

Deer Antler Regeneration: A Stem Cell-Based Epimorphic Process

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Full regeneration of deer antlers, a bona fide epimorphic process in mammals, is in defiance of the general rule of nature. Revealing the mechanism underlying this unique exception would place us in a better position to promote organ regeneration in humans. Antler regeneration takes place in yearly cycles from its pedicle, a permanent protuberance on the frontal bone. Both growing antlers and pedicles consist of internal (cartilage and bone) and external components (skin, blood vessels, and nerves). Recent studies have demonstrated that the regeneration of both internal and external components relies on the presence of pedicle periosteum (PP). PP cells express key embryonic stem cell markers (Oct4, Nanog, and SOX2) and are multipotent, so are termed antler stem cells. Now it is clear that proliferation and differentiation of PP cells directly forms internal antler components; however, how PP initiates and maintains the regeneration of external antler components is thus far not known. Based on the direct as well as indirect evidence that is presented in this review, I put forward the following hypothesis to address this issue. The full regenerative ability of external antler tissue components is achieved through PP-derived chemical induction and PP-derived mechanical stimulation: the former triggers the regeneration of these external components, whereas the latter drives their rapid elongation. Eventual identification of the putative PP-derived chemical factors would open up a new avenue for devising effective therapies for lesions involving each of these tissue components, be they traumatic, degenerative, or linked to developmental (genetic) anomalies. **Birth Defects Research (Part C) 96:51–62, 2012. © 2012 Wiley Periodicals, Inc.**

Key words: antler regeneration; stem cells

INTRODUCTION

The “Holy Grail” of modern regenerative biology and medicine is to achieve organ regeneration in mammals (Stocum, 2006; Carlson, 2007). The most dramatic type of organ regeneration is known as epimorphic, a phenomenon of de novo development of appendages distal to the level of amputation (Goss, 1983). To date, our knowl-

edge of epimorphic regeneration has primarily come from studies of amphibians, and a typical example is the growing back of missing limbs by newts. Notably, these animals have the ability to reprogram phenotypically committed cells at the amputation plane toward an embryonic-like cell phenotype (dedifferentiation) and to form a cone-shaped tissue mass, known

as a blastema (Mescher, 1996). Whether mammals have the capability to initiate analogous regeneration, and whether similar mechanisms would be involved if it were to occur, remains unclear. Deer antlers are the only mammalian appendages capable of full renewal, and therefore offer a unique opportunity to explore how nature has solved the problem of epimorphic regeneration in mammals (Goss, 1995; Kierdorf et al., 2009; Li et al., 2009). Through extensive studies, we and others have recently concluded that antler regeneration is not a blastema-based, but rather a stem cell-based, epimorphic process (Li et al., 2004, 2005, 2007a, 2010; Kierdorf et al., 2007; Rolf et al., 2008). Revealing the mechanism underlying this stem cell-based epimorphic regeneration would undoubtedly place us in a better position to promote tissue/organ regeneration in humans.

Antlers, despite being called head pieces, do not regenerate directly from the head of a deer but instead from the permanent cranial bony outgrowths, known as pedicles (Li, 1997). Deer are not born with pedicles; instead, these start to develop from frontal crests (behind and above the eye sockets) when deer approach puberty (Suttie et al., 1991; Suttie et al., 1995; Li et al., 2003). Initially, the developing pedicles are

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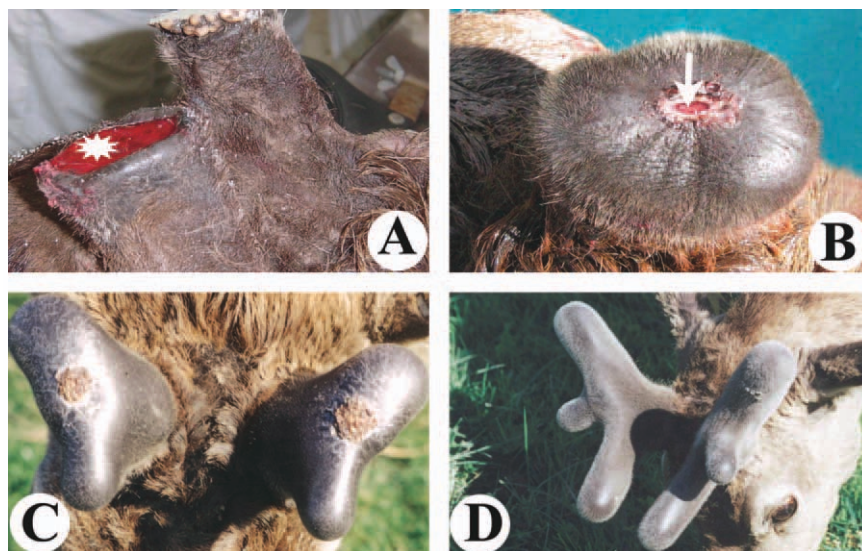


Figure 1. Antler regeneration from the permanent bony protuberance, the pedicle, in red deer. (A) Pedicle stump. Note the fresh casting surface (asterisk) immediately after casting the previous year's hard antler. (B) Late wound healing (arrow) stage antler bud. (C) Two-branch-stage antlers. (D) Three-branch-stage antlers.

covered by typical scalp skin. When they have grown to their species-specific height (around 5–6 cm in red deer), first antlers begin to generate spontaneously from the apices of these pedicles. This development can be seen externally by a change in the appearance of the skin from the typical scalp skin to a velvet-like soft pelage, called velvet skin or velvet. When the rutting season approaches, the antlers become fully calcified and the blood supply is occluded, which causes the demise of the velvet skin. The dead velvet is subsequently shed to expose the bare bone of the hard antlers. These are cast in the following spring, and regeneration of the second set antlers from their living pedicle stumps is immediately initiated. From then on, annual renewal of subsequent antlers enters a well-defined cycle: casting of previous hard antler (Fig. 1A) and regeneration of a new soft antler (Fig. 1B) in spring, with rapid antler growth (up to 2 cm/day) and maturation in summer (Fig. 1C, D), then full antler calcification and velvet shedding in autumn, followed by the bare bony antler phase in winter (Goss, 1983).

PEDICLE PERIOSTEAL CELLS ARE ANTLER STEM CELLS THAT GIVE RISE TO INTERNAL TISSUE COMPONENTS OF A REGENERATING ANTLER

Both pedicles and regenerating antlers are composed of internal (cartilage and bone) and external (skin, blood vessels, and nerves) tissue components (Li, 1997). Regeneration of antler internal tissue components has been studied reasonably extensively (Goss, 1961; Kierdorf et al., 2003; Li et al., 2005; 2007a,c; Mount et al., 2006; Rolf et al., 2008). Immediately after a hard antler falls off, the rim of pedicle skin surrounding the distal end of a pedicle stump encroaches upon the bone margin, the space that was formerly occupied by the periphery of the antler base. This rim of skin is shiny, only sparsely populated by hair, and it possesses the peculiar features of velvet skin, specifically a thicker epidermis and the de novo formation of hair follicles (Fig. 2A), which distinguishes it from the more proximal pedicle skin, typical of the scalp skin. Within days of hard

antler casting, wound healing nears completion by centripetal growth of velvet skin over the cast plane of a pedicle (Fig. 2B, C). At the same time, the distal part of pedicle periosteum (PP) becomes thickened through the active division of cells resident within it (Fig. 2B Inset). Toward the late wound healing stage, two crescent-shaped growth centers are formed directly from the thickening distal PP, one of which is located anteriorly (Fig. 2C, D) and the other posteriorly. Each center is made up of cartilaginous clusters that are capped by a layer of hyperplastic PP/perichondrium (Fig. 2D). Further augmentation of each growth center pushes up the anterior and posterior portions of the pedicle stump and leaves the central region behind. These posterior and anterior growth centers are the centers for the formation of the antler "main beam" and "first tine" (Li et al., 2005). This histological finding clearly indicates that the growth centers of a regenerating antler are formed exclusively from the proliferation and differentiation of distal PP cells of a pedicle stump, whereas pedicle skin only plays a role in wound healing to seal the cast plane of the pedicle stump.

Morphological and histological findings, while indispensable in the initial linking of PP with antler renewal, do not prove that a regenerating antler is derived exclusively from PP. To confirm this hypothesis, we carried out the following two experiments (Li et al., 2007a). In the first, the distal part of PP (one-third of the total length) was surgically removed before antler regeneration (Fig. 3A), to determine whether antler regeneration could still take place in its absence. Without the distal PP present, the pedicle skin was retracted along the pedicle shaft proximally to the level where it met the regenerating PP (Fig. 3B). Interestingly, an antler bud was formed/regenerated from the place where the distal ends of the regenerating periosteum and the retracting skin

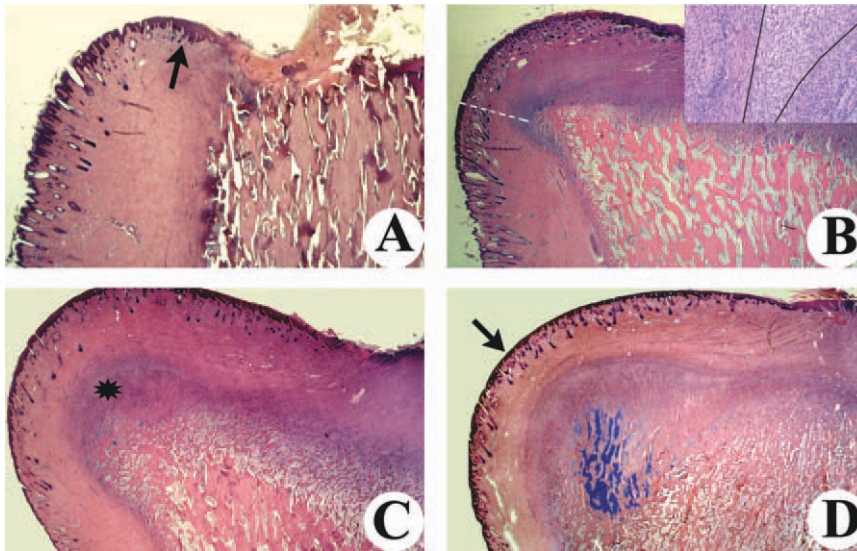


Figure 2. Histological examination of antler skin regeneration from the anterior side of a pedicle stump. (A) Sagittally cut section immediately after the hard antler casting. Note that at this stage the epithelium of the rim formed by distal pedicle skin has become thickened and acquired some velvet skin features, that is, development of new hair follicles (arrow). (B) Sagittally cut section at the early wound healing stage. Note that there is a clear demarcation (dotted line) between the pedicle skin and the newly healing skin, and the latter has clearly become antler velvet type thickened pedicle periosteum. (C) Sagittally cut section at the late wound healing stage. Note that the anterior growth center has become established (asterisk). (D) Sagittally cut section of an early regenerating antler bud. Note that wound healing process has completed, and the velvet type skin that covers the anterior growth center is pushed outwardly by the rapid expansion of the PP-derived tissue mass.

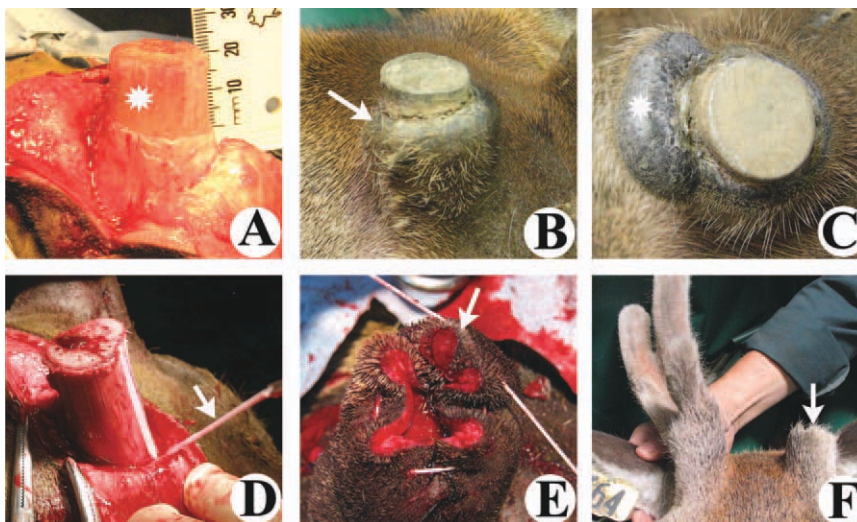


Figure 3. Antler regeneration from the partial- or total-PP-deleted pedicles (Fig. 3A, C, D, F; after Li et al., 2007a). (A) Partial PP deletion (asterisk) on a 2-year-old red deer stag. (B) Pedicle stump photographed 20 days after surgery. Note that skin from the pedicle had retracted proximally (arrow), which left the pedicle with a bare bony tip sticking out. (C) PP-deleted pedicle photographed 33 days after surgery. Note that an antler bud (asterisk) formed/regenerated on the pedicle shaft at the site where the regenerating PP and retracting pedicle skin met. (D) Total PP deletion (arrow) on yearling red deer stags. (E) Purse string suturing (arrow) was performed after total PP deletion. (F) No antler regeneration occurred on the total-PP-deleted pedicle (arrow), although the pedicle skin remained in place. The sham-operated pedicle formed a three-branched antler.

met on the pedicle shaft, which is markedly distant from the original regeneration site (Fig. 3C). In the second experiment, the PP was completely removed before antler regeneration (Fig. 3D). To stop pedicle skin retraction, a purse string tie was made following the total PP deletion (Fig. 3E). No antler regenerated even though the distal pedicle skin was firmly held in its original place (Fig. 3F). Therefore, these PP deletion experiments convincingly demonstrated that PP is the key tissue type not only for internal antler tissue formation but also for initiation of antler regeneration.

The PP deletion experiments, although convincing to show that PP is indispensable for antler regeneration, did not shed light on the possible role of pedicle skin in antler regeneration as pedicle skin is always associated with the process of antler regeneration. In fact, Waldo and Wislocki (1951) along with Goss (1992, 1995) stated that the cells in the dermal layer of the pedicle skin form the regenerating antler bud, and hence give rise to the whole antler. To address this issue, we carried out a membrane insertion experiment to physically separate pedicle skin from PP before antler regeneration (Fig. 4A) (Li et al., 2007c). Interestingly, a skin-less antler was regenerated (Fig. 4B) as pedicle skin failed to participate in the antler regeneration process once being separated from PP, even if the distal end of the pedicle skin had transformed into antler velvet (Fig. 4C) toward the end of the study at the place where the separating membrane was missing (Fig. 4D). Therefore, it is the PP, but not pedicle skin, that is the key tissue type for antler regeneration.

Now, one wonders how can such limited number of a single cell type (PP cells) reconstitute an entire antler? To answer this question, we characterized PP cells in vitro. First, we found that cells of both PP and antlerogenic periosteum, from which PP is derived (as described below), express the stem cell marker CD9 antigen (Fig. 5A). Critically, three so-called

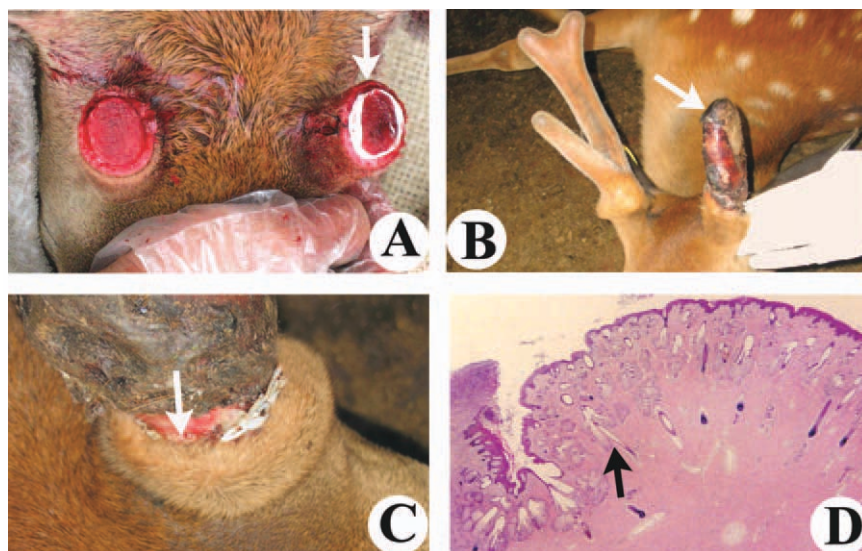


Figure 4. Membrane insertion and antler regeneration (Fig. 4A; after Li et al., 2007c). **(A)** Pedicle stump with the inserted membrane (arrow). **(B)** Skin-less antler (arrow) was regenerated from the membrane-inserted pedicle stump. **(C)** The same skin-less antler in Figure 5B, but at a later stage of regeneration. Note that some parts of the very distal end of the pedicle skin were observed to be in direct contact with the PP (arrow). **(D)** The distal pedicle skin from the direct contact parts to the PP. Note that the skin from these parts had acquired velvet skin features (arrow).

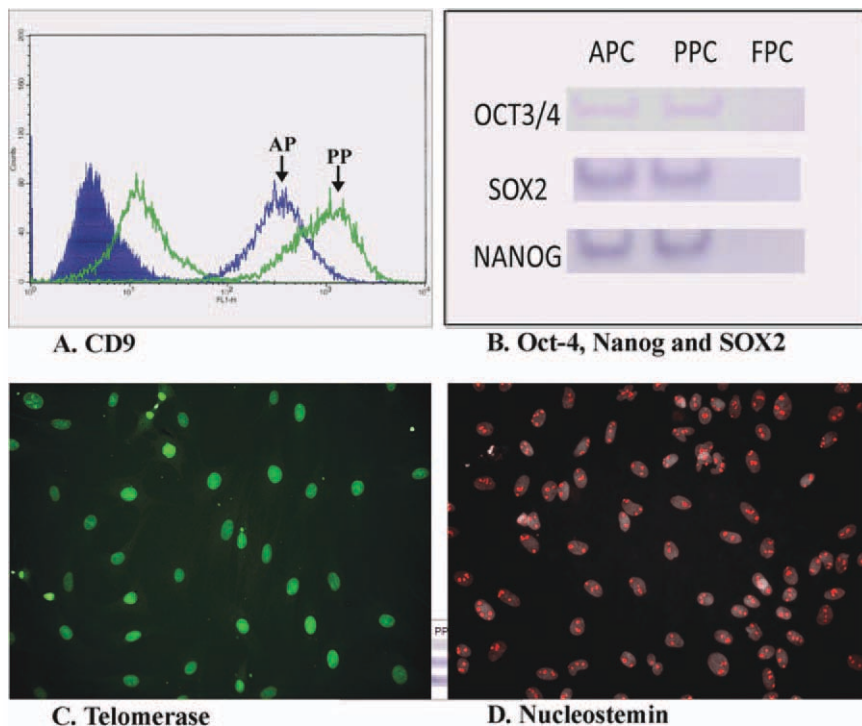


Figure 5. Expression of key embryonic stem cell markers (after Li et al., 2009). **(A)** CD9 in antlerogenic periosteum (blue trace) and PP (green trace) cell populations determined by flow cytometry. **(B)** Detection of the "pluripotency" genes Oct3/4, SOX2, and Nanog by Western blot analysis. APC, antlerogenic periosteal cells; PPC, pedicle periosteal cells; FPC, facial periosteal cells. **(C)** Expression of telomerase by antler stem cells detected by immunohistochemistry. **(D)** Expression of nucleostemin by antler stem cells detected by immunohistochemistry.

pluripotency genes, the POU domain family members Oct4, Nanog, and Sox 2 (Ginis et al., 2004), are present in both cell types (Fig. 5B). Additionally, we have shown elevated telomerase enzyme activity (Fig. 5C) and nucleostemin (Fig. 5D) in both cell types (Li et al., 2009). Telomerase activity has been linked to enhanced self-renewal in cells (Yang et al., 2008), which may explain how so few PP cells (3.3 million) that participate in each round of antler regeneration are capable of forming 10 kg or so of antler tissue mass within just 55–60 days. Expression of nucleostemin has been linked to the control of stem cell proliferation (Beekman et al., 2006) and newt limb regeneration (Maki et al., 2007). Furthermore, we found that PP cells can readily be induced in vitro to differentiate into cartilage (Fig. 6A), fat (Fig. 6B), muscle (Fig. 6C), and neuronal-like (Fig. 6D) cells. These results strongly suggest that the PP cell population not only function as tissue-specific "stem cells" in the adult organism but also retain characteristics of an embryonic origin throughout the lifetime of the animal. Therefore, PP cells are bona fide antler stem cells, and it is their proliferation and differentiation that give rise to internal antler tissue components and initiate the antler regeneration.

PEDICLE PERIOSTEAL CELLS ARE INDISPENSIBLE FOR INDUCING THE REGENERATION OF EXTERNAL ANTLER TISSUE COMPONENTS

The external tissue components of a regenerating antler consist of skin, blood vessels, and nerves. In contrast to the regeneration of internal antler tissue components, how antler skin, blood vessels, and nerves are regenerated has not been addressed so far, which has obviously hindered progress in the development of deer antlers as

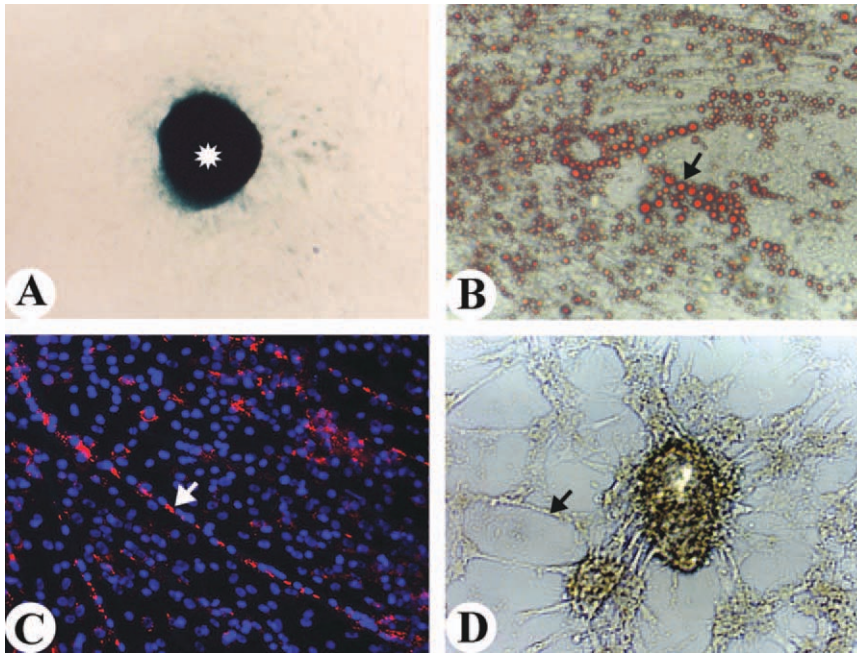


Figure 6. Multipotency of antler stem cells (after Li et al., 2009). **(A)** Cartilage nodule (asterisk) formed by antler stem cells in a micromass culture. **(B)** Adipocytes (arrow) differentiated from antler stem cells in the culture medium containing linoleic acid. **(C)** Myotube (arrow) formed from antler stem cells when cocultured with C2C12 cells. **(D)** Neuronal-like cells differentiated from antler stem cells when cultured in N2 medium. Note the extended neurites (arrow) from each cell body.

a mammalian epimorphic regeneration model.

Histological Makeup of Antler Skin, Blood Vessels, and Nerves

Studies have shown that antler external tissue components are not simple extensions of their pedicle counterparts. Antler velvet (Fig. 7A), in contrast to pedicle skin (Fig. 7B; a typical scalp skin), lacks a subcutaneous loose connective tissue layer but has a much thicker epidermis and the ability to form new hair follicles. These newly formed follicles lack associated arrector pili muscles and sweat glands but possess large multilobed sebaceous glands (Billingham et al., 1959; Goss, 1964; Bubenik, 1993; Li and Suttie, 2000; Li, 2010).

The arteries of antlers are peculiar in structure in that they have a conspicuously thickened wall and very small constricted lumen (Fig. 7C), compared with similar sized arteries (Fig. 7D) in pedicles (Waldo et al., 1949; Goss, 1983; Clark et al., 2006). The walls of

antler arteries possess no distinct elastic interna or externa and no ordinary media, but they are composed of elastic, muscular, and collagenous elements that are rather diffusely interwoven (Waldo et al., 1949). These authors postulated that the walls of antler arteries are constructed so as to offer more than usual resistance to being compressed or crushed as well as having the ability to close themselves by constricting promptly and firmly if they are severed.

Antler nerves consist of both myelinated and nonmyelinated fibers (Wislocki and Singer, 1946; Adams, 1979). The sensory nature of antler nerves has recently been confirmed by their neurochemical signature, in particular their immunoreactivity to calcitonin gene-related peptide, Substance P, NF200 peptides, and isolectin B4 (Rayner et al., 1981; Gray et al., 1992; Li et al., 2007b; Pita-Thomas et al., 2010). Based on these results, Nieto-Diaz et al. (in press) classified antler nerves into myelinated Abeta fibers, thin myelinated Adelta fibers, unmyeli-

nated peptidergic C fibers, and nonpeptidergic C fibers. There is no report thus far with regard to the difference in histological makeup of nerve fibers between antlers (Fig. 7E) and pedicles (Fig. 7F). However, functionally the growth rate of antler nerve fibers is truly remarkable and can extend their length to 2 cm/day, which far exceeds growth rates known in other animals (Wislocki and Singer, 1946; Goss, 1995), and is at least 10 times faster than that of pedicle nerves (based on the differential growth speed of pedicles and antlers (Li et al., 1994)).

Regeneration of Antler Skin

Regeneration of antler velvet skin commences with the wound healing over a pedicle stump by distal pedicle skin. This wound healing should be considered as a default process, because wound healing happens universally to restore continuity of interrupted tissue (Goss, 1980; Goad et al., 1996). The uniqueness in this case is, however, that when the centripetally migrating healing skin passes the point of distal PP, the skin begins to change in nature from scalp type to velvet type (Fig. 2A, B), suggesting chemical induction is involved from the PP to the covering skin. In addition, the PP-derived expanding tissue masses of the anterior and posterior growth centers push the overlying skin outwardly, and at the same time maintain the nature of the skin as velvet type (Fig. 2C, D). From these histological observations, one gains the impression that regeneration antler skin is realized through both the PP-derived chemical induction and PP-derived mechanical stimulation. The chemical induction alters the skin type from scalp skin to antler velvet, whereas the mechanical stimulation drives velvet skin rapid elongation (Li, 1997; Li et al., 2009).

Our aforementioned PP deletion experiments (Li et al., 2007a) further support the conclusion that is made based on our histology results. Removal of the distal PP abrogated antler regeneration

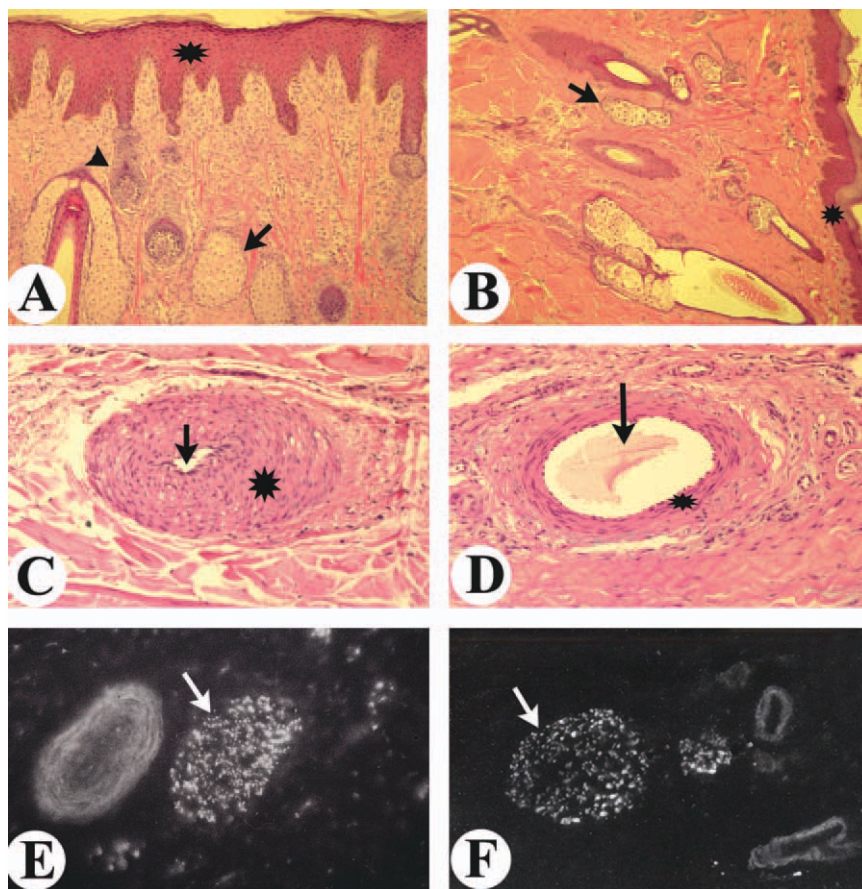


Figure 7. Comparisons of the histological makeup of skin, blood vessels, and nerves between pedicles and antlers. (A) Antler velvet. Note that epidermis is thick (asterisk) and possesses de novo developing hair follicles (arrow head), and sebaceous glands are large (arrow). (B) Pedicle skin. Note that epidermis is thin (asterisk), sebaceous glands are small (arrow). (C) Antler artery. Note that the artery has a considerably thick wall (asterisk) and narrow lumen (arrow). (D) Pedicle artery. Note that the artery has a thin wall (asterisk) and wide lumen (arrow). (E) Cross-section of antler nerve fibers. Note that the artery in the vicinity of the nerve has all the characteristics of an antler artery. (F) Cross-section of pedicle nerve fibers. Note that the artery in the vicinity of the nerve has all the characteristics of a pedicle artery.

from its original location (the distal end of a pedicle stump) and the retracting pedicle skin (due to absence of the distal PP, refer to section: pedicle periosteal cells are antler stem cells that give rise to internal tissue components of a regenerating antler) could only transform into antler velvet when it met regenerating PP in this case to initiate formation of an ectopic antler on the pedicle shaft, markedly distant from the expected location (Fig. 3C). Besides, no antler skin transformation and regeneration occurred after total PP removal, but instead a skin-less antler formed (Fig. 3F). These PP deletion experiments convincingly

demonstrated that transformation of antler velvet from pedicle skin and regeneration of antler skin fully relies on the presence of PP.

Additional experimental evidence is needed to support the claim that the PP dependency of antler velvet regeneration is composed of the PP-derived chemical induction and mechanical stimulation. One way to achieve this is to find a way to tease apart these two events, to show what one can do without the other. In this regard, we think the aforementioned membrane insertion experiment (Li et al., 2007c) can fulfil the role. In that study, it was noticed that the distal end of pedi-

cle skin was transformed into antler velvet toward the end of the study in some small places where the pedicle skin had direct contact with the adjacent PP due to partial lack of integrity of the separating membrane (Fig. 4C). Those direct contact segments of pedicle skin had acquired velvet skin features (Fig. 4D), but the transformed velvet skin did not give further growth. This finding clearly indicates that chemical induction from the PP had been conveyed to the distal pedicle skin where direct contact took place. However, this chemical induction did not stimulate the transformed velvet skin to grow or regenerate, possibly because the portion of the PP to which the skin was directly attached was no longer the growth center, as at that stage, the antler growth center had shifted to the antler tip. Consequently, mechanical stimulation may also be needed for velvet skin growth and regeneration.

Now the question remains, is it possible to generate new skin through mechanical stimulation? In fact, it is a general practice in clinical medicine to produce new skin through mechanical stretching (Fig. 8A) by subcutaneous insertion of an inflation-controlled tissue expander (Fig. 8B; (Austad and Rose, 1982; Timmenga et al., 1989; Brandy, 1991)). Austad and Rose (1982) concluded that skin expansion is a physiological process to accommodate an enlarging mass beneath it by increasing surface area. Francis and Marks (1977) found that stretching the skin stimulates epidermal proliferation only sufficiently to relieve tension. If mechanical stretch is an effective way to stimulate skin growth, it should not be surprising for us to claim that antler velvet regeneration and elongation are mainly driven by mechanical stimulation.

It is unknown thus far whether the enlarging internal antler components can create enough force to drive the enveloping skin not only to grow, but to grow at the rapid speed of up to 2 cm/day? To address this issue, we tested the

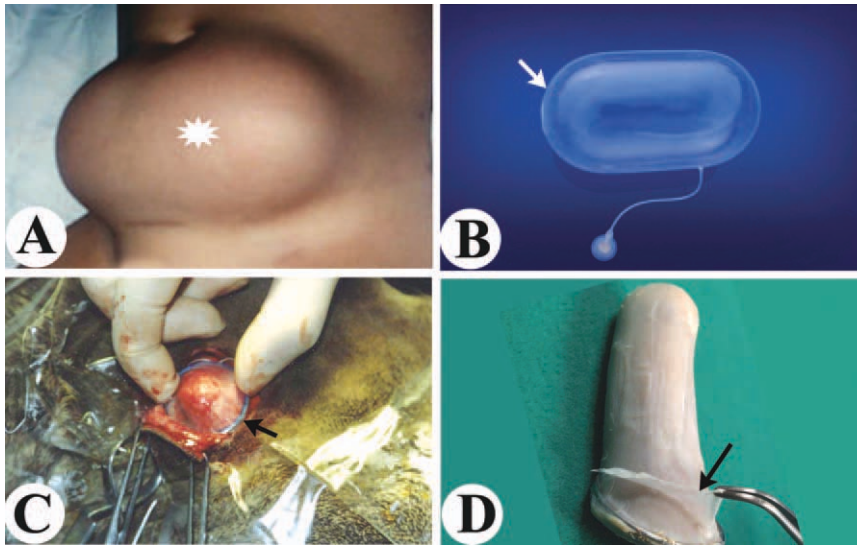


Figure 8. Mechanical stretch and skin formation. (A) Production of new skin (asterisk) by subcutaneous insertion of an inflation-controlled tissue expander. (B) Commercially available inflation-controlled tissue expander (arrow). (C) Membrane, which was attached to a stainless steel ring, was placed onto an exposed pedicle primordia (arrow). (D) The membrane was directly penetrated (arrow) by the growing antler tip.

results showed that the growing antler tip directly penetrated through the membrane (Fig. 8D). Because the breaking point of the membrane used was 1040 g/cm^2 (tested using a tenderometer), it follows that the antler growth force must be greater than 1040 g/cm^2 . Although we still do not know at this stage exactly how strong the mechanical force needs to be to naturally drive deer skin to grow, the observation that ectopically transplanted antlerogenic periosteum can readily push the overlying skin up to form a pedicle and antler skin should add weight to our argument that the mechanical force derived from the expansion of PP-derived tissue would be sufficient to drive skin formation.

Regeneration of Antler Blood Vessels

Regeneration of antler blood vessels begins from the stumps of their pedicle counterparts, which originate from the superficial temporal artery (Fig. 9A), the last branch of the external carotid artery (Waldo et al., 1949; Suttie et al., 1985a). Major blood vessels supplying pedicles and antlers are mainly located in the vascular layer (between the periosteum and the enveloping skin). However, appreciable amounts of blood flowing through the central bony core of antlers (Fig. 9B) and pedicles (Fig. 8C) have also been observed. The blood vessels that are located in the vascular layer of a growing antler are very close to the periosteum (Fig. 9B), whereas those of a pedicle are closely attached to the inner face of the dermis of skin (Fig. 9C). These claims are also evidenced by the ridges and grooves (blood vessel marks) on the shaft surface of hard antlers (Fig. 9D) and the smooth surface of bony pedicles (Fig. 9E). This topographical distribution of blood vessels in pedicles supports the assumption that major blood vessels would have stayed with the pedicle skin when the skin was separated from the attached PP by the insertion of an impermeable membrane (Li et al.,

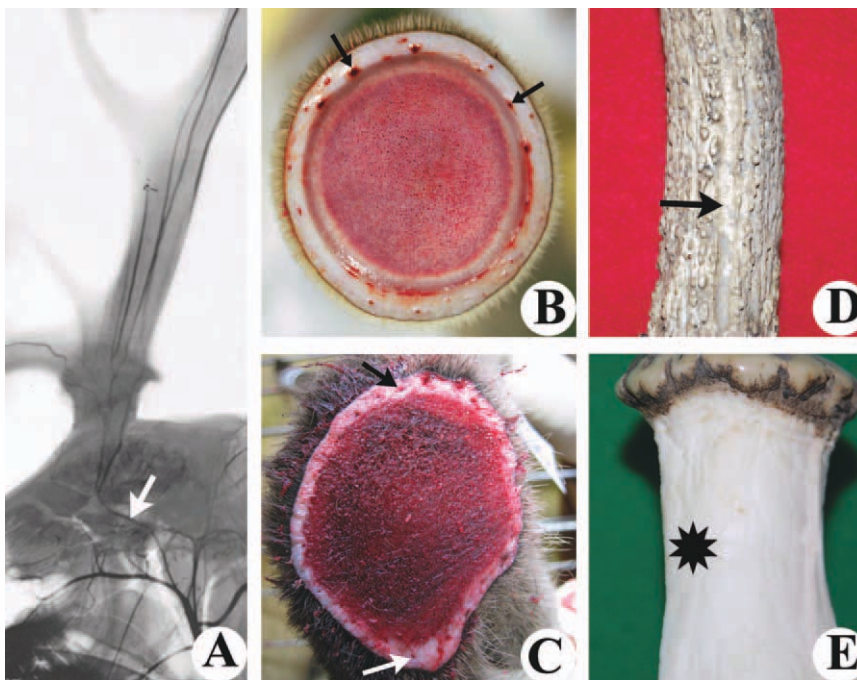


Figure 9. Blood supply of pedicles and antlers. (A) Angiogram to reveal blood vessels leading to a pedicle and an antler from the superficial temporal artery (arrow). (after Li and Suttie, 2012) (B) Crosscut surface at the mid section of an early growing spike antler. Note that the major blood vessels are located in the vascular layer (arrows). (C) Crosscut surface at the midsection of a 2-year-old pedicle. Note that some major blood vessels are located in the dermis of pedicle skin (arrows). (D) Bone surface of a hard antler shaft. Note the surface is covered with ridges and grooves (arrow, blood vessel marks). (E) Bone surface of a 2-year-old pedicle shaft. Note that the shaft has a very smooth surface.

strength of the growth force of internal antler tissue components by capping an early growing antler

bud with a Teflon membrane that was tied to a stainless steel ring (Fig. 8C) (unpublished data). The

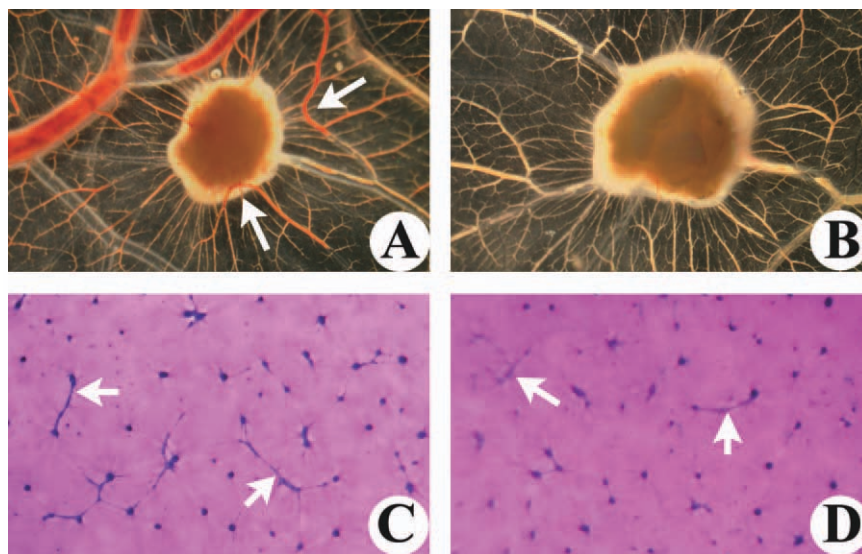


Figure 10. Angiogenic activities of PP tissue or its extracts. (A) and (B) CAM assay. (A) PP explant group. Note some of the nearby blood vessels were bending toward the PP tissue (arrows). (B) Facial periosteum explant group. Note that none of the nearby blood vessels were bending toward the explant. (C) and (D) Human umbilical vascular endothelial cells tube formation assay. Note that more tubes (arrows) were formed in the PP extract group (C) than those (arrows) in the facial periosteum extract group (D).

2007c). If that is true, the failure of the antler blood vessels to regenerate at the skin side must be, like pedicle skin, also caused by dissociation from the PP.

How the PP induces antler blood vessels to regenerate is open to speculation. However, based on our preliminary data, we propose that regeneration of antler blood vessels is also a result of PP-derived chemical induction and mechanical stimulation. To confirm the involvement of chemical induction, we carried out a chick embryonic CAM (chorioallantoic membrane) assay by directly placing small pieces of PP ($2 \times 2 \text{ mm}^2$) on the CAM (unpublished observation), and found that some of the nearby blood vessels were bending toward the PP tissue (Fig. 10A), whereas this phenomenon was not observed in the control facial periosteum group (Fig. 10B). Further, our human umbilical vascular endothelial cells tube formation assay (unpublished observation) demonstrated that the number of tubes formed in the dishes containing PP extracts (Fig. 10C) was significantly higher than the ones that contained facial periosteum extracts (Fig. 10D).

Based on these results, we tentatively conclude that PP has at least moderate angiogenic activities, and that the PP-derived chemical induction would play an important role in antler blood vessel regeneration.

There is no report thus far as to whether antler blood vessel elongation is caused by mechanical stretch, which is imposed by rapid expansion of the PP-derived tissue mass. However, classic studies showed that the vascular wall benefits from their high viscoelasticity during traction (Fink et al., 1999), which enables blood vessels to be lengthened considerably without suffering structural damage. Cohen and Ruiz-Razura (1992) used an intraoperative expander to repeatedly stretch the blood vessels and were thus able to double the length of vascular defects they could bridge without incurring thrombosis or aneurysms. Stretching and blood vessel wall growth may occur more or less simultaneously with a slight predominance of stretching in the distraction phase and a slight predominance of growth in the consolidation phase. This would explain why the comparison in wall thicknesses

between the lengthened and control sides only showed nonsignificant tendencies toward thinning in the distraction phase and thickening in the consolidation phase (Cohen and Ruiz-Razura, 1992). Li et al. (1999) found that there was an optimal lengthening rate for blood vessels when carrying out distraction of rabbit long bone. This rate was 0.7 mm/day, below which (0.3 mm/day) the angiogenic potential would not be maximized; whereas, above which (2.7 mm/day) angiogenesis would be inhibited or reduced. Lee et al. (2008) studied the adaptation of the endothelial cell shape in arteries under axial stretch using an organ culture model. The results showed that, when axially stretched, endothelial cells were initially elongated but eventually adapted and regained their normal shape. If lengthening of blood vessels due to mechanical stretch is a common feature, it would not be surprising if antler blood vessels rapidly elongate under the substantial mechanical stretch induced by rapid antler growth. However, the elongation rate of antler arteries is extraordinary in that it is at least 20 times over the optimal lengthening rate of somatic blood vessels (based on the antler growth speed).

Regeneration of Antler Nerves

Antler nerves regenerate from the stumps of pedicle sensory nerves, which are derived from the zygomaticotemporal (Fig. 11A) and supraorbital (Fig. 11B) branches of the trigeminal nerve (Wislocki and Singer, 1946; Adams, 1979; Suttie et al., 1985b; Li et al., 1993; Clark et al., 2006). After hard antler casting, the nerve stumps remain dormant in the vascular layer of a pedicle (Fig. 11C) and wait for the signal in the next spring to regenerate. It is well established that nerves follow the same route as blood vessels in antlers and pedicles (Wislocki and Singer, 1946). This phenomenon has been thought to be caused by nerve growth factor expression in arterial smooth muscle, which is positioned ahead of the axon growth cone in a growing ant-

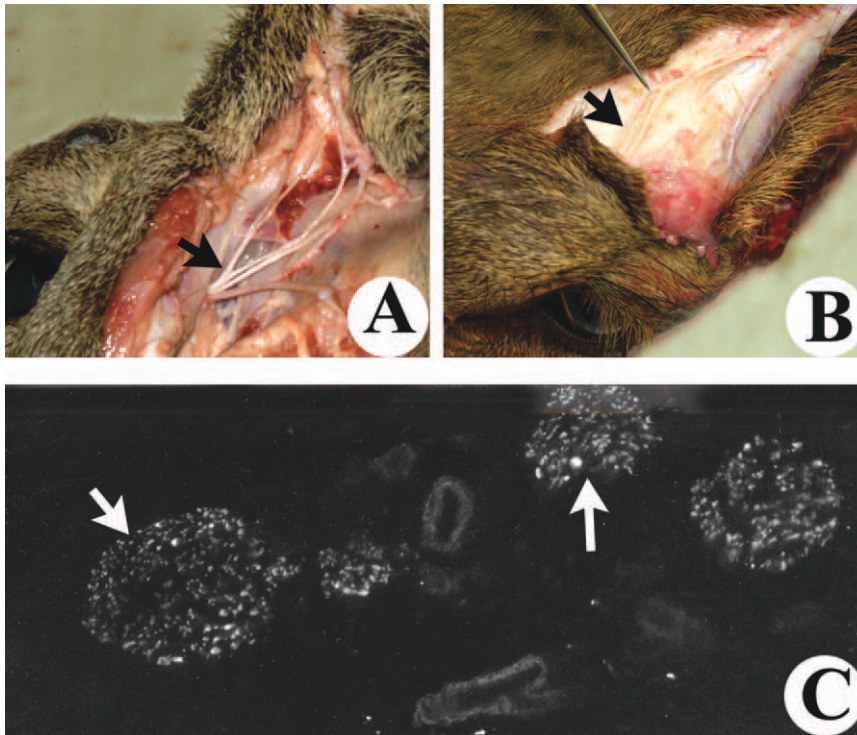


Figure 11. Innervation of pedicles and antlers. (A) Zygomaticotemporal branch (arrow) of trigeminal nerve. (B) Supraorbital branch (arrow) of trigeminal nerve. (C) Cross-sections of nerve bundles in the vascular layer of a pedicle after velvet skin shedding. Note that the blood vessels in the vicinity of these nerves have all the features of pedicle blood vessels.

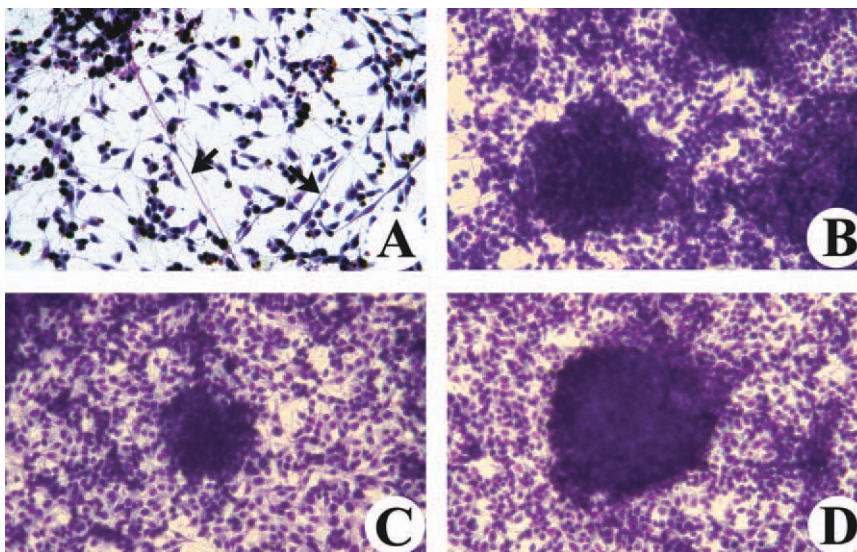


Figure 12. Neurogenic activities of PP extracts on SK-N-SH cells, a human nerve progenitor cell line. (A) PP-extract-treatment culture. Note that numerous neurites were formed by the differentiated SK-N-SH cells (arrows). (B) PP-extract-control culture (culture conditions were the same as for (A), but without PP extract). (C) FP-extract-treatment culture. (D) FP-extract-control culture (culture conditions were the same as for (C) but without FP extract). Note that no obvious neurites can be identified in the PP control, FP treatment, and FP control cultures, although cell aggregates are formed in all these cultures.

ler and provides a guidance cue to define the nerve track (Li et al., 2007b). Therefore, major pedicle nerves, like blood vessels, would have stayed with the pedicle skin when the skin was separated from the associated PP by insertion of an impermeable membrane (Li et al., 2007c). If that is true, the failure of the antler nerves to regenerate at the skin side must be, like pedicle skin and blood vessels, also caused by the dissociation from their attached PP. Further, we would like to postulate that regeneration of antler nerves is also a result of PP-derived chemical induction and mechanical stimulation.

To confirm the involvement of chemical induction, we conducted an *in vitro* assay to evaluate the effects of PP extract on the differentiation of human nerve progenitor cells SK-N-SH, using extract from deer facial periosteum as a control (unpublished data). The results clearly showed that numerous neurites radiated out from the aggregates of the differentiated SK-N-SH cells in the PP extract group (Fig. 12A, B), whereas no obvious neurite-like-cell processes could be identified from the SK-N-SH cells in the facial periosteum extract group (Fig. 12C, D). Consequently, the PP extract must have had strong neurogenic activities, supporting the hypothesis that PP-derived chemical induction plays a critical role in stimulating nerve regeneration during antler renewal, possibly through a paracrine pathway.

Direct experimental evidence demonstrating that PP-derived mechanical stretch is the main driving force for rapid antler nerve elongation is thus far lacking. Based on classic studies, however, mechanical stretch represents the most effective means for rapid and long-term axon growth during the period of animal growth, and axon tracts can remain intact structurally and functionally under extreme stretch-growth conditions. Pfister et al. (2004) reported that axon tracts could be stretch-grown at the rate of 8–10 mm/day and reach lengths of 10 cm from 100 μ m, while maintaining the morphology and density

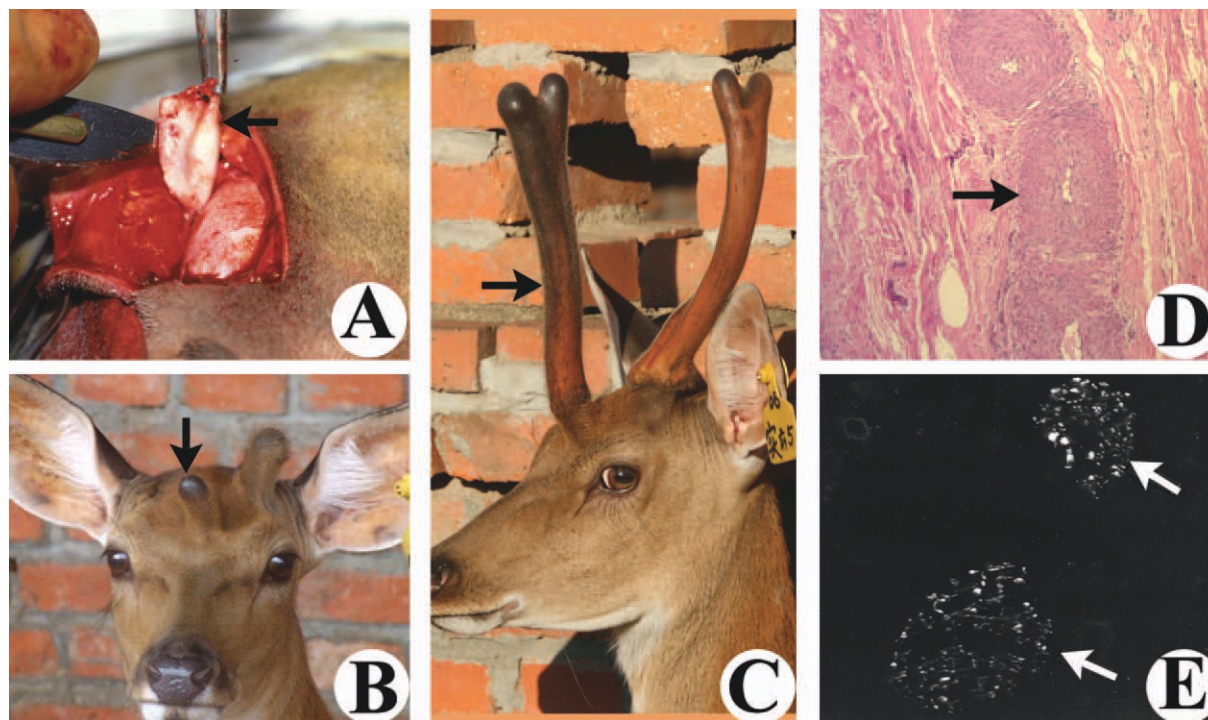


Figure 13. Origin of the external antler components. (A) Antlerogenic periosteum (arrow). (B) First year ectopic spike antler (arrow) initiated from the transplanted periosteum on the forehead region. (C) Regenerated ectopic antler (arrow) in the second year. (D) Blood vessels (arrow) in the vascular layer of the regenerated ectopic antler. Note these blood vessels have all the features of antler blood vessels. (E) Nerve fibers (arrows) in the vascular layer of the regenerated ectopic antler.

of the cytoskeletal constituents and organelles. Obviously, this extreme elongation rate is in sharp contrast to the 1 mm/day maximal nerve growth rate with growth-cone extension (Bray and Mills, 1991; Buettner, 1994). Therefore, mechanical stretch is the main driving force for rapid axon growth. Furthermore, direct electrophysiological examination under extreme stretch conditions showed that there was an increase in the density of Na^+ and K^+ channels in these stretched axons, and they also retained the ability to transmit active signals (Pfister et al., 2006). More convincing results of maintenance of nerve functionality were reported by Iwata et al. (2006), who used stretch-grown axons to span two populations of neurons and bridged a 1 cm lesion in the rat spinal cord. Their results showed that transplanted stretch-grown cultures maintained their pretransplant geometry, survived for at least 1 month, sent axons to integrate with viable host tissue at each end of the lesion, and trans-

mitted active signals across the lesion. If mechanical stretch growth is the most effective means for nerve elongation during animal growth and under experimental conditions, it is probably reasonable to assume that mechanical stretch, created by the fast expansion of PP-derived tissue mass, plays an indispensable role in rapid antler nerve regeneration and growth.

Based on the foregoing discussion, we would like to draw the tentative conclusion that antler regeneration is a stem cell (PP cell)-based process: direct proliferation and differentiation of the PP cells cause the internal tissue components (cartilage and bone) of a regenerating antler to form, whereas close association with PP or PP-derived tissue is the prerequisite for regeneration of external pedicle components (including skin, blood vessels, and nerves) to take place. This close association would facilitate the putative signal from PP to reach these external components and to induce them to transform into the antler counter-

parts (chemical induction); at the same time, the fast expansion of the PP-derived tissue mass would drive the external antler components to rapidly regenerate through mechanical stretch (mechanical stimulation).

FULLY REGENERATIVE ABILITY OF EXTERNAL ANTLER TISSUE COMPONENTS IS NOT INNATE BUT IS ACQUIRED

Although it may be convincing that regeneration of external antler tissue components is achieved through PP-derived chemical induction and mechanical stimulation (see above), the unparalleled regeneration speed of these external tissue components prompts a valid question: is this fully regenerative ability of external antler tissue components innate or acquired? The experiments directly addressing this question have not been forthcoming, but we believe it can be answered

indirectly by analyzing the previously conducted transplantation experiments using antlerogenic periosteum (Fig. 13A). An ectopic pedicle and antler can be readily induced by antlerogenic periosteum subcutaneously transplanted elsewhere on the deer body (Hartwig and Schrudde, 1974; Goss et al., 1985; Li and Suttie, 2001), such as on the deer forehead (Fig. 13B). These ectopic antlers also go through yearly regeneration cycles in a synchronized manner with the antlers at the original sites (Fig. 13C). Histology (Fig. 13D) and immunohistochemistry (Fig. 13E) studies showed that besides the presence of regenerated velvet skin, the regenerated ectopic antlers were also rich in blood vessels and nerve fibers. These regenerated tissue components, skin, blood vessels, and nerves, can only derive from those located in the ectopic site of transplantation. But under natural conditions, these components are incapable of full regeneration, and therefore it must be the transplanted antlerogenic periosteum that bestowed this ability on these tissue types. Thus, the ability to fully regenerate and the phenomenal growth rate of the skin, blood vessels, and nerves in a pedicle stump must be acquired through induction of the closely adjacent PP, rather than these tissues being constitutively capable of doing so.

Now, the question still remains: how can these external tissue components cope with such phenomenal elongation rates during the antler growth phase (up to 2 cm/day), well above the reported figures from studying corresponding somatic tissue types, without suffering structural and functional damage, if they are only ordinary somatic tissue types? We believe that the answer lies in their unique surrounding milieu, which contains the PP-derived factors. These putative factors would permit the external tissue components to become fully regenerative and to grow at that extraordinary speed. Eventual isolation and identification of these PP-derived factors

may open up a new avenue for devising effective therapies for lesions involving each of these tissue components, be they traumatic, degenerative, or linked to developmental (genetic) anomalies.

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