

EXPLORATION OF THE MECHANISM UNDERLYING NEOGENESIS AND REGENERATION OF POSTNATAL MAMMALIAN SKIN: DEER ANTLEER VELVET

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ABSTRACT

Transition from fetal to adult type skin in mammals accompanies the change in response to trauma from regeneration to scar formation. Any claims to have observed neogenesis/regeneration of even some of the skin appendages (such as hair follicles and sebaceous glands) in scar tissue are usually received with skepticism. In defiance of this general rule, deer antler velvet, a special type of mammalian integument, is not only capable of full regeneration, but regeneration on a grand scale. Therefore, antler velvet offers a unique opportunity to investigate how nature has solved the problem of regeneration of adult mammalian skin including its associated appendages. Antler velvet together with the tissue it envelops regenerates in yearly cycles from a permanent bony protuberance called a pedicle. Each spring, wound healing takes place over the cast plane (around 40 mm in diameter in red deer) of a pedicle stump created following the shedding of the previous year's hard antler. Interestingly, instead of forming a scar to seal the defect, as would be the case elsewhere on the deer or other mammals; antler velvet, demised due to occlusion of blood supply, is fully regenerated from the distal pedicle skin to heal the open wound. Characteristics that set velvet apart from other deer skin include a thicker epidermis, larger sebaceous glands, absence of sweat glands in some species, and successive stages in the initiation and development of hair follicles. The outcome of this wound healing obviously cannot be even partially attributed to skin contraction, as pedicle skin is firmly attached to the underlying tissue, a phenomenon akin to the human situation. Nor is it due to an impaired inflammatory response, as is the case in fetal wound healing, since without immune protection a pedicle wound with warm tissue fluids would have rapidly become infected with bacteria. A recent series of experiments has revealed that pedicle skin owes its full regeneration potential to the closely associated pedicle periosteum, working through soluble factors. Topical application of antler extracts significantly reduced scar size of full thickness skin excisional wounds on pigs, and on similar wounds on rats, stimulated a substantial increase in vasculature in the scar tissue. Eventual identification and isolation of these soluble factors from pedicle periosteum/growing antlers may have significant impact on the field of regenerative medicine as applied to human skin and its associated appendages.

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INTRODUCTION

Development of therapeutic strategies to restore tissue structure and function compromised by injury or disease has been the focus of modern regenerative medicine. To achieve this goal, regenerative medicine must be underpinned by regenerative biology, which seeks to understand the mechanism of regeneration through investigation of different model systems (Stocum, 2006). Among these systems, deer antler stands out as the only mammalian appendage that is capable of complete renewal. Therefore, it offers a unique opportunity to explore how nature has made regeneration possible in such a complex mammalian organ including tissues of bone, cartilage, blood vessels, nerves and full thickness of skin (Goss, 1983).

Antlers are male deer secondary sexual characters. As such their development is closely linked to male annual reproductive cycles (Bubenik, 1982; Li et al, 1988; Suttie et al, 1995). In spring when testosterone decreases to almost undetectable level, hard antlers grown the previous season drop off from the permanent bony protuberances, known as pedicles, and antler regeneration from these pedicle stumps immediately follows. In summer, regenerating antlers enter the most rapid growth period (up to 2 cm/day) while circulating testosterone level remains low. In autumn, sharply increasing testosterone causes full calcification of the antler, thereby terminating further antler growth and triggering antler skin shedding. In winter, exposed hard antlers are firmly attached to the living pedicles due to the effects of high level of circulating testosterone until the next spring when the hormone level drops again to the low threshold that triggers hard antler casting and a new round of antler regeneration (Li, 2003).

While in the growth phase, antlers are enveloped by a special pelage. This coat is distinctively different from the pedicle skin from which it derives, in that the hairs are shorter, thinner, more sparsely populated and growing out nearly at right angles to the surface, which tends to be lighter in color (Figure 1A-1D) than its pedicle counterpart. All these attributes of antler skin give it a velvety appearance; hence it is called “antler velvet”. Besides these differences to pedicle skin, the morphological attributes of velvet skin also vary between different deer species. In some deer, such as sika (Figure 1A) and fallow (Figure 1B), the velvet skin is pinkish, and velvet hairs are shorter and more sparsely populated leaving the velvet epidermis clearly visible. While in others, such as red deer the velvet skin is more brownish (Figure 1C); or wapiti, velvet skin more grayish (Figure 1D). The velvet surface of these deer species is more hairy so as to obscure the underlying epidermis.

Although antler velvet is a temporary integument (lasting up to 100 days), the demise of each year’s velvet (Figure 1E) in autumn has been shown to be a case of “murder”, not “suicide”, since when transplanted either to the deer’s hind leg (Billingham et al, 1959) or scalp (Goss, 1972), velvet skin has survived for several years (Figure 1F).

The most obvious features that can effectively differentiate antler velvet (Figure 1G) from its counterpart of deer body skin (Billingham et al, 1959; Bubenik, 1993; Goss, 1983) or pedicle skin (Figure 1F; Li and Suttie, 2000) at a histological level include a much thicker epidermis (e.g. thickness of epidermis in white-tailed deer: body skin vs antler velvet = 0.01-0.03 mm vs 0.149 mm; Bubenik, 1993); unique mature hair follicles that have larger sebaceous glands but lack arrector pili muscle and sweat glands; and different developmental stages of hair follicles (Figure 1G and 1H).

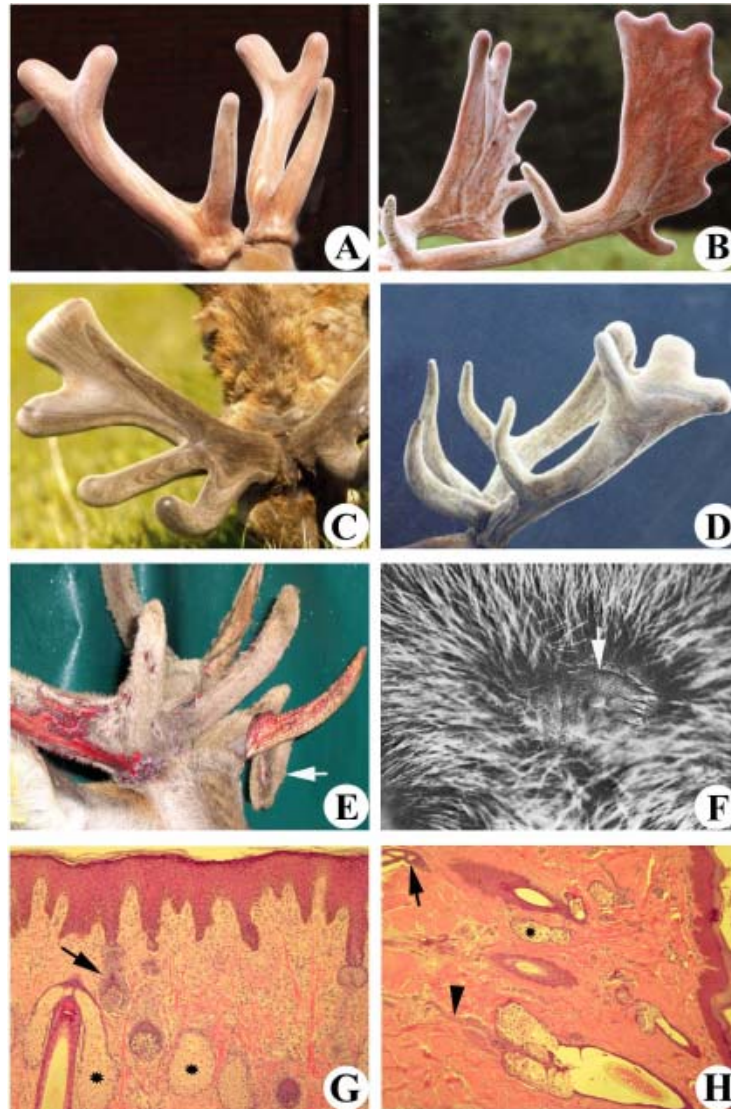


Figure 1. Deer antler and antler velvet. A. Sika deer antlers; B. Fallow deer antlers; C. Red deer antlers; and D. Wapiti antlers. Note that antler velvet of sika and fallow deer is pinkish, whereas red deer is brownish and wapiti is greyish. E. Antler velvet shedding. Just before the rutting season starts, velvet skin begins to peel off (arrow) the antler bone mainly due to the occlusion of the blood supply. F. Autotransplant of velvet skin to the scalp in a sika deer (Reproduced with permission from Goss, 1983. *Deer Antlers. Regeneration, Function and Evolution*. P144). The grafted velvet survived for several years and retained its original features (arrow). G. Histological section of apical velvet skin of red deer, haematoxylin and eosin stain. Note that antler velvet has a thick epidermis, big sebaceous glands (asterisks), and neogenesis of hair follicles (arrow). H. Histological section of pedicle skin of red deer, haematoxylin and eosin stain. Note that pedicle skin has a thinner epidermis, small sebaceous glands (asterisk), sweat glands (arrow) and arrector pili muscle (arrow head).

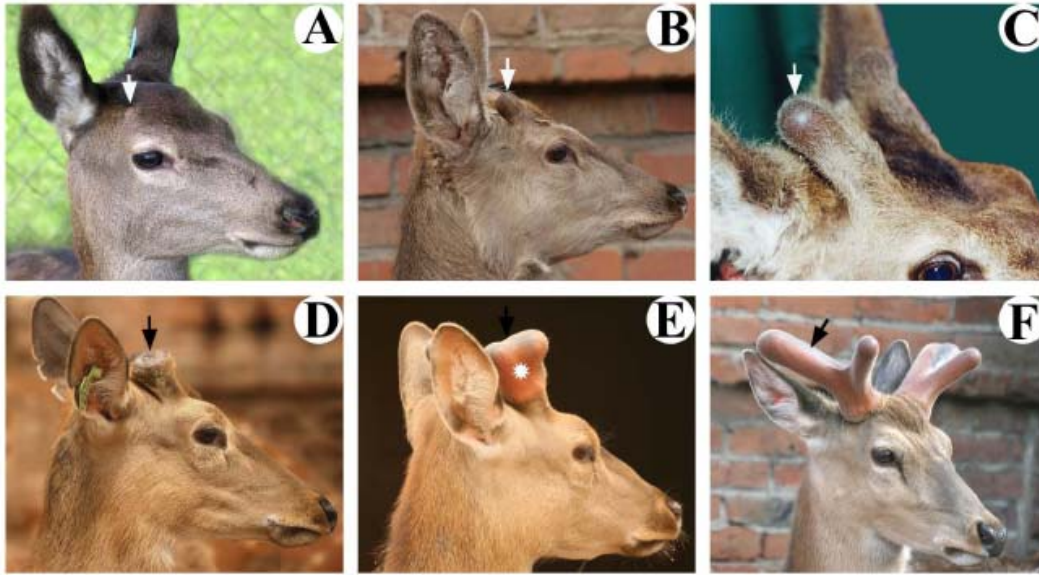


Figure 2. Morphogenesis of antler velvet. A-C, antler velvet generation in red deer. A. Scalp skin overlying a frontal crest (arrow). B. Scalp skin overlying an incipient pedicle (arrow). C. Early antler bud (arrow). Note that the apical skin of the bud is shinier and hair is more sparsely populated than the skin covering the pedicle, from which it derives. D-F. Antler velvet regeneration in sika deer. D. The pedicle stump (arrow) immediately after the previous antler casting. Note that the stump is enveloped by typical scalp skin. E. Incipient regeneration antler (asterisk). F. Two-branch antler (arrow). Note that these antlers are covered with typical velvet skin.

TRANSFORMATION OF DEER ANTLER VELVET

1. Morphogenesis

Deer are not born with pedicles, these start to develop from the frontal crests (Figure 2A) when deer approach puberty and reach threshold body weight (5-7 month old and about 56 kg in red deer), which is normally by late winter or early spring. Initially, the developing pedicles are covered by typical scalp skin (Figure 2B). When pedicles grow to their species-specific height (around 5-6 cm high in red deer), first antlers begin to generate spontaneously from the apices of these pedicles in spring or early summer. This generation can be seen externally by a change in skin appearance from typical scalp to antler velvet (Figure 2C). The development of pedicles is a once in a lifetime event, while antlers are deciduous and form anew once a year.

Once a hard antler drops off from its pedicle due to a decrease in the circulating testosterone level in the next spring, a pedicle stump is created. Within hours of casting, a scab is formed at the site of bleeding in the centre of the cast plane of the pedicle stump, and the plane is surrounded by a ring of shiny skin (Figure 2D, 9A). This ring of skin subsequently gives rise to the future antler velvet, and is readily distinguishable from typical scalp skin that envelops the pedicle shaft. As the regeneration process advances, the skin ring migrates further centripetally and distally, then converges in the center of the early regenerating antler bud to complete the wound healing. Even before completion of wound

healing, a regenerating antler bud begins to push up from underneath. The antler bud at this stage is completely covered with typical velvet skin (Figure 2E). Soon after, the antler main beam and brow tine (first antler branch above deer's brow) emerge from the antler bud and form a typical two branch antler (Figure 2F).

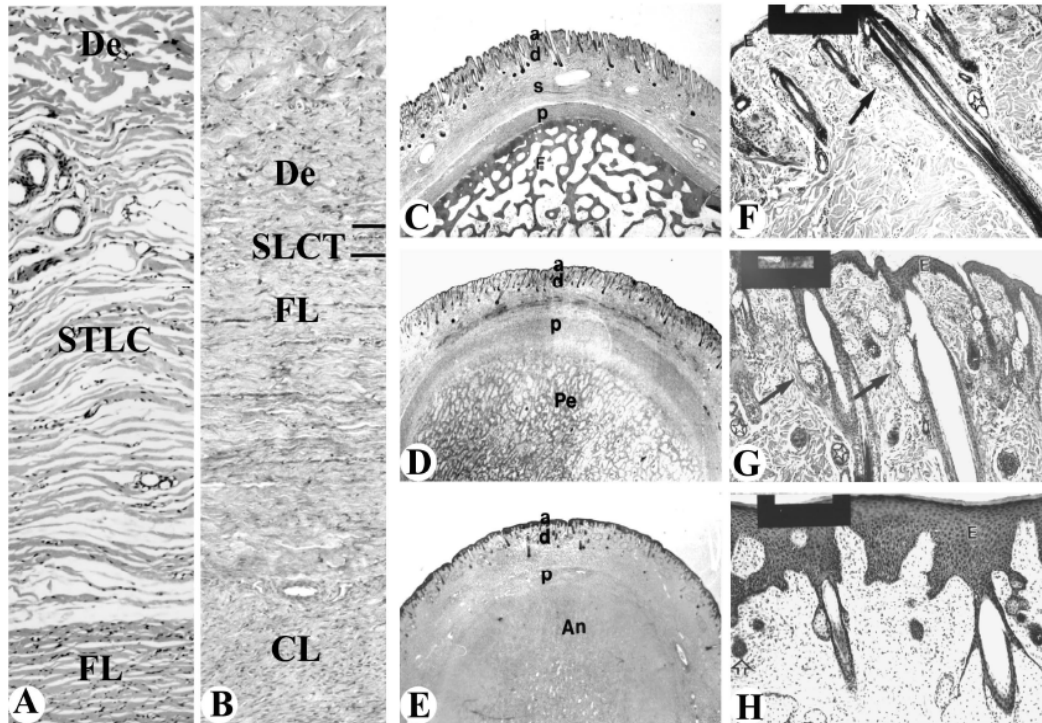


Figure 3. Histogenesis of antler velvet in antler generation. Vertical sections of the apical skin during the period of transformation from pedicle skin to antler velvet. A and B. Subcutaneous loose connective tissue (SLCT). A. Before pedicle initiation. Notice that the SLCT is a very loose and thick layer. B. At an early stage of antler generation. Notice that the SLCT layer has become a thin strip. C. Scalp skin overlying a frontal crest (F). Notice that the epidermis layer is undulated. D. Skin overlying a mid growth stage pedicle to show the relative flat epidermis. E. Velvet overlying an incipient antler to show the thick and relative flat epidermis. F-H. Apical epidermis and its associated appendages. F. Before pedicle initiation. Notice that the epidermis (E) is thin and undulated; the sebaceous gland is small and monolobar with an obvious arrector pili muscle (arrow) attached. G. At mid pedicle growth stage. Note that the epidermis becomes thicker but is still undulated, and sebaceous glands become bigger than those at the earlier stage but still with obvious arrector pili muscles (arrows). H. At an early antler generation stage. The epidermis is relatively flatter and thicker, and the sebaceous glands become even bigger than those of the mid pedicle growth stage but without arrector pili muscles attached. De, dermis; FL or CL, fibrous layer or cellular layer of antlerogenic periosteum/perichondrium. d, dermis; s, subcutaneous loose connective tissue; p, periosteum/perichondrium; Pe, pedicle; An, antler. Star, sweat gland.

2. Histogenesis

In Antler Generation

Li and Suttie (2000) carried out a detailed histological analysis of pedicle skin formation and antler velvet transformation in red deer. At the initiation of pedicle formation the subcutaneous loose connective tissue (SLCT) overlying the frontal crest is a very loose and thick layer, within which the vascular system is located (Figure 3A). As pedicle growth proceeds, the SLCT layer becomes thinner and denser and eventually turns into a thin dense strip (Figure 3B) when the pedicle is over 35 mm high (around the time of initiation of skin transformation to antler velvet). At pedicle initiation stage, epidermis overlying a frontal crest is undulated in shape (Figure 3C). When the pedicle grows over 25 mm high (mid pedicle growth stage), undulation configuration of the epidermis has almost disappeared (Figure 3D). When the pedicle is over 35 mm high, the epidermis is essentially a flat layer (Figure 3E). The change in epidermis thickness is observed at the stage when the epidermis becomes relatively flat. At the pedicle initiation stage, the epidermis is thin (Figure 3F). As pedicle growth proceeds, the epidermis becomes increasingly thicker (Figure 3G). When the pedicle is over 35 mm high, the epidermis becomes about 10 times thicker than that at pedicle initiation stage (Figure 3H). Antler velvet transformation does not occur until the apical pedicle skin becomes intimately associated with the underlying mesenchymal tissue (Figure 3E and 3F), and is mainly associated with alteration in the skin appendages. This alteration includes the loss of arrector pili muscle and sweat glands, and the gain of the large bi- or multi-lobed sebaceous glands (Figure 3F-3H).

Based on the results of this histological study (Li and Suttie, 2000), we conclude that formation of the pedicle skin and antler velvet consists of three histologically distinguishable stages. These stages are 1) compression of the apical SLCT layer when pedicles grow to about 15 mm high, 2) straightening of the apical undulated epidermis when pedicles are about 25 mm high, and 3) neogenesis of the overlying skin and its associated appendages when pedicles grow over 30 mm high. These observations suggest that the factor that drives pedicle skin formation and expansion may be the mechanical force derived from the rapid expansion of underlying mesenchymal tissue. When a pedicle starts to grow, the first sign of histological change is the compression of the SLCT, followed by straightening of the overlying undulated epidermis. Formation of new pedicle skin and its associated appendages is not initiated until the overlying epidermis is fully stretched, which is also demonstrated by our BrdU labeling experiments (Li and Suttie, 2000).

Based on their study, 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. Consequently, the impetus that drives rapid growth of pedicle skin and antler velvet, which is commensurate with the elongation of a pedicle and antler, would be the mechanical force derived from the expansion of underlying mesenchyme.

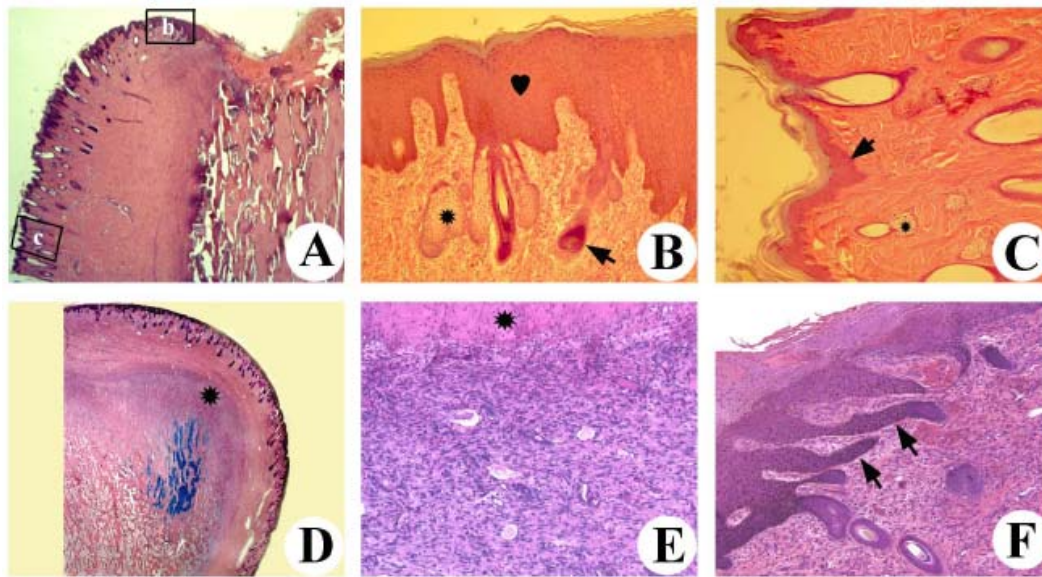


Figure 4. Histogenesis of antler velvet in antler regeneration. A. Sagittally cut histological section of a fresh pedicle stump, anterior portion. Note that the distal pedicle skin is fused to the subdermal pedicle periosteum and has acquired some velvet skin features. B. Higher magnification of the box labelled “b” in Figure 4A to show the thick epidermis (heart), big sebaceous glands (asterisk) and neogenesis of hair follicles (arrow) of the distal pedicles skin. C. Higher magnification of the box labelled “c” in Figure 4A to show thin epidermis (arrow), small sebaceous glands (asterisk) of the shaft pedicle skin. D. Sagittally cut section of the posterior portion of a pedicle stump at late wound healing stage to show the newly established growth centre (asterisk). E. Histological section of the central region of a pedicle stump at middle of the wound healing stage. Note that this region was still devoid of skin (asterisk). F. Histological section of the leading edge of wound healing skin over the cast plane to show the “tongue-like” structures formed along the undersurface of healing epidermis.

In Antler Regeneration

Detailed histological examination of the process of velvet skin regeneration (Li et al, 2005) further confirmed our previous morphological findings that at the time of a previous hard antler casting, the distal pedicle skin, which is seamlessly fused with the subdermal PP, has already acquired some velvet skin features (Figure 4A). These include a thickened epidermis, bigger sebaceous glands and neogenesis of hair follicles (Figure 4B). However, the pedicle skin at the more proximal level (on the pedicle shaft) still retains typical scalp skin structures, such as a thin and undulated epidermis, small sebaceous glands and existence of sweat glands (Figure 4C). The skin that subsequently seals the cast plane of the pedicle stump and covers the growing antler bud is the typical antler velvet.

If the rapid growth of pedicle skin and antler velvet in antler generation is caused by the mechanical force derived from the expansion of the antlerogenic periosteum (AP) tissue underneath, what would be the factor that drives the velvet skin elongation at the initial stage of antler regeneration? Superficially, an alternative stimulus to mechanical stretch might be postulated. This is because the situation in antler generation, i.e. skin overlying expanding mesenchymal tissue, has not been created at the initial regeneration stage. However, a careful histological analysis shows that velvet expansion even if at the initial stage of antler regeneration may be still caused by mechanical stretch.

Firstly, in contrast to the currently held view that the upwardly expanding antler growth centre is located in the central region of a pedicle stump (Goss, 1995; Kierdorf et al, 2003), so peripherally regenerating distal pedicle skin/initial antler velvet would not be able to get stretched mechanically at the initial stage. However, our results showed that the growth centers for the main beam and brow tine are established at the posterior and anterior corners, rather than in the central region, of a pedicle stump (Figure 4D). Before the central region is sealed by centripetally migrating skin (Figure 4E), these corners have been totally covered by the regenerating antler velvet for some days. Secondly, at the time of a hard antler casting the distal pedicle skin is fused to the subdermal PP, and its epidermis not only seals the broken end of pedicle dermis caused by velvet skin shedding, but the leading edge of the epidermis is firmly abutted to the PP and subperiosteal bone (Figure 4A). The thickening PP at the distal end would certainly create mechanical pressure to the enveloping distal pedicle skin, and the skin would have to grow in order to release the pressure. Thirdly, at the early initiation stage of antler regeneration the undersurface of healing epidermis at the leading edge forms specific angled “tongue-like” structures, which appear to act as “pegs” to firmly anchor the leading edges of the migrating epidermis to the underlying connective tissue (Figure 4F). The rapidly expanding PP-derived tissue mass at the anterior and posterior corners gradually pushes up and forms the incipient brow tine and main beam. During this period, the newly formed velvet skin over the areas of anterior and posterior corners becomes substantially stretched (Figure 4D). Interestingly, under such a mechanical pressure from the underlying rapidly expanding tissue, the leading edge of the healing skin is not only still attached to the underneath connective tissue but also keeps extending toward the centre of the pedicle stump/antler bud. Consequently, the expansion of both pedicle skin and antler velvet in both antler generation and regeneration is likely to be caused by mechanical force derived from the underlying rapidly expanding mesenchymal tissue. This conclusion explains how antler velvet is capable of rapid growth so as to keep pace with the rapid elongation of antler bone.

ANTLER VELVET TRANSFORMATION IN ANTLER GENERATION

The transformation from pedicle skin into antler velvet during first antler generation has been considered a unique zoological phenomenon, as this process happens in postnatal life and involves mature skin (Goss, 1983). Significant progress has been made in recent years toward unveiling the mechanism underlying this transformation process. AP transplantation experiments (Goss and Powel, 1985; Hartwig and Schrudde, 1974; Li and Suttie, 2001) have clearly established the role of this antlerogenic tissue in the induction. Removal of AP (Figure 5A) prior to the skin transformation abolishes antler formation and the skin transformation in the original place (Figure 5B); whereas, subcutaneous transplantation of the AP elsewhere on the deer body, such as the forehead (Figure 5B) or foreleg (Figure 5C) induces ectopic antler development and skin transformation. It seems probable that the mechanical force built up from the expanding AP tissue underneath plays an indispensable role in this transformation process, but it is unlikely to be able to accomplish the process alone, because mechanical stretch can only drive skin neogenesis but cannot alter skin type (Austad et al, 1982; Johnson et al, 1988). Consequently, molecular induction from the underlying AP-derived tissue would have to be involved.

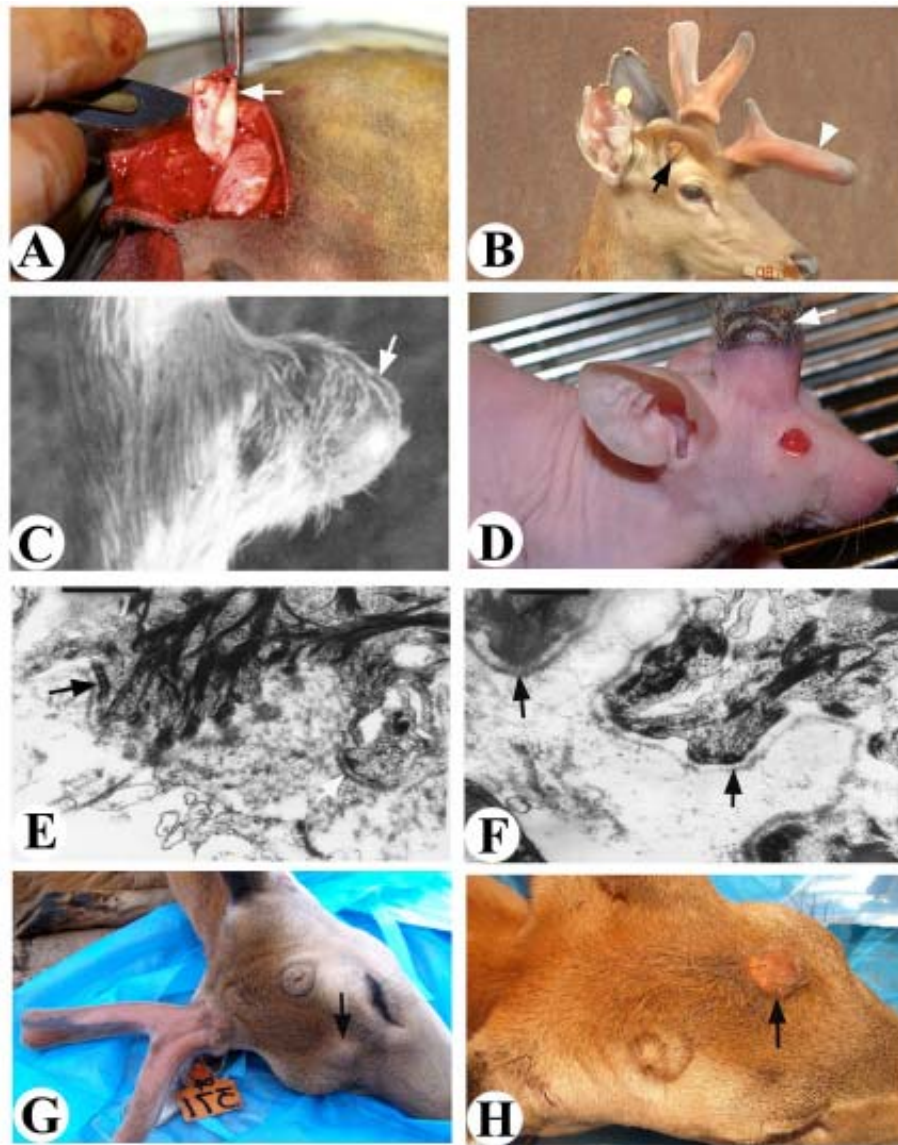


Figure 5. Mechanism of velvet transformation. A. Surgical removal of antlerogenic periosteum (AP, arrow) from the frontal crest of a prepubertal deer for transplantation. B. Original antler growth region (arrow) failed to give rise to an antler after losing AP; whereas the ectopically grafted AP transformed the deer forehead skin into antler velvet (arrow). C. Grafted AP transformed the deer foreleg skin into antler velvet (arrow). D. Xenografted AP transformed the co-transplanted deer scalp skin into antler velvet (arrow). E and F. Electron microscopy micrographs of apical skin before (F) and during (E) the process of transformation from pedicle skin into antler velvet. Note that intact basement membrane (arrows) was detected before the transformation (F); whereas, a fragmented basement membrane (arrows) was found during the transformation (E). G and H. Dome-shaped bulges were formed two years after the subcutaneous AP transplantation and membrane insertion on the forehead region. Note that the bulge was still covered with the scalp skin (G) in the impermeable membrane group; whereas, the bulge was covered with typical antler velvet (H) in the permeable membrane group.

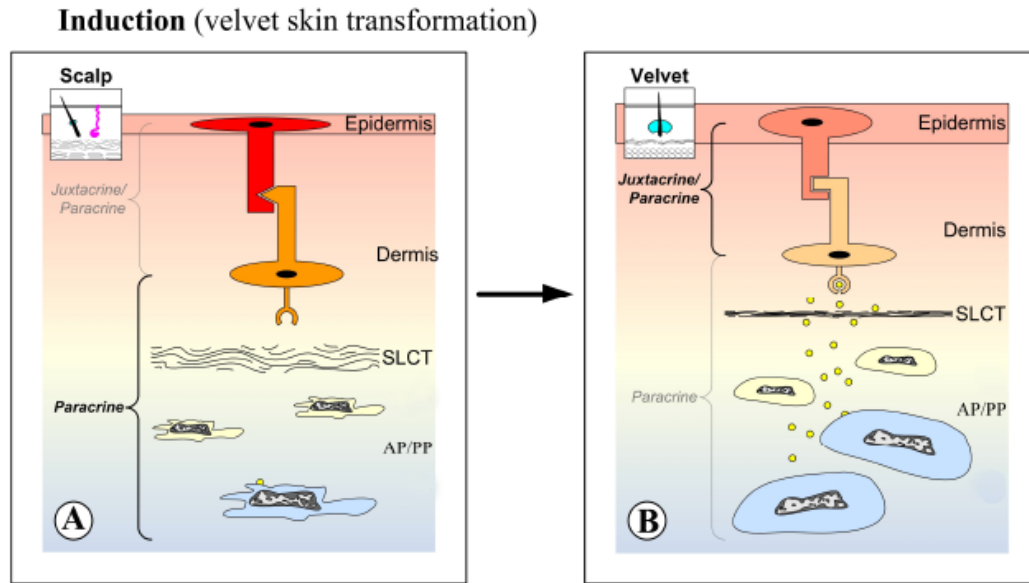


Figure 6. Schematic drawing of the induction of transformation from pedicle skin into antler velvet. A. Prior to the initiation of skin transformation, the inducer (AP or PP) and the responder (skin epidermis) are interposed by a wide and loose layer of SLCT. B. Once the two interactive tissue types become closely contacted, i.e. SLCT layer is compressed into a narrow strip, the antlerogenic cells (AP/PP) release the instructive diffusible molecules, which traverse the compressed SLCT and its associated partial dermal layer to act on the dermal cells resident in the dermis or in hair follicles. The altered dermal cells exert their influence via paracrine and juxtacrine mechanisms on the overlying epidermis, which then is transformed into antler velvet.

When carrying out his AP transplantation experiments, Goss (1987) noticed that ectopic antlers could form only if the tissue derived from the grafted AP becomes closely associated with the overlying skin, which lead him to propose (1990) that this close association is a prerequisite for the putative morphogens to reach the skin from the AP and to effect on the epidermis. The results of our histological examination (Li and Suttie, 2000) on pedicle skin formation and antler velvet transformation (Figure 3) support Goss's claim. Interestingly, we succeeded in transforming deer scalp skin into antler velvet on the head of a nude mouse (Figure 5D). This was achieved when AP was tightly sutured together with deer scalp skin, from which SLCT and its associated partial dermal layer (about half thickness of the dermal layer) were trimmed off. This result not only provides further evidence for the idea that the close association between AP and the skin is indispensable, but also demonstrates that SLCT layer and its associated partial dermal layers are not essential for skin transformation.

The chemical induction hypothesis is further supported by our electron microscopy study (Li and Suttie, 2000). The results showed that, at the early skin transformation stage, disruption of the basement membrane integrity (Fig 5E) of apical pedicle/antler skin occurred; whereas prior to that stage, the basement membrane in the apical skin was well intact (Figure 5F). It is known that an intact basement membrane between epidermis and dermis can effectively block the skin interactions and hence inhibit regeneration (Neufeld and Aulthouse, 1986). Neufeld et al (1996) reported that the basal lamina (the top layer of basement membrane) is initially absent from the amputation surface of an animal appendage and re-established with complete continuity by the late bud stage of regeneration. They concluded

that the ability to delay basal lamina closure until after a blastema (a cone shaped cell mass over an appendage stump and endowed with potential to form a replacement structure) has fully formed is the feature that distinguishes regenerative from non-regenerative appendages. The finding that disruption of the basement membrane integrity of the apical pedicle skin during the period of transformation to antler velvet at least partially explains why this process can proceed without the basement membrane being physically broken, a process that happens in antler regeneration.

To further test the chemical induction hypothesis of antler velvet transformation and investigate the nature of this induction, we recently (Li et al, 2008) carried out a membrane insertion experiment. In the study, an impermeable or permeable (0.45 μm pore size) membrane was inserted between the ectopically grafted AP (on deer forehead in this case) and the overlying scalp skin. Two years after the operation, the skin in the impermeable membrane group still retained the original scalp skin features (Figure 5G), whereas, the skin in the permeable membrane group had convincingly transformed from scalp skin into antler velvet (Figure 5H). These results clearly demonstrate that the skin transformation relies on AP induction, and this induction is realized through diffusible molecules, as permeable membrane insertion did not stop the skin transformation although significantly delayed the process (for a year).

A model that explains the mechanism underlying skin transformation is represented here by a schematic drawing (Figure 6). Prior to the initiation of antler generation or regeneration, a wide layer of subcutaneous loose connective tissue (SLCT) is interposed between the two interactive tissue types: AP or PP and the covering skin (Figure 6A). Once the two interactive tissue types come into close contact, i.e. SLCT layer is compressed into a narrow strip, the cells of AP in the case of antler generation or PP in antler regeneration release the instructive diffusible molecules, which traverse the compressed subcutaneous loose connective layer (SLCT) and its associated partial dermal layer to act in a long-distance-paracrine manner on the dermal cells resident in the epidermis-associated partial dermal layer (also see Figure 8). The activated dermal cells exert their influence via paracrine and juxtacrine (Rendl et al, 2008) mechanisms on the overlying epidermis, which then transforms into antler velvet (Figure 6B). The eventual identification and isolation of these putative inductive molecules from AP/PP or their derived-tissue may help us to devise a therapy for better quality of wound healing or even complete tissue regeneration for humans.

ANTLER VELVET TRANSFORMATION REQUIRES INTERACTION WITH PEDICLE PERIOSTEUM

In order to determine the tissue origin for the regeneration antlers, a variety of techniques have been used in a number of experiments in antler research. A pedicle stump consists of two main tissue types: skin and bone. Based on the histological examination, Wislocki (1942) concluded that it was the proliferating deeper portion of the healing skin corium, rather than adjacent periosteal tissue, which restored the surface of the pedicle cast plane and gave rise to the osteogenic germinal bed for subsequent antler regeneration. Goss (1972; 1984; 1995) reinforced this view with the observation that following previous hard antler casting, the skin around the upper margin of the pedicle thickened and gave rise to cells that healed over the

exposed pedicle bone and provided a potential source of cells for the formation of regenerating antler buds. Consequently, these authors considered that it was really the centripetally migrating pedicle dermal cells that give rise to regenerating antlers. This conclusion aligned the process of early antler regeneration with the classic model of epimorphic regeneration (*de novo* formation of the lost part to the level amputation of an appendage stump), in which a blastema is formed from cells migrating over the amputation plane of a limb stump (Mescher, 1996).

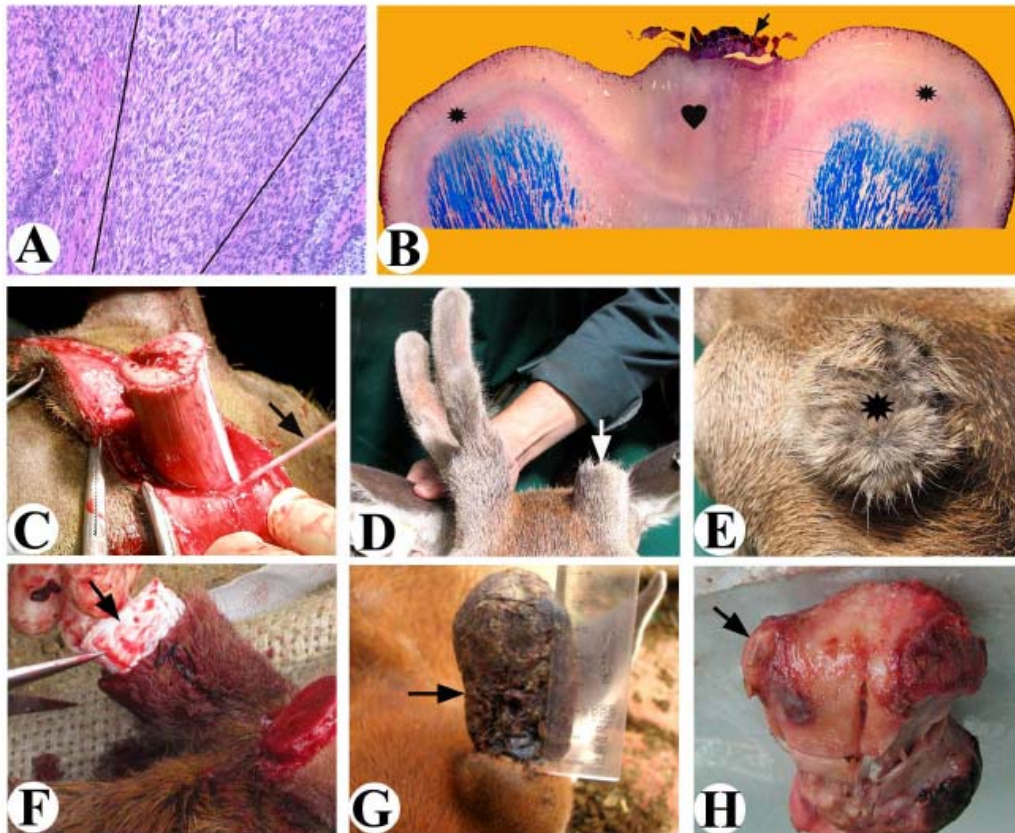


Figure 7. Role of antlerogenic tissue in the skin transformation. A. Thickening hyperplastic perichondrium, which is derived from the distal pedicle periosteum and is going to form a growth centre for antler regeneration. B. Sagittally cut section of an early regenerating antler bud to show the two independent growth centres formed from the hyperplastic distal periosteal cells at the anterior and posterior portions (asterisks). Note that in the central region and under the scab, only granulation tissue (heart) is observed. C. PP deletion (arrow) from a pedicle stump prior to antler regeneration. D. Outcome of the PP deletion. Note that no antler regeneration occurred from the PP-deleted pedicle stump (arrow), although a 3-branched-antler was formed from the sham-operated pedicle. E. The apex of the PP-deleted pedicle stump was covered with typical scalp skin (asterisk) till end of the antler growth season. F. Membrane insertion (arrow) into the space between PP and the enveloping skin of a pedicle stump before initiation of antler regeneration. G. Skin-less antler (arrow) was regenerated from the membrane-inserted pedicle stump, and was covered with a thick layer of scab. H. Rudimentary branch (arrow) on a skin-less antler was revealed after removing the scab layer.

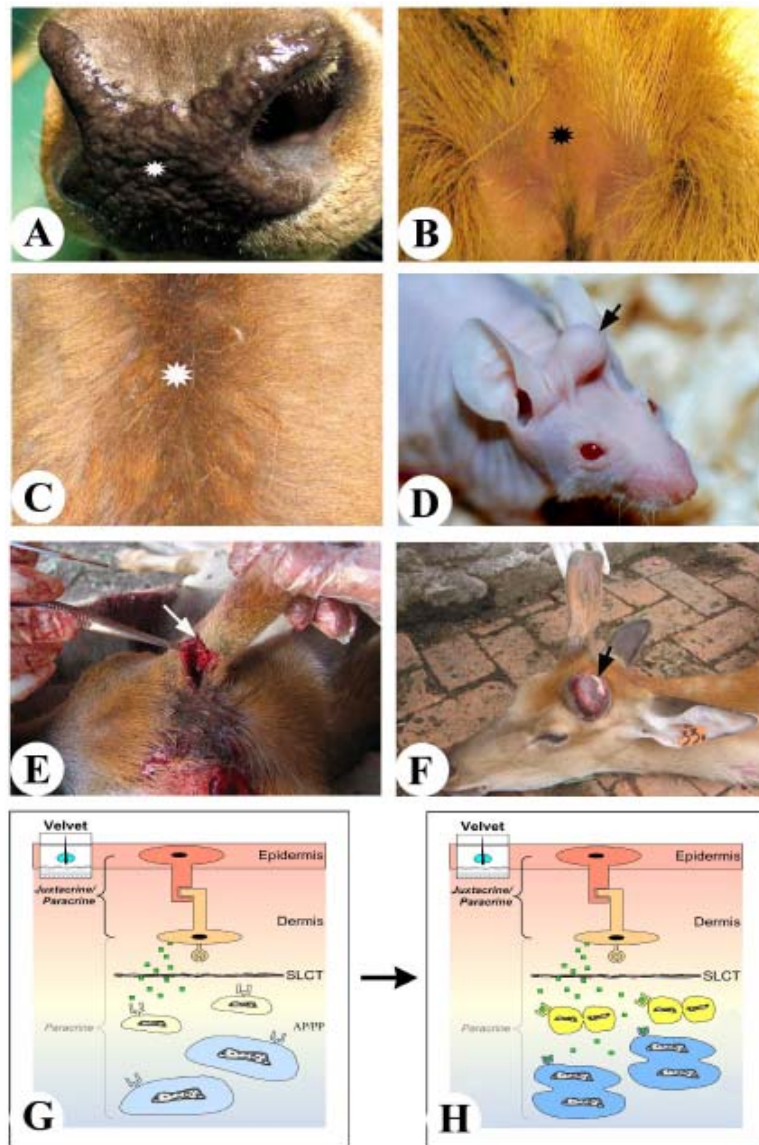


Figure 8. Role of velvet skin in antler regeneration. A-D. Four skin types were found to be incompetent to respond to AP induction for transformation into antler velvet. A. Deer snout (asterisk). B. Deer tail ventral surface (asterisk). C. Deer back (asterisk). D. Nude mouse skin. Note that a pedicle-like protuberance (arrow) formed from the subcutaneously xenografted AP on a Nude mouse head, and the protuberance is enveloped by typical nude mouse skin (Reproduced with permission from Li et al, 2001). E. The junction (arrow) of the tight and loose association between pedicle skin and PP, which was located by making a vertical skin incision along the pedicle shaft. F. Pedicle (arrow) of the membrane inserted side remained antler-less at the end of the antler growth season (Reproduced with permission from Li et al, 2007). G and H. Schematic drawing of the instructive feedback from the AP/PP primed skin (velvet skin). G. Once transformed, cells of the antler velvet epidermis and dermis send the feedback signals, which diffuse through the partial dermal layer and the compressed SLCT layer to act on the AP/PP cells. H. The feedback signals drive the AP/PP cells into a mode of rapid proliferation to initiate antler generation or regeneration.

However, recently Kierdorf et al (2003) and Li et al (2004) found that the distal PP increased in thickness during the period of early antler regeneration (Figure 7A). Further, Li et al (2005) showed that the growth centers for both antler main and brow tine formation were formed exclusively from the proliferation of the distal pedicle periosteal cells *in situ* at the posterior and anterior corners respectively (Figure 7B), rather than in the centre of a pedicle stump. In contrast, the migrating dermal cells, which healed the central region of the exposed pedicle bone, only formed granulation tissue under the scab and did not participate in antler growth centre formation (Figure 7B). Consequently, they suggested that regenerating antlers are the derivatives of pedicle periosteal cells, rather than pedicle dermal cells. This suggestion is consistent with the previous discovery that antlerogenic periosteum, from which PP derives, exclusively holds the potential to form a pedicle and a first antler (Hartwig and Schrudde, 1974).

Although each group has plausible argument to back up its theory, all of these arguments are based on static histological analysis. Decisive functional analyses were required. Therefore, we conducted two *in vivo* experiments. In the first experiment (Li et al, 2007a), PP was totally removed from a pedicle stump (Figure 7C) to determine if antler regeneration could still take place in the absence of PP. Convincingly, when PP deletion was carried out at the critical time, the PP-less pedicles failed to give rise to a regenerating antler, although the sham-control pedicle formed a 3-branched-antler (Figure 7D). At the end of that antler growth season the apex of the PP-less-pedicle was still covered with the typical pedicle skin (Figure 7E). Therefore, pedicle periosteum is unequivocally the key tissue type for antler regeneration.

While the PP deletion experiment convincingly shows that PP is indispensable for antler regeneration, it does not shed light on the role played by pedicle skin, as antler velvet is always an integral part of each regenerating antler. To determine the role of the skin in this case, one must find a way to effectively separate these two tissue components prior to the initiation of antler regeneration. Hence, we carried out a second *in vivo* experiment (Li et al, 2007b). In this study, a piece of impermeable membrane was inserted into the space between the PP and the enveloping skin of a pedicle stump (Figure 7F). Interestingly, antler regeneration took place even without participation of pedicle skin and formed a skin-less antler, which was covered with a thick layer of scab (Figure 7G). One such skin-less antler even developed a rudimentary branch (Figure 7H), which was revealed after removing the scab. Consequently, pedicle skin/antler velvet is dispensable for antler regeneration from the initiation stage onwards.

ROLE OF VELVET SKIN IN ANTLER REGENERATION

That pedicle skin/antler velvet does not play an essential role in antler generation and regeneration at the initiation stage does not necessarily mean that skin is not required prior to that stage. Indeed, the AP transplantation experiments by Goss (1987) showed that skin specificity is required for feedback to AP and subsequent antler generation. Subcutaneous AP transplantation elsewhere on the deer body but on the nose (Figure 8A), tail (Figure 8B) and back (Figure 8C), induced ectopic antler formation. Recently, we found (Li et al, 2001) that nude mouse skin was not competent to interact with the underlying xenografted AP, and

hence failed to transform into antler velvet (Figure 8D). AP grafts to the back or tail of the deer and head of the nude mouse developed into bony nodules that bulged upward from the skin beneath it. However these nodules remained loose even when their enlargement caused the skin to be elevated (Figure 8D). Most of the AP grafts to the nose of the deer did not grow and all remained loose, although some became hardened. The possible explanation as to why these skin types are not competent remains obscure. There is reason to believe that hair follicles, including dermal papilla cells, might be involved in this process. Because in these studies, three out of the four unresponsive skin types: nose snout, ventral surface of the tail and nude mouse skin, are almost devoid of hair follicles. Although deer back skin is adorned with hairs, it is the most sparsely hair populated skin on the deer body (Bubenik, 1996) and these hairs are predominantly guard hairs (Figure 8C; personal observation). Furthermore, our recent xenograft transplantation experiment using the nude mouse model (Li *et al*, in press) showed that AP could effectively induce the epidermis of the co-transplanted deer skin to transform into antler velvet, and this transformation was not affected when the interposed SLCT layer and its associated partial dermal layer were missing, so long as the epidermis-associated partial dermal layer (containing hair follicles) was intact.

Likewise, antler regeneration also requires PP to be primed by the competent enveloping skin through physical and chemical interactions. While developing a technique to sample PP tissue, Li and Suttie (2003) noticed that there is a difference in the degree of association between the enveloping skin and the PP along the shaft of a pedicle. Skin of the proximal portion of a pedicle is loosely attached to the PP; whereas on the distal portion of the pedicle, the skin is tightly bound. As close contact between AP and the overlying skin is a prerequisite for antler generation (see above), we hypothesized that antler regeneration requires the close association between the PP and the enveloping skin, and this association may facilitate whatever the inductive molecules might be to reach the target. Hence, the distal closely associated region of a pedicle stump must be in a more advanced state of antler regeneration than the proximal loosely attached region. In another words, PP in the loosely associated region has not been primed by the adjacent skin for regenerating an antler. To test this hypothesis, we employed the membrane insertion approach again (Li *et al*, 2007b). Before carrying out the membrane insertion, an artificial pedicle stump was created by sawing off the distal part at the junction between the tightly and loosely associated regions. The junction was located along the pedicle shaft by making a vertical skin incision to identify the very point where transition occurs from tight to loose contact between the PP and the overlying skin (Fig 8E). When the PP was separated from its enveloping skin by inserting a piece of impermeable membrane at the loosely attached region, antler regeneration was effectively stopped, while the sham-control pedicle gave rise to a normal spike antler (Figure 8F). These results are in sharp contrast to those when the separation was at the tightly bound region, in that a skin-less antler was regenerated from PP tissue alone (Figure 7G).

Overall, competent skin is required for both antler generation and regeneration, and this requirement is not in differentiating into antler tissue types, but in priming the closely attached antlerogenic tissue (AP or PP) to initiate antlerogenesis by secretion of putative diffusible molecules. Upon receiving these morphogens, antlerogenic cells enter the mode of fast proliferation and differentiation to rapidly build up antler tissue. This proposal is presented by a schematic drawing (Figure 8).

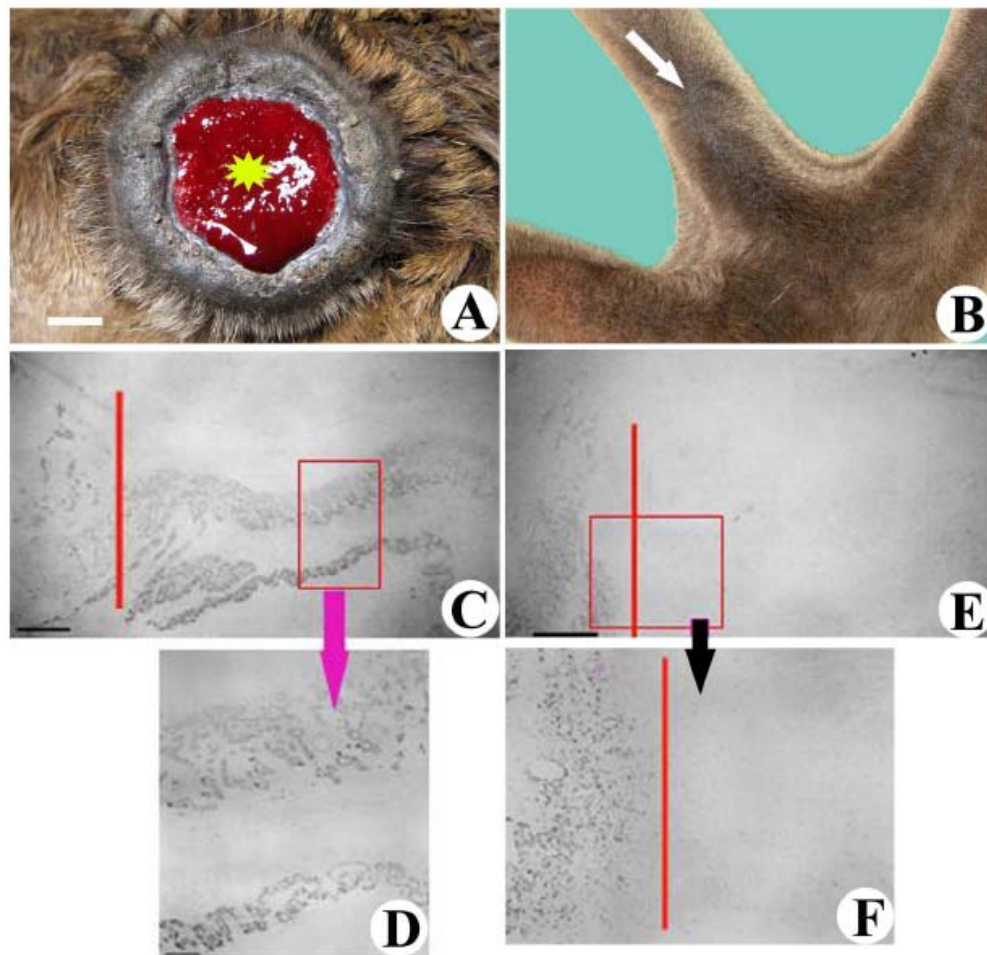


Figure 9. Pedicle wound healing and effects of antler extracts on wound healing. A. Fresh pedicle stump to show the size of the open wound. Note that some bleeding (asterisk) occurred on the casting surface after the hard antler casting. B. The final outcome of the wound healing over a pedicle stump cast surface. Note that the scar formed from the wound healing is almost invisible (arrow) in most cases. C-F. Laminin antibody stained histological sections, cut through the healing area of the full thickness skin punch wound (8 mm in diameter) on the backs of rats (Clark et al, unpublished data). Vertical bar indicates the boundary between the existing and the healing skin tissues. C. Wound treated with low molecular weight antler extract. Note that dense blood vessel networks were formed in the healing tissue in this group. D. Higher magnification of the box in 9C. E. Placebo treated wound. Note that the healing tissue is almost devoid of blood vessels. F. Higher magnification of the box in 9E.

TRANSLATION OF THE FINDINGS TO TREAT WOUND HEALING

In addition to the phenomenon of full antler regeneration, the initial stage of this regeneration, i.e. wound healing, over the cast plane of a pedicle stump also provides a novel model of wound repair. Following casting of a previous hard antler, a pedicle stump is created. The diameter of the cast plane can be up to 60 mm (Figure 9A) in a moderate size deer species, such as red deer or wapiti. The outcome of healing over such an open wound in

most cases is the formation of an almost invisible scar (Figure 9B). It is well established (Billingham et al, 1959) that in skin that is loosely associated with the underlying structure, such that over the trunk of most mammals, contracture will significantly reduce the size of the wound so that only a small scar remains. In contrast, in skin that is tightly attached to the subdermal tissue, such as that over the most parts of human body and animal ears, contracture will not be able to proceed to reduce the wound size, so a prominent scar is usually the final outcome. Interestingly, pedicle skin at the distal end, which is fused to the subdermal PP (Figure 4A), heals the huge open wound and leaves an almost undetectable scar. In this case, skin regeneration rather than contraction must account for the high quality of wound healing. Consequently, substances that drive this skin regeneration would have to be involved. We suggested that these substances could be isolated from regenerating antlers and used as wound healing products in clinical application.

On this basis, we developed an advanced method (Haines, 2004) to make low molecular weight (LMW) antler extracts (<10 kD) aimed at enhancing wound healing quality. Topical application of the LMW antler extract on full skin thickness punch wounds (20 mm in diameter) on the backs of pigs significantly improved the rate of wound healing compared to controls and, during the early phase, influenced the expression of a number of genes important to the healing process (Haines et al, unpublished data). A similar experiment in rats (Clark et al, unpublished data) showed that improvement of wound healing is at least partially achieved through the stimulation of angiogenesis, as the healing tissue in the extract-treated group contained ample blood vessels (Figure 9C and 9D), whereas the control group was almost devoid of blood vessels (Figure 9E and 9F).

Wounds, in particular persistent or chronic wounds, are difficult to heal as they lack a blood supply to nourish the wound, mediate the healing process and minimize scar formation. We envisage that our LMW antler extracts, may be of clinical benefit, by improving the rate and quality of the healing of chronic wounds in which an impaired blood flow is a contributing factor, such as in leg ulceration.

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