following antler growth season (454–456 days after AP transplantation), a small bulge was observed after shaving the hair at the IT site in Deer 7 (Fig. 7A) and only barely palpable bulges were detected at the IT sites in Deer 8 and Deer 9 (Fig. 7D, G). Mechanical wounding (Fig. 7B, D, E, and H) did not stimulate antler development at any of these transplantation sites by the end of the second antler growth season (Fig. C, F and I).

PP Transplantation

Morphology. Two months after PP transplantation, small bulges were formed at the IT sites, whereas no visible or palpable growth occurred at the ST sites of the three deer by the end of the first antler growth season. Therefore, mechanical wounding was performed by making a cross-shaped cut at each transplantation site (Fig. 8A, D and G) at the time of the second antler growth season (376 days after PP transplantation). However, mechanical wounding did not stimulate the bulges at the IT sites to grow to protuberances or transform into antlers (Fig. 8B, E and H), nor did it induce growth of bulges or protuberances at the ST sites (Fig. 8C, F and I).

In Deer 10, although a 5 mm high bulge formed at the IT site, the bulge was located at the margin of the site and its apical skin retained scalp skin characteristics (rather than velvet) (Fig. 8B), suggesting that it was unlikely to be antler tissue. The bulges at the IT sites in Deer 11 and Deer 12, although less impressive, all developed from the central transplantation region (Fig. 8E and H).

Histology. Histological examination confirmed the morphological observation that all the bulges formed at the IT sites of the three deer were covered by the typical scalp skin (thin epidermis



and small sebaceous glands; Fig. 9). A small ossification center was also found in each of these bulges (Fig. 9A, D and F). In contrast, only the ST site of Deer 11 had an observable ossification center (Fig. 9E), and no bone tissue could be found at the ST sites of either Deer 10 or Deer 12 (Fig. 9C and G). Critically, the PP-derived ossification center of each deer had fused with the surrounding dermis at the IT site, and only very small blood vessels were occasionally encountered in close proximity to these PP-derived ossification centers (Fig. 9H, I and J). Interestingly, the 5 mm high bulge at the IT site of Deer 10 was solely composed of skin and soft connective tissue (Fig. 9B).

DISCUSSION

Whether regeneration recapitulates development is not only a fundamental question but also highly relevant to regenerative medicine. Research to date (Goss and Powel, '85; Faucheux et al., 2004; Li et al., 2007a,b, 2008) has convincingly demonstrated full recapitulation in deer antlers, which is the only case of mammalian epimorphic regeneration. However, it was unclear whether the PP (stem cells for antler regeneration) recapitulates AP (stem cells for antler development) in antler induction because the subcutaneously transplanted PP failed to induce antler development (Li et al., 2007a). It cannot be concluded that PP does not recapitulate AP in antler induction based on that negative result, though, because it is not clear that it was caused by the inability of the PP-derived tissue to interact with the overlying skin. Instead, it may have been caused by a failure to form the close association between these two tissue types that is required for antler induction (Goss, '90; Li and Suttie, 2000),

because the transplanted PP did not expand from the initial grafting mass sufficiently to create significant mechanical pressure on the overlying skin. By use of the novel IT approach in this study, we have now clarified this ambiguity and demonstrated that even when closely associated with skin (Fig. 9A, D and F) for two antler growth seasons, PP was not able to initiate antler development. Therefore, PP does not have the ability to interact with the skin at an ectopic site, and hence does not recapitulate AP in antler induction (at least not within the experimental period of 2 years).

IT vs. ST

The proof-of-concept experiment using AP in this study successfully demonstrated that IT is a valid approach for ectopic antler induction. Because the IT approach established a close association between the grafted periosteal tissue and skin without relying on significant expansion of grafted periosteal tissue, the minimum amount of tissue required to be transplanted in order to induce ectopic antler formation, should be substantially fewer than the ST approach. This study confirmed the earlier report that ST of 1/4-AP or less could not initiate an ectopic antler development within 2 years (Goss and Powel, '85), whereas 1/8-AP or more was, without exception, sufficient for antler induction using the IT method. Based on these results, the minimum grafting AP tissue in IT for successful antler induction is estimated to be between 1/8- and 1/12-AP. This threshold mass of AP required for antler formation may represent the minimum number of AP cells that are able to successfully establish inductive interactions with the skin.

Figure 6. Histology of the tissue samples collected from the 1/8-AP Group. Tissue sections were sagittally cut through the center of each outgrowth/transplantation site. Hematoxylin and eosin/Alcian blue counterstaining. The scale bar (10 mm) shown in (A) applies to all figures, but in each case represents a different length that is indicated below in the legend for each figure. (A–F) Deer 4. (A) A single growth center was formed from the fused AP explants at the IT site. The center was located on the tip of the protuberance and covered by a typical velvet skin (for a detailed structure, refer to (B–F)). The protuberance consisted of four zones distoproximally: hyperplastic mesenchyme (HM), precartilagel (PC), osseocartilagel (OC), and bone (BO). Bar = 2.14 mm. (B) Higher magnification of the area labeled "b" in (A) to show the multicell-layered hyperplastic mesenchyme (HP). Bar = 0.10 mm. (C) Higher magnification of the area labeled "c" in (A) to show the cartilaginous (arrows) and osseous tissue (asterisk) in the osseocartilagel zone. Bar = 0.10 mm. (D) Higher magnification of the area labeled "d" in (A) to show the thickened epidermis (asterisk) and enlarged sebaceous glands (arrow). Bar = 0.15 mm. (E) Higher magnification of the area labeled "e" in (A) to show the unique phenomenon in which the lower part of a hair follicle, immediately above the growing center, was bent away from the growth direction of the AP-derived tissue (arrow). Bar = 0.17 mm. (F) Higher magnification of the area labeled "f" in (A) to show that the skin enveloping the lower part of the protuberance remained the typical scalp skin features: thin epidermis (arrowhead), existence of sweat glands (asterisk), and arrector pili muscle (arrow). Bar = 0.21 mm. (G–I) Deer 5. (G) A dominant and an accessory growth centers were formed from the fused AP explants at the IT site. The former was located on the tip of the protuberance (arrow) and covered by a typical velvet skin (refer to (H)), and the latter was located at the lower left corner but associated with the scalp skin (arrowhead). Bar = 1.96 mm. (H) Higher magnification of the area labeled "h" in (G) to show the typical velvet skin features, including thickened epidermis (asterisk) and enlarged sebaceous glands (arrow). Bar = 0.11 mm. I. Higher magnification of the area labeled "i" in (G) to show the multicell-layered hyperplastic mesenchyme (HP). Bar = 0.18 mm. (J, K) Deer 6. (J) A single growth center (arrow) was detected from the fused AP explants at the IT site. The center was located in the rostral end of the "ridge-like" protuberance and covered by typical velvet skin (for detailed structure, refer to (K)). Bar = 1.97 mm. (K) Higher magnification of the area labeled "K" in (J) to show the typical velvet skin features, including thickened epidermis, neogenesis of a hair follicle (arrow), and enlarged sebaceous glands (arrow). Bar = 0.17 mm.

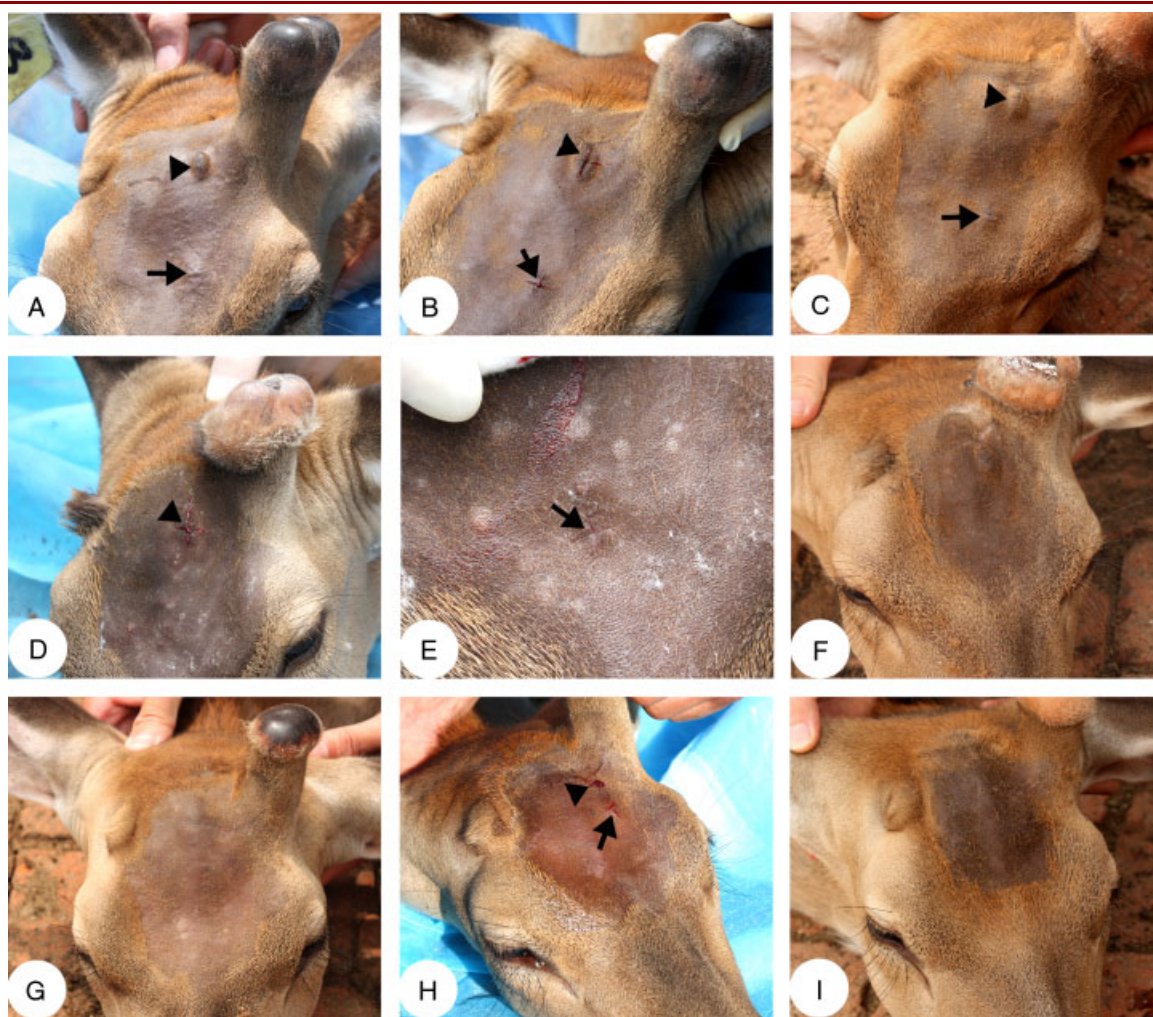


Figure 7. Morphology of the outgrowths formed in the 1/12-AP Group. (A–C) Deer 7. (A) A small bulge was formed at the IT site (arrowhead), but nothing could be visualized at the ST site (arrow) in the mid of second antler growth season. (B) Both IT (arrowhead) and ST (arrow) sites were wounded by making a cross-shaped cut using a scalpel. (C) No further growth was observed at either the IT (arrowhead) or the ST site (arrow) by the end of the second antler growth season. (D–F) Deer 8. (D) No visible outgrowth was found at the IT site, which was wounded by making a cross-shaped cut (arrowhead) early in the second antler growth season. (E) The ST site was wounded by making a cross-shaped cut (arrow). (F) Mechanical wounding did not stimulate any visible growth at either the IT or the ST site by the end of the second growth season. (G–I) Deer 9. (G) No outgrowth could be detectable at any of the transplantation IT sites in the middle of the second antler growth season. (H) Both IT (arrowhead) and ST (arrow) transplantation sites were wounded by making a cross-shaped cut. (I) No visible growth was detected at either of the transplantation sites by the end of the second growth season.

Evidence of bypassing the need for tissue expansion for the formation of close association between the intradermally grafted AP and the skin can be morphologically observed in Deer 3 of the 1/4-AP group (Fig. 3J). In this deer, the small protuberance (around 5 mm in height) at the IT site transformed into an antler, whereas the similar sized protuberance at the ST site remained covered by typical scalp skin. ST may have an advantage over IT in the reestablishment of circulation to the grafted AP tissue, as

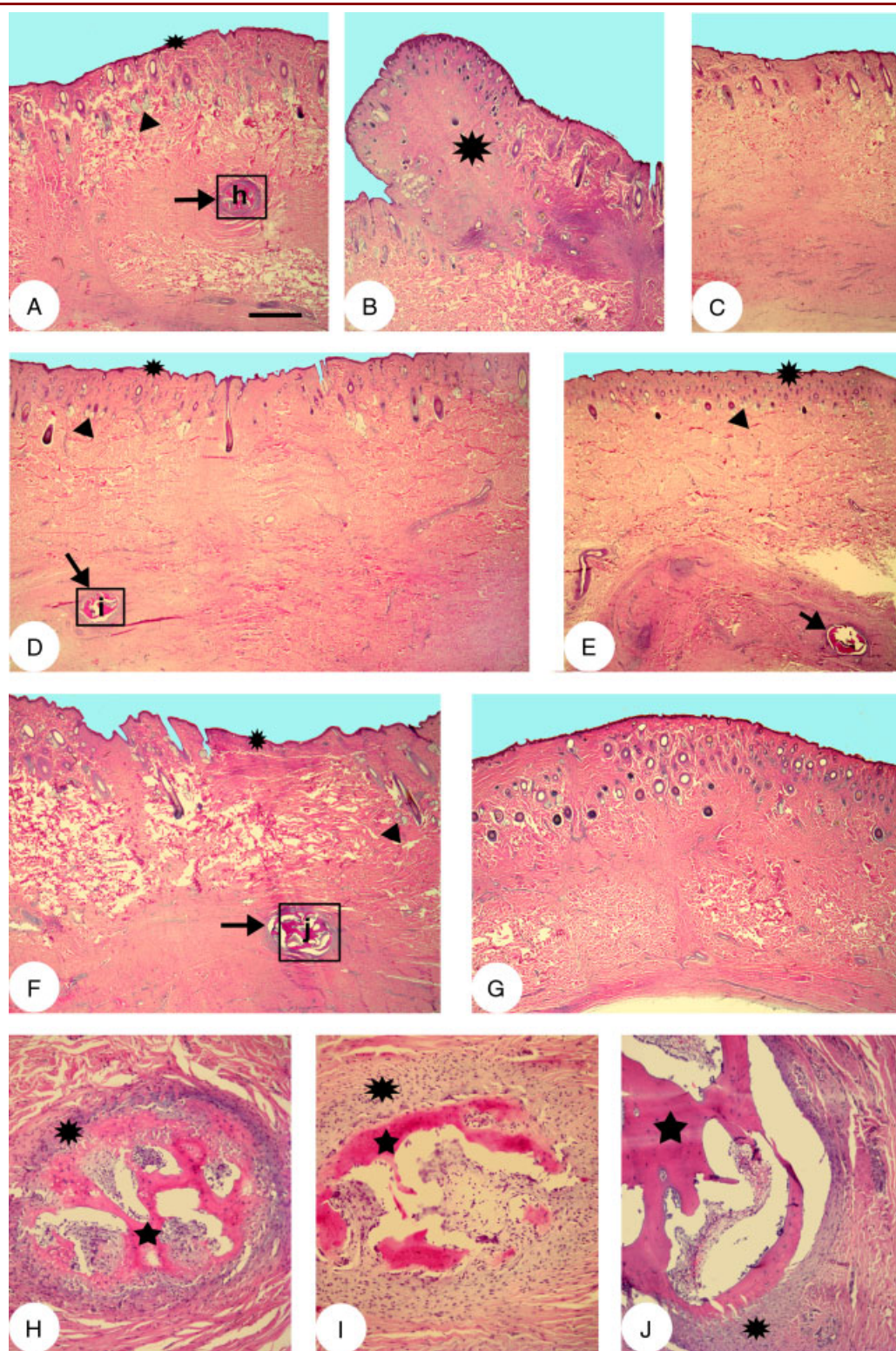
in ST, the AP is directly grafted in the vascular layer. This is supported by the observation that some protuberances on the ST sites, such as in Deer 1 of the 1/4-AP group (Fig. 3A), were initially bigger than those at the IT sites. The requirement for reestablishment of circulation to the transplanted tissue may also explain the variable sizes of protuberances that were formed in this study from equal quantities of AP tissue grafted using the same transplantation approach.



Figure 8. Morphology of the outgrowths formed in the PP Group. (A–C) Deer 10. (A) Early in the second antler growth season, the bulge at the IT site was less impressive than 2 months after transplantation. A cross-shaped cut was made to wound the bulge (arrow). (B) A bulge (arrow) was formed at the margin of the IT site (asterisk) by the end of the second antler growth season. (C) No outgrowth could be detected at the ST site (arrow) by the end of the second antler growth season despite mechanical wounding. (D–F) Deer 11. (D) A bulge had formed at the IT site early in the second antler growth season. Note that the bulge was covered by seemingly velvet-like skin (asterisk, sparse hair, and shiny surface), and was wounded by making a cross-shaped cut (arrow). (E) The bulge did not morphologically transform into an antler (arrow, note the typical scalp skin) by the end of the second antler growth season. (F) No outgrowth was detectable at the ST site (arrow) by the end of the second antler growth season. (G–I) Deer 12. (G) A small bulge was formed at the IT site (asterisk) but not at the ST site 2 months after PP transplantation. The bulge was wounded by making a cross-shaped cut (arrowhead). (H) A widespread bulge with low height (around 3 mm) was stimulated by the mechanical wounding of the IT site (asterisk) by the end of the second antler growth season. (I) No outgrowth was found at the ST site (arrow) by the end of the second antler growth season.

An interesting phenomenon was observed in Deer 4 of the 1/8-AP group, which had the longest protuberance in this study. Hair follicles in the apical velvet skin were in direct contact with, and bent away from, the AP-derived growth center tissue (Fig. 6A and E). Potentially, this was caused by the growth force of the AP-derived tissue from directly underneath the hair follicles. One possible explanation, assuming the phenomenon was not purely

incidental, is that the intensity of the interaction between the skin and AP might have been enhanced by direct contact between the hair follicles and AP-derived tissue, leading to extra growth. In support of this supposition, hair follicles have been demonstrated to be indispensable in order for the skin to be competent to interact with the grafted AP and successfully induce antler formation (Goss, '87).



AP vs. PP

Successful validation in this study of the IT approach, and determination of the minimum mass of AP required to be grafted for ectopic antler induction, enabled objective selection of the amounts of PP tissue to test for antler induction using this new method. The three quantities of PP tissue chosen (62, 78, and 84 mg) represented at least twice the minimum amount of AP tissue ($1/8\text{-AP} \approx 30\text{ mg}$) required to be grafted for successful antler induction. However, none of these quantities initiated ectopic antler formation despite fusion between the PP tissue and the skin. Therefore, we conclude that PP is unable to recapitulate AP in induction of antler development, at least not within the 2-year experimental period. However, the following points remain to be properly explained.

PP may have only impaired inductive potential for antler development compared with AP. Therefore, the amounts of PP tissue grafted in this study, although at least twice as much as that of AP, may still be not sufficient for successful antler induction using the IT approach. Secondly, 2 years may not be sufficient time for the establishment of interactions between the PP and skin. Third, the number of antler stem cells (mainly PP cellular layer cells) resident in the grafted PP may be below the threshold that can effectively initiate antler development, because PP cellular layer is thinner than that of AP. However, these arguments are unlikely based on our results, because only a small ($< 1\text{ mm}$) ossification center (from all IT and one of three ST sites) or no bony tissue (from two of three ST sites) was observed, irrespective of the amount of PP initially grafted and the transplantation method used (IT or ST). Interestingly, these small ossification centers did not give any indication of having the potential for further growth and inducing the overlying epidermis to transform to antler velvet (Fig. 9A, D, F). For sites where no bony tissue was detected we suggest that either the ossification centers were missed when sectioning, owing to their

small sizes, or that the bony tissue never developed from the minced PP explants. Therefore, the amount of PP tissue grafted and the observation period following transplantation are unlikely the factors that account for the absence of antler development. Likewise, we do not think that antler stem cell number in the grafted PP is a contributing factor either. This is because the timing of PP sampling (around a month before initiation of antler regeneration) just coincides with the thickening PP cellular layer (Kierdorf et al., 2003; Li et al., 2005). More importantly, in an inductive milieu (distal end of a pedicle stump) 3.3 million PP cells can produce up to 10 kg antler tissue mass just within 60 days (Li et al., 2009), and the cells in each grafted PP in this study would be many times 3.3 million; however, the grafted PP in some cases did not even give rise to bony tissue. Consequently, PP cells, the stem cells for antler regeneration, have lost the ability to initiate antler development once differentiated from AP.

It is of interest as to why AP tissue can readily expand through rapid growth when being grafted to an ectopic site, whereas PP tissue cannot. Careful examination of the histological results indicates that the drastic difference in size and quantity of blood vessels surrounding each tissue type may be the answer. Major blood vessel trunks were readily seen around the AP-derived tissue (Figs. 4C, H and 6F), whereas only small capillaries were occasionally found around the PP-derived tissue (Fig. 9J). Consequently, the inability of the PP tissue to initiate new and/or attract existing blood vessels toward it may account for its failure to undergo significant expansion. In situ, PP can initiate rapid growth, probably because pedicles are well nourished by the major blood vessels from superficial temporal arteries that already exist (Wislocki, '42; Suttie et al., '85). However, the question remains whether the grafted PP failed to initiate antler development owing to the lack of sufficient blood supply, rather than an inability to interact with the skin. In other words, the

Figure 9. Histology of the tissue samples collected from the transplantation sites of the PP group. Tissue sections were sagittally cut through the center of each outgrowth/transplantation site. Hematoxylin and eosin/Alcian blue counterstaining. The scale bar (10 mm) shown in (A) applies to all figures, but in each case represents a different length that is indicated in the legend below for each figure. (A–C) Deer 10. (A) A small ossification center (arrow) was detected in the place where the PP explants were inserted at the IT site, and the PP-derived center had fused to the surrounding dermal tissue, but the overlying epidermis still retained typical scalp skin features (thin epidermis (asterisk) and small sebaceous glands (arrowhead)). Bar = 1.18 mm. (B) The bulge located at the margin of the IT site was only made up of soft tissue (asterisk) and no PP-derived bone tissue was found. Bar = 1.61 mm. (C) No ossification center/bone tissue was found at the ST site. Bar = 1.58 mm. (D, E) Deer 11. (D) A small ossification center (arrow) was detected in the place where the PP explants were implanted at the IT site and the PP-derived center had fused to the surrounding dermal tissue, but the overlying epidermis still retained typical scalp skin features (thin epidermis (asterisk) and small sebaceous glands (arrowhead)). Bar = 1.20 mm. (E) A small ossification center (arrow) was detected underlying the dermis at the ST site (arrow) and the PP-derived center had fused to the surrounding tissue (arrow). No velvet skin was observed in this case. Bar = 1.55 mm. (F, G) Deer 12. (F) A small ossification center (arrow) was detected in the middle of the dermis at the IT site and the PP-derived center had fused to the surrounding dermal tissue, but the overlying epidermis still retained typical scalp skin features (thin epidermis (asterisk) and small sebaceous glands (arrowhead)). Bar = 1.19 mm. (G) No ossification center was found at the ST site. Bar = 0.97 mm. (H–J) Higher magnification of the ossification centers of the areas labeled "h," "i," and "j" in (A, D, and F), respectively. Note that the minced PP explants had fused together and formed trabecular bone (stars) surrounded by a dense cellular layer (asterisks). All these centers had fused to the surrounding dermal tissue. Bar = 0.14 mm in (H); 0.13 mm in (I); 0.16 mm in (J), respectively.

interactions between the PP-derived tissue and the skin may have taken place, but did not result in antler development owing to a lack of nutrients to sustain growth of the PP-derived tissue. Again this is unlikely, as the overlying epidermis 2 years after transplantation did not show any signs of transformation toward that of antler velvet (i.e. hair follicle down growth toward AP-derived tissue, epidermal thickening, and sebaceous gland enlarging).

It is unclear why PP can readily interact in situ with the distal pedicle skin (a type of deer scalp skin), but cannot do so with the ectopic scalp skin. Extra antler growth was naturally encountered from a pedicle shaft (Kierdorf and Kierdorf, 2002), suggesting under unusual conditions yet to be defined that PP can initiate antler growth. However, this induction of extra antler growth may be happened at the same time of original antler development and, at least, it resulted again from the interactions with the in situ pedicle skin. Mechanical traumatization created by casting of the earlier antler should not be the reason because, in this study, mechanical wounding using a scalpel was also conducted on both the IT and ST sites, including both the ectopic scalp skin and grafted PP in the early antler growth season. Besides, Goss ('95) failed to stimulate an extra antler to grow from the shaft of a pedicle by traumatizing both pedicle skin and bone. Consequently, we conclude that once differentiated from AP, PP has been restricted for antler regeneration and has lost the potential to initiate antler development.

ACKNOWLEDGMENTS

We thank the staff of Biotechnology Laboratory and the deer crew from the Institute of Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, China; Dr. Colin Mackintosh, Dr. Dawn Clark, Mrs. Shirley Martin, and the deer crew from the AgResearch Invermay Agricultural Centre, New Zealand, for the help with the tissue implantation surgery and subsequent observation.

LITERATURE CITED

- Carlson B. 2007. Principles of regenerative biology. New York: Academic Press. p 1–379.
- Dor Y, Stanger BZ. 2007. Regeneration in liver and pancreas: time to cut the umbilical cord? *Sci STKE* 2007:pe66.
- Faucheux C, Nicholls BM, Allen S, Danks JA, Horton MA, Price JS. 2004. Recapitulation of the parathyroid hormone-related peptide-Indian hedgehog pathway in the regenerating deer antler. *Dev Dyn* 231:88–97.
- Gardiner DM, Bryant SV. 1996. Molecular mechanisms in the control of limb regeneration: the role of homeobox genes. *Int J Dev Biol* 40:797–805.
- Goss RJ. 1961. Experimental investigations of morphogenesis in the growing antler. *J Embryol Exp Morphol* 9:342–354.
- Goss RJ. 1983. Deer antlers. Regeneration, function and evolution. New York, NY: Academic Press.
- Goss RJ. 1987. Induction of deer antlers by transplanted periosteum. II. regional competence for velvet transformation in ectopic skin. *J Exp Zool (Mol Dev Evol)* 244:101–111.
- Goss RJ. 1990. Of antlers and embryos. In: Bubenik G, Bubenik A, editors. Horns, pronghorns, and antlers. New York: Springer. p 299–312.
- Goss RJ. 1995. Future directions in antler research. *Anat Rec* 241: 291–302.
- Goss RJ, Powel RS. 1985. Induction of deer antlers by transplanted periosteum. I. graft size and shape. *J Exp Zool (Mol Dev Evol)* 235:359–373.
- Hartwig H, Schrudde J. 1974. Experimentelle untersuchungen zur bildung der primären stirnhaare beim Reh (*Capreolus capreolus* L.). *Z Jagdwiss* 20:1–13.
- Jaczewski Z. 1982. The artificial induction of antler growth in deer. In: Brown RD, editor. Antler development in Cervidae. Kingsville, TX: Caesar Kleberg Wildl. Res. Inst. p 143–162.
- Kierdorf U, Kierdorf H. 2002. Pedicle and first antler formation in deer: anatomical, histological, and developmental aspects. *Z Jagdwiss* 48:22–34.
- Kierdorf U, Stoffels E, Stoffels D, Kierdorf H, Szuwart T, Clemen G. 2003. Histological studies of bone formation during pedicle restoration and early antler regeneration in roe deer and fallow deer. *Anat Rec* 273A:741–751.
- Li C, Suttie JM. 1994. Light microscopic studies of pedicle and early first antler development in red deer (*Cervus elaphus*). *Anat Rec* 239:198–215.
- Li C, Suttie J. 2000. Histological studies of pedicle skin formation and its transformation to antler velvet in red deer (*Cervus elaphus*). *Anat Rec* 260:62–71.
- Li C, Suttie JM. 2001. Deer antler generation: a process from permanent to deciduous. In: Sim JS, Sunwoo HH, Hudson RJ, Jeon BT, editors. Antler science and product technology. Canada: Banff. p 15–31.
- Li C, Suttie JM. 2003. Tissue collection methods for antler research. *Eur J Morphol* 41:23–30.
- Li C, Harris AJ, Suttie JM. 2001. Tissue interactions and antlerogenesis: new findings revealed by a xenograft approach. *J Exp Zool (Mol Dev Evol)* 290:18–30.
- Li C, Suttie JM, Clark DE. 2005. Histological examination of antler regeneration in red deer (*Cervus elaphus*). *Anat Rec A Discov Mol Cell Evol Biol* 282:163–174.
- Li C, Mackintosh CG, Martin SK, Clark DE. 2007a. Identification of key tissue type for antler regeneration through pedicle periosteum deletion. *Cell Tissue Res* 328:65–75.
- Li C, Yang F, Li G, Gao X, Xing X, Wei H, Deng X, Clark DE. 2007b. Antler regeneration: a dependent process of stem tissue primed via interaction with its enveloping skin. *J Exp Zool A Ecol Genet Physiol* 307:95–105.
- Li C, Yang F, Xing X, Gao X, Deng X, Mackintosh C, Suttie JM. 2008. Role of heterotypic tissue interactions in deer pedicle and first

- antler formation—revealed via a membrane insertion approach. *J Exp Zool (Mol Dev Evol)* 310:267–277.
- Li C, Yang F, Sheppard A. 2009. Adult stem cells and mammalian epimorphic regeneration—insights from studying annual renewal of deer antlers. *Curr Stem Cell Res Ther* 4:237–251.
- Michalopoulos GK, DeFrances MC. 1997. Liver regeneration. *Science* 276:60–66.
- Mount JG, Muzylak M, Allen S, Althnaian T, McGonnell IM, Price JS. 2006. Evidence that the canonical Wnt signalling pathway regulates deer antler regeneration. *Dev Dyn* 235:1390–1399.
- Otu HH, Naxerova K, Ho K, Can H, Nesbitt N, Libermann TA, Karp SJ. 2007. Restoration of liver mass after injury requires proliferative and not embryonic transcriptional patterns. *J Biol Chem* 282:11197–11204.
- Rolf HJ, Kierdorf U, Kierdorf H, Schulz J, Seymour N, Schliephake H, Napp J, Niebert S, Wolfel H, Wiese KG. 2008. Localization and characterization of STRO-1 cells in the deer pedicle and regenerating antler. *PLoS ONE* 3:e2064.
- Simon HG, Kittappa R, Khan PA, Tsilfidis C, Liversage RA, Oppenheimer S. 1997. A novel family of T-box genes in urodele amphibian limb development and regeneration: candidate genes involved in vertebrate forelimb/hindlimb patterning. *Development* 124:1355–1366.
- Stocum D. 2006. *Regenerative biology and medicine*. New York: Academic Press.
- Suttie J, Fennessy P, Mackintosh C, Corson I, Christie R, Heap S. 1985. Sequential cranial angiography of young red deer stags. In: Fennessy P, Drew K, editors. *Biology of deer production*. Royal Society of New Zealand. p 263–268.
- Vlaskalin T, Wong CJ, Tsilfidis C. 2004. Growth and apoptosis during larval forelimb development and adult forelimb regeneration in the newt (*Notophthalmus viridescens*). *Dev Genes Evol* 214:423–431.
- Wislocki GB. 1942. Studies on the growth of deer antlers. I. on the structure and histogenesis of the antlers of the Virginia deer (*Odocoileus virginianus borealis*). *Am J Anat* 71:371–451.