Antler Transformation is Advanced by Inversion of Antlerogenic Periosteum Implants in Sika Deer (*Cervus nippon*)

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ABSTRACT

Deer antlers offer a unique model for the study of tissue-specific stem cells and organogenesis, as antler stem cells are confined to the antlerogenic periosteum (AP), a tissue that can be readily located (overlying a frontal crest) and experimentally manipulated. AP consists of an upper fibrous layer and a lower cellular layer. Tissue transplantation and membrane insertion experiments demonstrated that antler formation is triggered by the interactions between AP and the overlying skin. Interestingly, fairly normal antlers can be induced to grow by an inverted AP implant (the AP cellular layer facing the skin) at an ectopic site, raising the question whether the initial inductive signal is derived from the fibrous layer or cellular layer or both. To answer this question, in this study we used eight sika deer stag calves and selected one side of future antler growth region for implanting inverted AP and the contralateral side for noninverted AP as the control. The results showed that implantation of the AP discs in an inverted orientation generated pedicles with final height (17 $\pm$ 5.1 mm), less than half the height of those formed from the noninverted AP implants (45 $\pm$ 11.7 mm). Critically, antler transformation was initiated from a shorter pedicle, which was formed from the region where the AP cellular layer was brought in close proximity to the overlying skin. Therefore, the AP cellular layer, as opposed to the AP fibrous layer, is likely to be the main source of the initial inductive molecules for antlerogenesis. Anat Rec, 293:1787–1796, 2010. © 2010 Wiley-Liss, Inc.

Key words: antler; pedicle; antlerogenic periosteum; sika deer; implantation

INTRODUCTION

Development of individual organs in animal embryos involves the formation of tissue-specific stem cells that sustain cell renewal of their own tissue for the lifetime of the organism (Slack, 2008). Model organ systems in which stem cells can be precisely defined and readily manipulated are required if stem cell self-renewal, multipotency, positioning information, and interactions with the niche in their development are to be elucidated. In

Grant sponsor: Jilin Provincial Department of Science and Technology; Grant number: 20080901.

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Received 21 February 2010; Accepted 5 May 2010

DOI 10.1002/ar.21221
Published online 20 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

this regard, initial formation of deer antlers provides a novel example, where antler stem cells are confined to a small area of periosteum located in the future pedicle and antler growth region in prepubertal deer (Li and Suttie, 1994; Kierdorf et al., 2009; Li et al., 2009b). Thus far, experimental manipulation of the periosteum has yielded invaluable information for antlerogenesis.

Deer antlers are cranial appendages that are subject to annual renewal from permanent bony protuberances, known as pedicles. Deer are not born with pedicles, but they start to develop when male deer reach puberty. It has been convincingly demonstrated that the potential to form a pedicle and an antler mainly resides in the periosteum overlying the frontal crest of a prepubertal deer (Hartwig and Schrudde, 1974). Therefore, this periosteum is termed antlerogenic periosteum (AP; Goss and Powel, 1985). Histologically, the AP consists of an upper fibrous layer and a lower cellular layer, but these layers are much thicker than those of its somatic counterpart, such as facial periosteum (Kierdorf et al., 1994; Li and Suttie, 1994). Cells resident in AP have recently been demonstrated to express key embryonic stem cell markers (OCT4, Nanog, and SOX2) and induced to differentiate *in vitro* into multiple cell lineages (chondroblasts, adipocytes, myoblasts, and neuronal-like cells) and, therefore, are called antler stem cells (Li, 2009; Li et al., 2009b)

Since the discovery of AP, a series of *in vivo* experiments have been conducted to further the understanding its role in antler development. Through a combination of tissue deletion and transplantation, Goss and Powel (1985) determined the minimum threshold of AP size required for antler induction in fallow deer to be 15 mm in diameter. Subsequently, Goss (1987) discovered that ectopic antler induction not only requires the existence of AP but also instructive feedback from the overlying skin, and all tested deer skin can provide such feedback except three: ventral surface of the tail, back, and nose snout skins. This finding lead Goss (1990) to propose that interactions between AP and the overlying skin are indispensible for triggering antler growth, which was later confirmed by means of a membrane insertion approach (Li et al., 2008). Interestingly, when the AP discs were subcutaneously grafted upside down, fairly normal antlers were induced (Goss, 1991) suggesting that not only the naturally adjacent fibrous layer but also the spatially distant AP cellular layer can also successfully establish the interactions with the overlying skin to initiate antler formation. Nonetheless, this study raises a question: if either layer can initiate the interactions with the skin to form antlers when being placed close to skin, then which layer (the AP fibrous layer or cellular layer or both) would likely be the source for these initial interactive molecules? This could not be assessed in that study (Goss, 1991) because neither noninverted AP implants (control) were included nor the timing of antler transformation and height of full-grown pedicles from the inverted AP implants were recorded.

It is critically important to pinpoint AP cell types from which the initial interactive molecules are secreted, if the mechanism underlying antlerogenesis is to be understood. The best way to study this would logically be to implant one layer (cellular or fibrous) at a time to see which one could induce antler formation. However, practically this is not feasible as AP tissue is too thin ($\sim$2 mm in thickness for sika deer) to be accurately sliced into cellular and fibrous layers. To overcome this problem, in a previous study, we cotransplanted the inverted AP and deer skin (cellular layer facing the skin) onto a nude mouse to determine whether the rate of transformation in deer skin from scalp to velvet in this type of cotransplantation would be similar, faster or slower than that in the noninverted AP cotransplantation (fibrous layer facing the skin; Li et al., 2009a). Although that study failed to pinpoint the AP cell type for the initial interactive molecules, because the nude mice used in the study died prematurely from infection, it did give an indication that the AP cellular layer may be the major source of these molecules. Specifically, the skin overlying the inverted AP implants possessed antler velvet characteristics; whereas, the skin overlying the noninverted AP implants still kept its original scalp skin features.

In this study, we took an approach modified from the aforementioned nude mouse model to address this issue by placing sampled AP back to its original site of each deer in an inverted orientation on one side and in a noninverted orientation on the other and then comparing the timing of antler transformation, which was assessed by difference in height of the full-grown pedicles from the inverted AP and the noninverted AP sides and number of days between surgery and antler transformation. A significant advancement in the timing of antler transformation with inverted AP, as opposed to noninverted AP, would suggest that the key molecules were primarily derived from the cellular layer.

MATERIALS AND METHODS

Animal

Eight male sika deer calves (*Cervus nippon*) were selected during the period of pedicle initiation (Table 1) and continued to be maintained inside an enclosure from birth to the termination of the experiment. This study only involved superficial surgery and had approval from the Animal Ethics Committee of the Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences.

Design

One of the two future antler growth regions of each deer was randomly assigned for inverted AP implantation and the other for noninverted AP implantation as a control. The inversion was carried out by reversing the medial and lateral sides and keeping anterior and posterior axes in register. Tissue sampling from three deer was planned for the comparison of the histogenesis of pedicle formation and antler transformation between the inverted AP and noninverted AP implants. The biopsied pedicles and/or antlers together with those from deer that died accidently within the first antler growth season were termed "one-growth-season" appendages, whereas those retained into the second antler growth season were termed "two-growth-season" appendages. The purpose for including the second antler growth season in this study was to give an extended period for those AP implants that did not activate in the first growth season to initiate pedicle and antler formation and to observe the process of antler regeneration from the pedicles on the inverted AP sides.

TABLE 1. Design and results of the Inverted AP implantation experiment

Deer	Inverted side	Pedicle height at surgery (mm)		Antler initiation after surgery (day)		Final height in first season P(A) (mm)		Final shape in second season	
		Inverted	Noninverted	Inverted	Noninverted	Inverted	Noninverted	Inverted	Noninverted
1	L	<5	<5	148	NG	11 (3)	0 (0)	Spike	NG
2	L	<5	<5	57	NT	15 (4)	29 (0)	–	–
3	R	7	7	88	65	19 (5)	52 (8)	–	–
4	L	<5	<5	57	NT	20 (5)	28 (0)	–	–
5	L	6	6	80	69	20 (4)	51 (88)	2 Tines	2 Tines
6	L	<5	<5	NT	NG	8 (0)	0 (0)	–	–
7	R	<5	<5	389	119	8 (0)	45 (62)	Spike	2 Tines
8	R	<5	<5	NG	83	0 (0)	31 (5)	NG	2 Tines
Mean ± SD						17 ± 5.1	45 ± 11.7		

AP, antlerogenic periosteum; L, left; R, right; P(A), pedicle(antler); NG, no growth; NT, no transformation; –, not applicable; SD, standard deviation.

Surgery

Surgery was carried out under general anaesthesia (xylazine hydrochloride, 1.5–2.0 ml/100kg body weight) during the period of pedicle initiation (4 April), when the deer were around 8–10 months old. Each operation site was thoroughly shaved, and then sterilized with 70% alcohol and 1% iodine tincture. Detailed AP sampling has been reported elsewhere (Li and Suttie, 1994, 2003). Briefly, a crescent-shaped incision was made through the scalp skin 2 cm medial to the frontal crest, ensuring that the laterally-located major blood vessels and nerves were avoided. The skin was then separated from underneath bone by blunt dissection and reflected laterally to expose the AP (Fig. 1A). An incision in the periosteum ($\sim 8 \times 16$ mm^2) was made along the longitudinal axis of each crest, and AP was subsequently peeled off from the crest by using rat-toothed forceps (Fig. 1B). For noninverted implantation, the peeled AP was replaced in the area from which it was removed (Fig. 1C). For the inverted implantation, the peeled AP was grafted upside down into the original area (Fig. 1D). The skin flap was then put back and sutured using nonabsorbable 2-0 black silk (Ethicon, New York) before the anaesthesia was reversed using Nikethamide (1.5–2.0 ml/100 kg body weight).

Observation

Animals were observed daily for 7 days after surgery, weekly thereafter and photographed when necessary. The sutures were taken out on 6 May (32 days after surgery), and the heights of the pedicles were measured at the same time (Table 1). Subsequent measurements were carried out whenever the deer were anaesthetized for manipulation.

Tissue Sampling

The appendages formed from both sides of Deer 2 and 4 in the "one-growth-season" group were biopsied from the base (Fig. 2C) at day 74 after surgery using a scalpel (because tissue was still soft); and those from Deer 3 and 6 were sawn off from the base (because tissue became hard) immediately after death at day 269 and 98, respectively. Subsequently, these appendages were sagittally divided into two even halves for histology.

Histology

Sagittally-divided halves of each appendage in the "one-growth-season" group were fixed in 10% buffered formalin immediately after removal. The tissue samples were then decalcified in 10% formic acid, embedded in paraffin wax, sectioned at 5 μm, and counterstained with haematoxylin and eosin and alcian blue.

RESULTS

At the time of removing sutures (30 days after surgery), pedicle initiation had only started in Deer 3 and 5. Incipient pedicles from the inverted AP sides were slightly shorter than those from the noninverted AP sides in these two deer (Table 1), suggesting that the reestablishment of circulation for the latter have been faster for the noninverted AP.

There was no clear pattern with regard to the number of days between surgery and antler transformation in these two types of implantation, inverted or noninverted, in this study (Table 1). In marked contrast, the full-grown pedicles (mean length: 17 ± 5.1 mm) from the inverted AP implants were no more than one half the height of those from the noninverted AP implants (mean length: 45 ± 11.7 mm; Table 1). The height of the full-grown pedicles from the latter was within the species-specific range in sika deer (~50 mm; Li et al, 1988), suggesting that the manipulation (removal–replacement) did not disrupt the memory of species-specific pedicle height for whatever the underlying mechanism is.

"One-Growth-Season" Appendages

Morphology. Deer 6 died on 11 July (98 days after the surgery) due to a long chronic illness. No obvious growth had occurred at the noninverted AP side, but 8 mm incipient pedicle had initiated at the inverted AP side (Table 1). Tissues including scalp skin and the underlying AP-derived tissue from both sides were sampled immediately after death for histology evaluation.

Initiation of antler transformation took place on the pedicles derived from the inverted AP implants in both Deer 2 (Fig. 2A) and 4 (Fig. 2D) around 57 days after surgery (Table 1). Seventy-four days after surgery, the transformation process on the inverted AP side pedicle from Deer 4 was completed having its apical skin

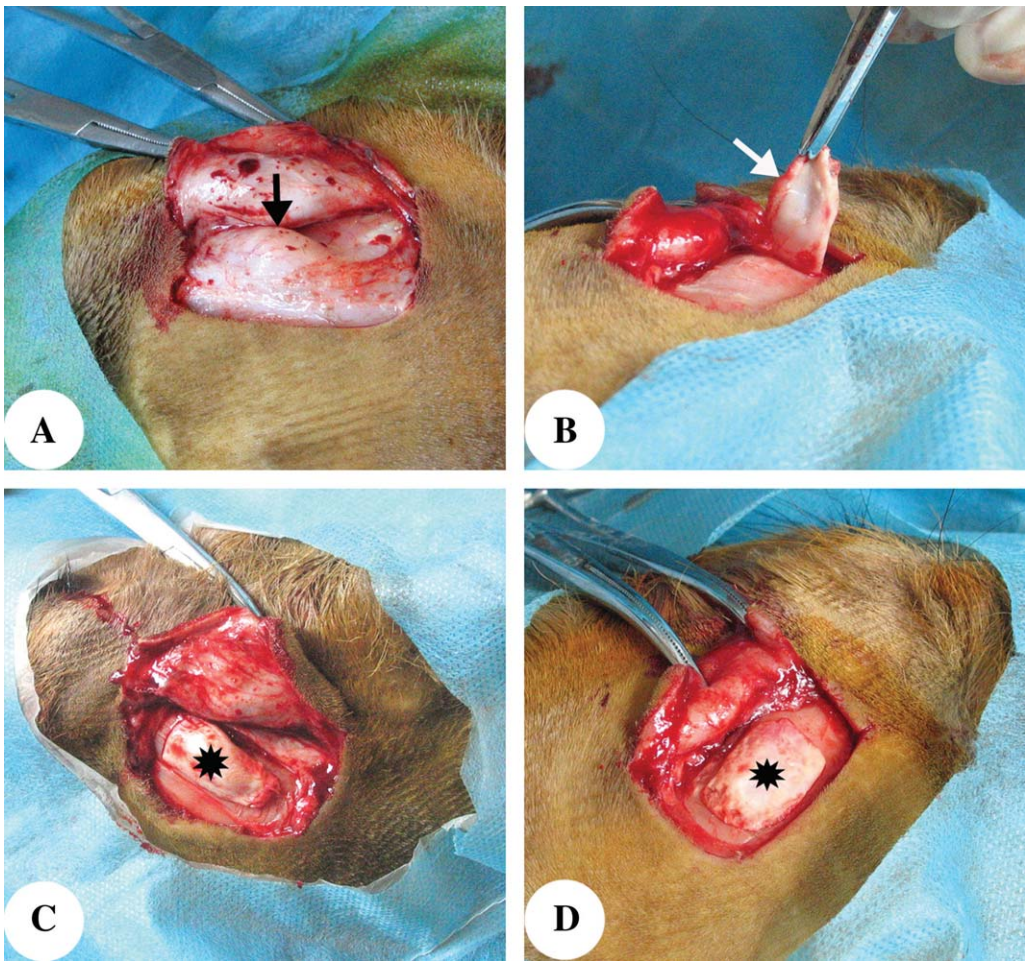

Fig. 1. Removal and implantation surgery of AP on 8–10 months old sika deer stag calves. **A**: Skin overlying a frontal crest was reflected laterally after cutting to expose the AP (arrow). **B**: AP overlying the crest was peeled off (arrow) from the underlying bone using a pair of rat-toothed forceps. **C**: The detached AP was replaced onto the area (asterisk) from which it was removed. **D**: The detached AP was replaced upside down onto the area (asterisk) from which it was removed. The inversion was carried out by reversing the medial and lateral sides and keeping anterior and posterior axis in register.

possessing typical velvet features (hair sparely populated shiny skin, Fig. 2E), but Deer 2 was still in the transition state (Fig. 2B). No antler transformation was observed on either of the pedicles derived from the noninverted AP implants in these two deer (Fig. 2B,F), although at this stage these pedicles were marginally longer than the total length of pedicles and antlers derived from the inverted AP implants (Table 1). All four appendages were biopsied from the base (Fig. 2C) for histological evaluation.

At the time of removing sutures, the pedicle (14 mm) derived from the noninverted AP implant was longer than the inverted AP implant (9 mm) in Deer 3 (Fig. 2G), whereas pedicles from both sides were of similar height (7 mm) at the initial surgery (Table 1). Antler transformation took place from the pedicles of both the noninverted AP (65 days after surgery) and inverted AP (88 days after surgery) sides in Deer 3; although the transformation occurred in the former when its pedicle reached 52 mm in height (species-specific), in the latter, its pedicle only reached 19 mm in height (Fig. 2H,I). Deer 3 died of misadventure in the mid of winter (269 days after surgery), and no histology for its appendages was carried out, as they were totally calcified at the time of the deer death.

Histology. At the time of the death of Deer 6 (98 days after surgery), the noninverted AP implant still consisted of typical cellular and fibrous layers, and no obvious growth was detected from the AP (Fig. 3A), although its cellular layer had firmly attached to the underlying crest bone. In contrast, the inverted AP implant had started to initiate pedicle growth and had formed a "bean-shaped" trabecular bony ossicle, which was wrapped by an uninterrupted layer of periosteum consisting of seemingly normal cellular and fibrous layers (Fig. 3B,D). Proximodistally, the structure could be divided into 4 zones: the originally grafted periosteum, newly differentiated cartilage and trabecular bone, and newly formed periosteum. The cartilage layer was discrete and narrow, and trabecular layer was wide

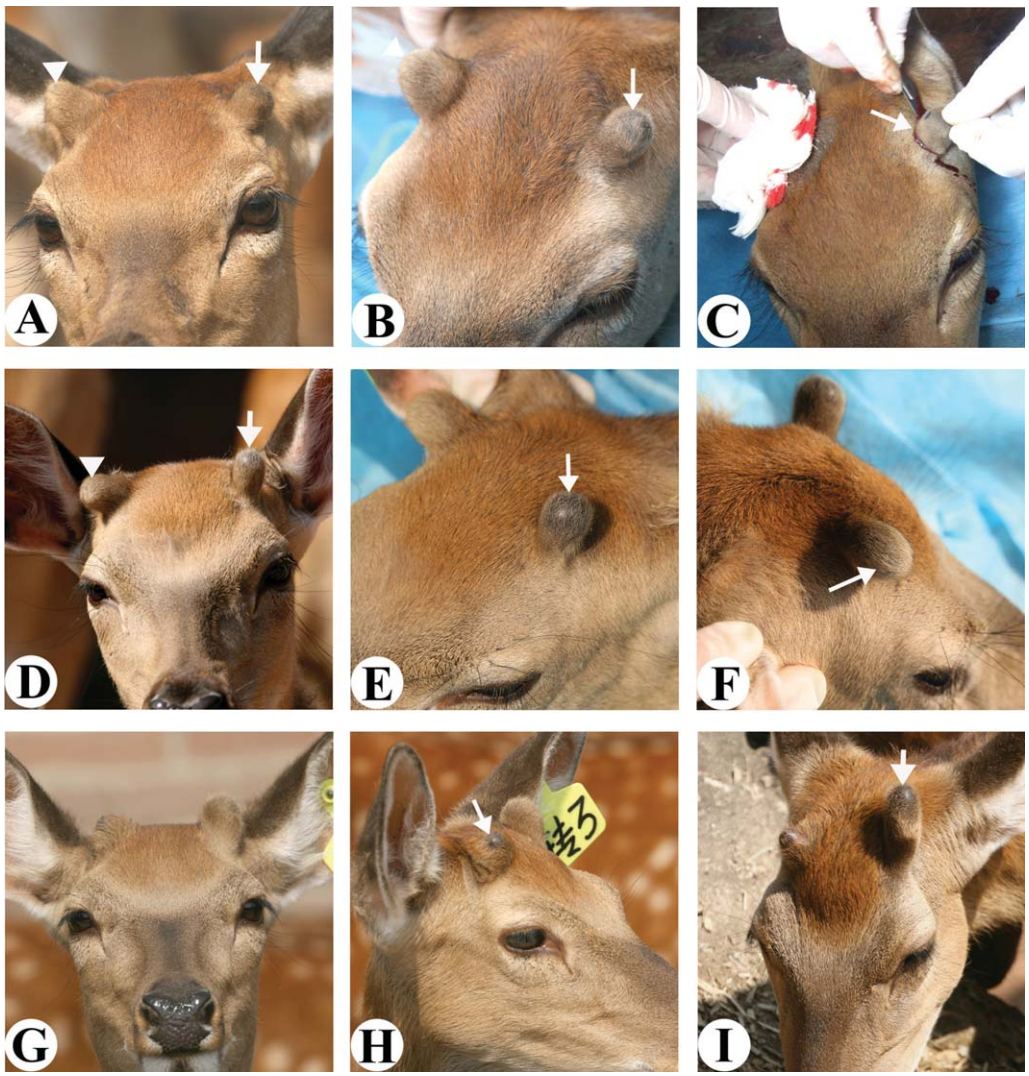

Fig. 2. Status of pedicle/antler formation from the inverted AP and the noninverted AP implants in the one-growth-season group. A–C: Deer 2. **A**: Fifty-seven days after surgery, antler transformation (arrow) had started on the 15 mm high pedicle from the inverted AP side; whereas, no sign of antler transformation could be observed on the apex of the 20 mm high pedicle from the noninverted AP side (arrowhead). **B**: At tissue biopsy (74 days after surgery), antler transformation was well on the way although not completed (arrow) on the inverted AP side; but had not started on the 29 mm high pedicle from the noninverted AP side (arrowhead). **C**: Biopsy of the two appendages from their base using a scalpel (arrow). D–F. Deer 4. **D**: Fifty-seven days after surgery, antler transformation (arrow) had completed on the 20 mm high pedicle from the inverted AP side; whereas, no sign of antler transformation could be observed on the apex of the 25 mm high pedicle from the noninverted AP side (arrowhead). In **E**, at tissue biopsy (74 days after surgery), antler growth (arrow, evidenced by shiny skin and sparsely populated hairs) had taken place from the inverted AP side; whereas, in **F**, no velvet skin could be observed on the 28 mm high pedicle from the noninverted AP side (arrow). G–I: Deer 3. **G**: At the time of taking sutures out, pedicle formation had started from both the inverted AP and noninverted AP sides; no antler transformation could be detected from either of the pedicles. In **H**, Antler transformation had occurred on the 19 mm high pedicle from the inverted AP side (arrow); whereas, in **I**, only took place when the pedicle reached 52 mm high from the noninverted AP side (arrow).

and spread (Fig. 3B,C). It cannot be determined from the present results, whether the newly formed periosteum was derived through migration of both the existing (originally grafted) AP fibrous and cellular cells or via *in situ* proliferation of the lining cells (progeny of the AP cellular layer cells) of the most distal bony trabeculae. This is because the new periosteum was the continuum of the original counterpart (Fig. 3B), and at the same time, its cellular layer was seamlessly merged with the lining cells of the underneath bony trabeculae (Fig. 3D).

At the time of tissue sampling, a 29-mm high pedicle on the noninverted AP side in Deer 2 had developed, but no antler transformation occurred. The histological makeup of the pedicle (Fig. 3E) matched those of normal late developmental stage pedicles (Li and Suttie, 1994), distoproximally i.e., apical scalp skin, hyperplastic mesenchyme, continuous and discrete cartilage columns, and trabecular bone. In contrast, antler transformation on the inverted AP side had taken place from the 15-mm high pedicle. Interestingly, although the newly formed

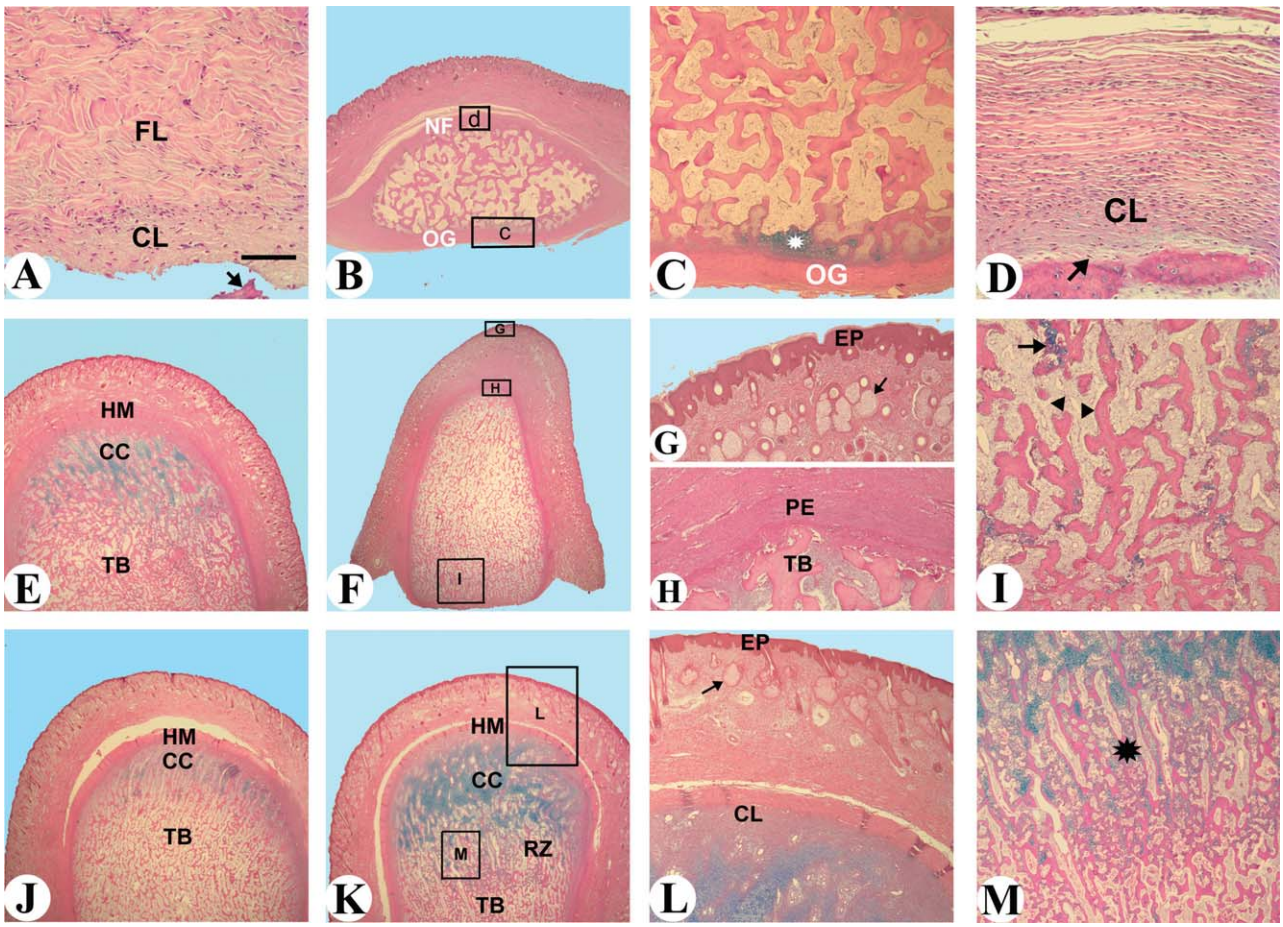

Fig. 3. Histological examination of the appendages formed from both the inverted AP and the noninverted AP implants in the one-growth-season group. Haematoxylin and eosin and alcian blue counterstaining. The 10 mm bar used for Fig. 3A also applies to every other figure in Fig. 3, but represents different lengths that are indicated below. **A**: Cross section of the AP from the noninverted AP side at the death of Deer 6. Note that no growth can be detected. CL, cellular layer; FL, fibrous layer. Arrow points to trabecular bone. Bar = 20 μm. **B**: Cross section of the ossicle formed from the inverted AP implant in Deer 6. Note that the ossicle was wrapped by an uninterrupted layer of periosteum that is composed of originally grafted (OG) AP at base and newly formed (NF) periosteum on top. Bar = 1.76 mm. **C**: Higher magnification of the area labelled with "C" in Fig. 3B to show that cartilage tissue (blue color) was formed by the cellular layer cells of the original AP in that area. Bar = 75.9 μm. **D**: Higher magnification of the area labelled with "D" in Fig. 3B to show that the CL of the newly formed periosteum was in seamless transition with the lining cells (arrow) of the underneath bony trabeculae. Bar = 20 μm. **E**: Sagittally cut longitudinal section of the pedicle formed from the noninverted AP implant in Deer 2. Note that the histological makeup is comparable with that of a normal late stage developmental pedicle distoproximately: hyperplastic mesenchyme (HM), cartilage columns (CC), and trabecular bone (TB). Bar = 2.27 mm. **F**: Sagittally cut longitudinal section of the appendage formed from the inverted AP implant in Deer 2. Note that the histological makeup is comparable with that of a normal late stage developmental pedicle or an early stage antler, but in an inverted order (also refer to Fig. 3H,I). Bar = 3.37 mm. **G**: Higher magnification of the area labelled with "G" in Fig. 3F to show that the apical skin had acquired velvet features: such as thickened epidermis (EP), large multilobed sebaceous glands (arrow), and absence of sweat glands. Bar = 21.5 μm. **H**: Higher magnification of the area labelled with "H" in Fig. 3F to show that under the apical periosteum (PE) still lay mature trabecular bone (TB), although the overlying skin had transformed into antler velvet (Fig. 3G). Bar = 21.5 μm. **I**: Higher magnification of the area labelled with "I" in Fig. 3F to show that the remodelling zone of cartilage (arrow) and newly formed slender bone trabeculae (arrowheads), which were differentiated from the originally grafted AP cellular layer cells. Bar = 28.4 μm. **J**: Sagittally cut longitudinal section of the pedicle formed from the noninverted AP implant in Deer 4. Note that the histological makeup is comparable with the counterpart of Deer 2 (Fig. 3E): consisting of hyperplastic mesenchyme (HM), cartilage column (CC), and trabecular bone (TB). Bar = 2.27 mm. **K**: Sagittally cut longitudinal section of the antler formed from the inverted AP implant in Deer 4. Interestingly, the histological structure of this antler is very different from the one that formed from the inverted AP implant in Deer 2 (Fig. 3F,H), but comparable with the one derived from the noninverted AP side in the same deer (Fig. 3J). Apically positioned antler growth centre of the antler had well formed at this stage, which consisted of hyperplastic mesenchyme (HM), cartilage columns (CC), remodelling zone (RZ), and trabecular bone (TB). Bar = 2.38 mm. **L**: Higher magnification of the area labelled with "L" in Fig. 3K to show the nature of apical velvet skin (thickened epidermis (EP), large sebaceous glands (arrow) and absence of sweat glands), and underlying hyperplastic mesenchyme [thickened cellular layer (CL)]. Bar = 80.1 μm. **M**: Higher magnification of the area labelled with "M" in Fig. 3K to show the remodelling zone. Note that the earlier formed cartilage had been destroyed via chondroclasia (asterisk). Bar = 130 μm.

and apically positioned periosteum had fused to the overlying skin (Fig. 3F) and the skin had transformed into antler velvet (Fig. 3G), the tissue immediately underlying the periosteum was mature trabecular bone (Fig. 3H) rather than expected cartilage, an essential component of an antler growth centre. In marked contrast, a substantial amount of newly formed tissue was observed above the cellular layer of the originally grafted inverted AP (Fig. 3I).

Antler transformation was more advanced in Deer 4 compared with Deer 2. Although histologically the appendage developed from the noninverted AP implant in Deer 4 was typical of a pedicle (Fig. 3J), the one from the inverted AP implant had convincingly transformed into antler (Fig. 3K) as shown from distal to proximal by apical velvet skin, hyperplastic mesenchyme (Fig. 3L), continuous and discrete cartilage columns, osseocartilage, remodelling zone, and trabecular bone (Fig. 3M). Therefore, the histological structure of the inverted AP-derived antler in Deer 4 was comparable with the similar developmental stage as that of the naturally formed pedicles and antlers.

"Two-Growth-Season" Appendages

In the second antler growth season, two antlers formed in both Deer 5 and 7; whereas only one antler developed in Deer 1 and 8, although in Deer 1 from the inverted AP implant and in Deer 8 from the noninverted AP implant, respectively. No visible growth occurred on their counter-lateral sides in either of the deer during the experimental period.

Pedicle and/or antler did not visibly grow on either side of the future antler regions in Deer 1, during the first antler growth season (Fig. 4A). However, in the late autumn, the skin over the inverted AP implant was shed exposing a bare bony button (Fig. 4B), suggesting true antler transformation did occur from an almost invisible pedicle (11 mm). In the next spring, a normal size spike antler was regenerated from this stunted pedicle after casting of that hard button (Fig. 3C). In contrast, in Deer 8, a normal pedicle (31 mm) and a small spike antler (50 mm) were developed from the noninverted AP implant in the first antler growth season (Fig. 3D,E). In the second antler growth season, a two-branched-antler with a reversed anterior and posterior polarity was regenerated after casting of the spike antler (Fig. 3F).

Similar to Deer 8, Deer 7 also formed a normal pedicle and a spike antler from the noninverted AP implant in the first antler growth season (Fig. 4G); unlike Deer 8, Deer 7 developed a two-branched-antler in the second season. Deer 7 initiated a small incipient pedicle (8 mm) from the inverted AP implant in the first antler growth season (Fig. 3H) and a normal spike antler from that shortened pedicle (8 mm in height) in the second season (Fig. 3I).

Deer 5 was the only animal from "two-growth-season" group that developed pedicles and antlers on both the noninverted AP and inverted AP sides in the first antler growth season (Fig. 3K) and formed two-branched antlers in the second season (Fig. 3L), although the height of the full-grown pedicle from the inverted AP side (20 mm) was less than half the height of the noninverted AP side (51 mm).

DISCUSSION

Height of Full-Grown Pedicles and Timing of Antler Transformation

As an organ, first antler development relies on the interactions between mesenchyme (antlerogenic tissue, i.e., AP) and epithelium (epidermis of the overlying skin; Goss, 1990; Li et al., 2008). To enable establishment of these heterotypic tissue interactions, the interactive tissues (AP-derived apical pedicle tissue and its overlying skin) must become intimately associated (Li and Suttie, 2000). To maintain this close tissue contact, AP-derived pedicle tissue must continuously grow to provide a constant mechanical tension to the overlying skin, as mammalian skin has a tendency to release the mechanical pressure by forming new skin to accommodate the expanding tissue mass below (Austad, 1988). This incessant growth of the interior pedicle tissue is triggered and sustained by the high level of circulating androgen hormones (Li et al., 2003b). The time length of this tissue association required for successful establishment of these interactions varies among deer species, which explains why different deer species develop pedicles of different heights (5–55 mm; Li et al., 2003a).

It is known that full-grown sika deer pedicles measure 40–50 mm high (Li et al., 1988). Interestingly, in this study, AP manipulation (peeling off and grafting back) did not alter the height (45 $\pm$ 11.7 mm) and full-grown pedicles were formed from the noninverted AP implants. However, the final pedicle height (17 $\pm$ 5.1 mm on average) at which antler transformation started to take place was significantly shortened when AP was implanted back in an inverted orientation (cellular layer facing the overlying skin). In other words, with the AP cellular layer contacting the overlying skin, antler transformation was significantly advanced compared with the natural orientation, with the AP fibrous layer facing the skin. Therefore, the AP cellular layer is likely to be the source of the initial interactive molecules. If this statement is true, to establish the tissue interactions under the natural situation, the initial interactive molecules secreted by the AP cellular layer need to diffuse through the overlying AP fibrous layer before reaching their target, overlying dermal and epidermal cells. Consequently, the AP fibrous layer may be required in this system to act as a physical barrier to control the timing of establishment of the tissue interactions, hence specifying the species-specific pedicle height.

Here, one may argue that antler transformation on the inverted AP sides was indeed advanced compared with the noninverted AP sides, but only spatially (reduced full-grown pedicle height) and not temporally (time length from implantation surgery to antler transformation). Because, in the study (Table 1), antler transformation on each inverted AP side from the only three deer (Deer 3, 5, and 7) that developed antlers on both sides took longer time than that on their corresponding noninverted AP sides, particularly in Deer 7 over three times longer (389 days vs. 119 days). Therefore, it would be premature to conclude that the AP cellular layer provides major source of the initial interactive molecules. It is true based on the data (Table 1) that antler transformation on the inverted AP side was temporally delayed in these three deer. However, a close examination reveals that this temporal delay was caused by the

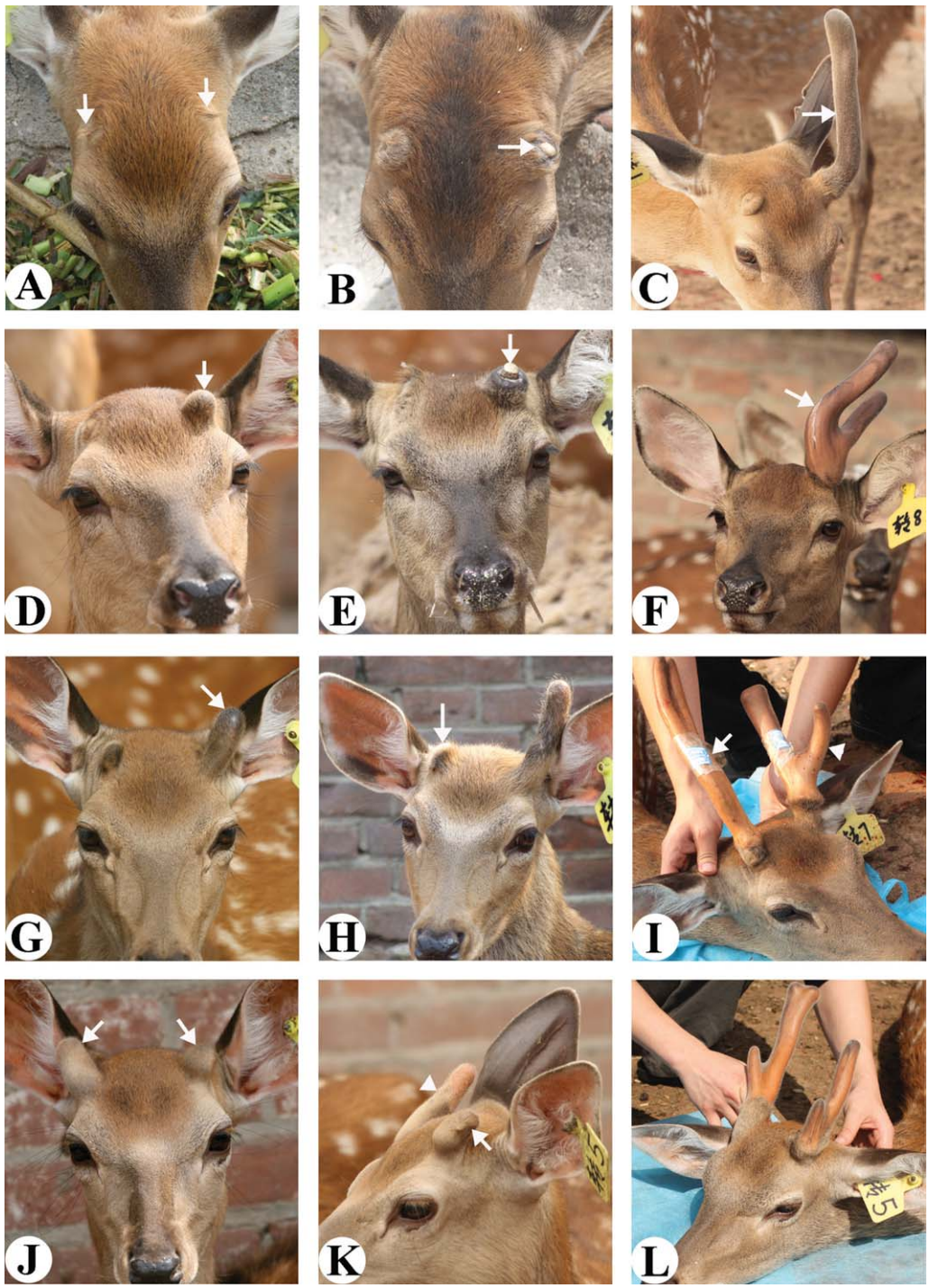

Fig. 4. Status of pedicle/antler formation from the inverted AP and the noninverted AP implants in the two-growth-season group. A–C: Deer 1. **A**: No sign of any development from either side of presumptive antler growth regions (arrows) in the late summer. **B**: A hard button (arrow) was revealed on the inverted AP side after shedding of the overlying skin in the late autumn, suggesting the true antler had differentiated from that side. **C**: A reasonably normal spike antler (arrow) was formed from the inverted AP side in the second antler growth season after casting that hard bony button. D–F: Deer 8. **D**: A pedicle developed from the noninverted AP side (arrow), and antler transformation took place when the pedicle reached 31 mm high 83 days after surgery. **E**: The antler (arrow) was totally calcified in the late autumn and removed from its base for the safety reason. **F**: A two-branched antler (arrow) was regenerated in the second antler growth season from the noninverted AP side after casting of that previous bony remnant. G–I: Deer 7. **G**: A pedicle was developed on the noninverted AP side and antler (arrow) transformation took place when the pedicle reached 45 mm high 119 days after surgery. **H**: An 8-mm high pedicle (arrow) was formed on the inverted AP side in the late autumn. **I**: A normal spike antler (arrow) was transformed on the 10 mm high pedicle (formed in the first antler growth season) from the inverted AP side; whereas, a two-branched antler was regenerated on the pedicle from the noninverted AP side (arrowhead). J–L: Deer 5. **J**: Pedicles were developed from both the inverted AP and noninverted AP sides at the time of taking sutures out. **K**: Antlers were transformed from both sides of the pedicles, but on the inverted AP side it took place when the pedicle reached 20 mm high (arrow); whereas, on the noninverted AP side it occurred when the pedicle reached 51 mm high (arrowhead). **L**: Antler regeneration took place from both sides of the pedicles and both formed two-branched antlers.

delayed initiation of pedicle growth on the inverted AP sides, rather than by the impaired pedicle growth rate. For example, in Deer 7 true pedicle development on the inverted AP side did not start until the second antler growth season, although on the noninverted AP side it commenced in the first antler growth season (differs by a year). Besides, in Deer 2 and 4, at the time of completion of antler transformation on the inverted AP sides, there was no indication on the noninverted AP sides that antler transformation was commencing. Consequently, the time length between implantation surgery and antler transformation may be more a reflection of the timing of reestablishment of proper circulation to the AP implants, rather than a reliable indicator of the timing of antler transformation. This claim is also supported by a phenomenon that antler transformation from an ectopically formed pedicle can be delayed for several years (Kierdorf and Kierdorf, 2000). Nonetheless, the data of this study (Table 1) do suggest that reestablishment of proper circulation to an inverted AP implant is slower than to a noninverted AP implant, as there is an overall delay of pedicle initiation on the inverted AP sides compared with that on the noninverted AP sides.

Histogenesis of Appendages Developed from Inverted AP Implants

In a previous histological study (Li and Suttie, 1994), we reported that a pedicle and subsequent antler are built up by proliferation and differentiation of the AP cellular layer cells. Initially, the cellular layer cells differentiate into osteoblasts and then trabecular bone is formed (intramembranous ossification); when a pedicle reaches ~10 mm high, some of the cellular layer cells start to change differentiation direction toward a chondroblast lineage, and the mixture of bone and cartilage is formed (transitional ossification); when a pedicle reaches ~35 mm high, all cellular layer cells differentiate into chondroblasts and then uniform cartilage is formed (modified endochondral ossification). As the newly formed cartilage and bone tissue is gradually built up below the scalp skin, AP is continuously pushed upward, which is by then called reserve mesenchyme (Banks and Newbrey, 1982). From the mid stage of the transitional ossification onward, apically positioned mesenchyme becomes closely associated with the overlying skin by totally compressing the interposed subcutaneously loose connective tissue (Li and Suttie, 2000), and subsequently, the interactions between reserve mesenchyme and the overlying skin are established for triggering antler transformation.

To generate a pedicle and an antler, an inverted AP implant also has to form sufficient tissue mass to create the close association with the overlying skin. This study showed that the inverted AP cellular layer cells (facing the skin) first formed trabecular bone and then switched differentiation direction to form cartilage (Fig. 3B,C). Unexpectedly, instead of being the earliest differentiated mature trabecular bone directly in contact with the overlying skin, a layer of newly formed periosteum was found to interpose between the trabecular bone and the overlying skin, even at the beginning of transitional ossification (Fig. 3B,D). Interestingly, even after antler transformation has almost completed (as judged by skin morphology and histology), from the inverted AP formed 15 mm high pedicle in Deer 2; beneath the newly formed apical periosteum still lay mature trabecular bone (Fig. 3H), and elongation of the appendage was still driven by the proliferation and differentiation of the cellular layer cells of the original AP implant located at the base (Fig. 3F,I). One possible explanation is that this phenomenon is caused by insufficient blood supply from the reestablished circulation. Nevertheless, a well formed apical antler growth centre does not seem to be indispensible for antler transformation to take place. In marked contrast, the histological makeup of the growth centre (Fig. 3K) of the antler formed from the inverted AP-derived pedicle (20 mm high) of Deer 4 was perfectly comparable with that of similar growth stage normal spike antler.

Unquestionably, more tissue samples from more different pedicle and early antler developmental stages are required to answer the following questions: (1) Where do the cells responsible for the new periosteum formation come from: direct differentiation of the lining cells of the earliest formed mature bony trabeculae from the AP cellular layer cells, or migration of the originally implanted the AP fibrous and cellular layer cells?, (2) When do the cellular layer cells of the newly formed and apically located periosteum start to replace the role of those of the originally implanted AP in driving antler elongation?, and (3) How can antler transformation be advanced from the inverted AP implants compared with noninverted AP implants, given that in the former an extra step is required for rebuilding the apical periosteum before the interactions between the AP-derived tissue and the overlying skin take place?

Overall, this study convincingly demonstrated that implantation of AP discs in an inverted orientation, i.e., bringing the naturally distant AP cellular layer to immediately adjacent to the overlying skin, did generate pedicles with the final height less than half the height of those formed from the noninverted AP implants (the AP fibrous layer facing the skin). In other words, antler transformation can be significantly advanced if the AP cellular layer becomes closely associated with the overlying skin. Therefore, the AP cellular layer, as opposed to the AP fibrous layer, is likely to be the main source of the initial inductive molecules for antlerogenesis.

ACKNOWLEDGMENTS

The authors thank the staff of Biotechnology Laboratory and the deer crew from Institute of Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, China, for the help with the tissue implantation surgery and subsequent observation as well as Drs. Stephen Haines and Colin Mackintosh for the critical comments to the manuscript.

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