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The regenerating antler blastema: the derivative of stem cells resident in a pedicle stump

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1. ABSTRACT

Antlers of the deer are the only mammalian organs that can fully grow back once lost from their pedicles, hence offer the only opportunity to learn how nature has bestowed mammalian epimorphic regeneration. Investigations have demonstrated that it is the proliferation and differentiation of pedicle periosteal cells (PPCs), but not dedifferentiation of the local differentiated cells, that give rise to the antler blastema. PPCs express key embryonic stem cell markers and can be induced to differentiate into multiple cell lineages, so are termed antler stem cells. Further research has found that PPCs can initiate antler regeneration only when they have interacted with cells of the pedicle skin. Histologically, the process of early antler regeneration resembles that of healing of a mouse leg stump wound. However what sets these two apart is the difference in proliferation potential between the PPCs and the periosteal cells of the long bone. We believe that if we can impart a greater proliferation potential to the long bone periosteal cells, we might be able to achieve the dream of regenerating limbs in mammals.

2. INTRODUCTION

The 'Holy Grail' of modern regenerative medicine is to grow back lost organs/appendages, which is known as epimorphic regeneration (1,2). To realize this dream, however, regenerative medicine must be underpinned by regenerative biology, which seeks to understand the mechanism of regeneration through investigation of different model systems (3). Our current knowledge of epimorphic regeneration is largely gained from the studies on lower vertebrates (4), particularly amphibians (urodeles and anurans). Suitable mammalian models of organ regeneration are lacking, but are highly desired if successful strategies are to be devised for the restoration of damaged organs or limbs of humans. One plausible mammalian model is the use of deer antlers.

Antler regeneration occurs in a well-defined yearly cycle under hormonal control (Figure 1): in most species (e.g. Cervus species such as red deer and sika deer), the casting of the previous hard antler (calcified bone) from the permanent bony protuberance, known as the "pedicle", takes place in early spring; the stump heals and new soft antler starts to regenerate; the antler grows rapidly for a period, but then the process of calcification starts to accelerate, blood supply to the velvet skin ceases and the velvet is shed in late summer/autumn; hence the stag presents a hard bony antler for the rut (mating season); the hard antler is retained over winter and cast in the next spring to trigger a new round of antler regeneration (1,5,6).

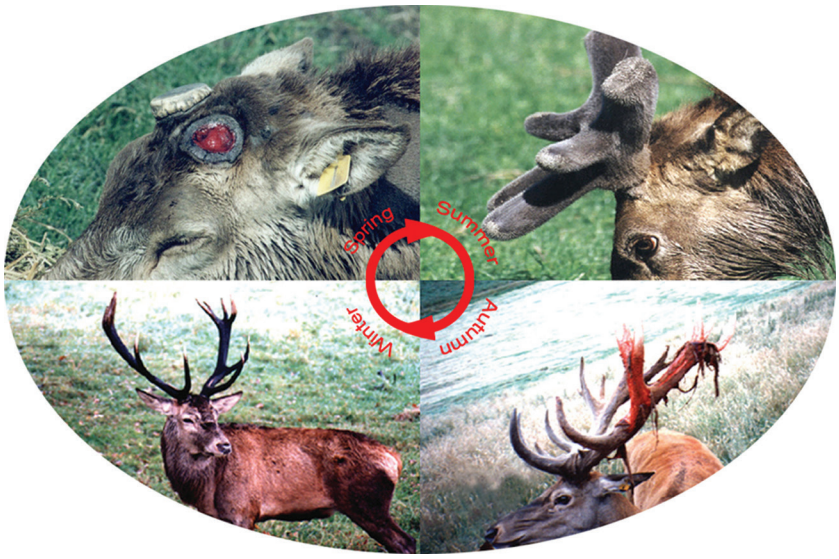

Figure 1. Annual antler regeneration cycle in red deer. In spring, hard antlers drop off from the pedicles, and antler regeneration immediately follows. Rapid antler growth occurs in summer. Growing antlers are enveloped with velvet skin. In autumn, antlers become fully calcified and velvet skin starts to shed. In winter, hard antlers are firmly attached to their pedicles and subsequently cast in the next spring, which triggers a new round of antler regeneration.

Deer antlers are unique mammalian organs in that once lost they can grow back fully, and hence offer the only opportunity to learn how nature has solved the problem of mammalian organ regeneration (5,7-11). Despite this, regeneration of antlers has largely gone unnoticed in the field of regeneration as a research model to attain the Holy Grail – to grow a new limb in a mammal.

3. FULL DEPENDENCY OF PEDICLE PERIOSTEUM (PP)

What tissue/cell type(s) from a pedicle stump gives rise to regenerating antlers? This is a question that has challenged and frustrated generations of antler researchers. Based on histological examinations, both Wislocki (12) and Goss (13,14) stated that the dermis of the pedicle skin was the main source of cells for regenerating antlers; that is, regenerating antlers are dermal derivatives. However, Goss (15) later revised his view by stating that "as histologically complex structures, antlers must surely have multiple origins for such tissue components as the epidermis, dermis, cartilage, bone, blood vessels, and nerves." Interestingly, recent studies do not support these claims, but show convincingly that regenerating antlers are derived from the pedicle periosteum or PP (6,16-18). Regenerating antlers are composed of internal (cartilage and bone) and external (skin, blood vessels, and nerves) tissue components (1,19,20).

3.1. Cartilage and bone

Regeneration of antler internal tissue components has been well-studied (10,16-18,21). Immediately after a hard antler falls off, the centre of depressed bony tissue of the pedicle stump is surrounded by a rim of shiny skin (with very sparse hairs). PP, a tissue that is closely attached to the shiny skin rim, thickens through the active division of cells resident within it. Subsequently, at the late wound healing stage two crescent-shaped growth centres are formed directly from the thickening distal PP, one located anteriorly for the formation of "brow tine" and the other posteriorly for the "main beam" (18). Therefore, it is the proliferation and differentiation of the PP that form the internal tissue components of regenerating antlers.

Mindful of the danger of making a conclusion about a dynamic process solely on the basis of point samples, we have conducted a number of in vivo functional analyses. A pedicle stump mainly comprises three tissue components: bone, periosteum and skin. The first experiment was designed to determine whether the periosteum is indispensable for antler regeneration (17). In this experiment, the PP tissue was removed completely from a pedicle stump prior to the initiation of antler regeneration. Notably, the pedicles lacking PP failed to give rise to a regenerating antler (Figure 2A). Therefore, antler regeneration relies on the presence of the PP.

The second experiment was to ascertain whether the bone component is required for antler regeneration (17). In this work, only the distal part of the PP was deleted leaving the distal end of the PP at a point along a pedicle shaft that is markedly distant from the original antler regeneration site (i.e. the cast plane of a pedicle stump), to see if antler regeneration could occur at that particular point. Early regenerating antler buds did indeed form on the pedicle shafts where the distal

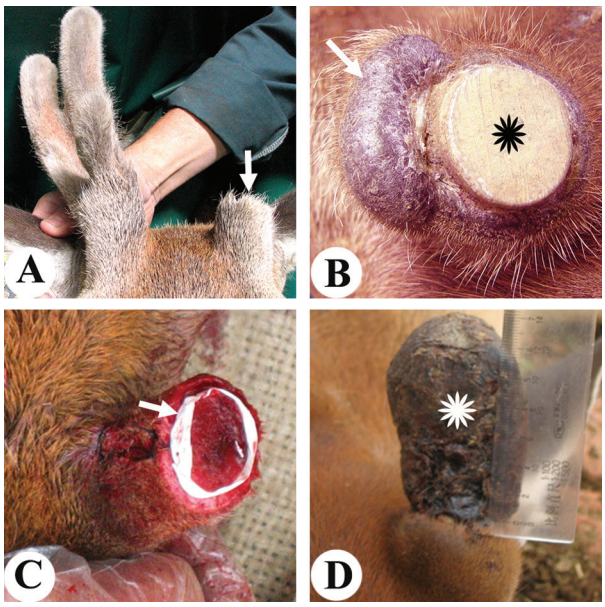

Figure 2. Experimental manipulation and antler regeneration. A. Total deletion of PP. Note that the PP-less pedicle failed to regenerate an antler (arrow), although the sham-operated pedicle formed a 3-branch-antler. B. Partial deletion of PP. Note that an antler bud (arrow) regenerated (or generated) from the pedicle shaft where the distal ends of PP and its enveloping skin met, and where is markedly distant from the original regeneration site (asterisk). C. Membrane insertion (arrow) between the PP and the enveloping skin. D. A skin-less antler (asterisk) regenerated from the membrane-inserted pedicle stump.

ends of PP and its enveloping skin met (Figure 2B). In this case, the pedicle bone was effectively excluded from participating in the process of antler regeneration, but antler regeneration was successful.

The third experiment was to find out if the skin component is required for antler regeneration (22). In the trial, an impermeable membrane was inserted into the space between the PP and the pedicle skin prior to antler regeneration (Figure 2C). Notably, antler regeneration still took place although the pedicle skin failed to participate (Figure 2D) in the process due to separation from the PP. Overall, these experiments provided unequivocal evidence that PP is the key tissue type that gives rise to the internal tissue components, cartilage and bone, of regenerating antlers.

3.2. Skin

The external tissue components of the antler are not simple extensions of their pedicle counterparts. In contrast to the pedicle skin that is a typical scalp skin, antler velvet (skin) has much thicker epidermis but lacks a subcutaneous loose connective tissue layer, and is able to form new hair follicles. The follicles of velvet skin are absence of arrector pili muscles and sweat glands, but possess big multi-lobed sebaceous glands (23-27). This uniqueness of antler velvet may be partially attributed to the complete absence of the subcutaneous loose connective tissue layer.

Regeneration of the velvet skin starts when the wound healing initiates by the distal pedicle skin over a pedicle stump. Wound healing is a natural process and occurs universally to restore continuity of the interrupted skin (14,28). The specificity in this case is, however, when the healing pedicle skin passes the distal end of PP, the healing skin starts to change in property from scalp to velvet type. This suggests that chemical signal from the PP to the migrating skin is involved (26). Subsequently, the expanding tissue mass, the PP cell-derivative of the growth centres, pushes the overlying skin outward and, at the same time, maintains the velvet skin nature. Therefore, it seems that both PP cell-derived chemical induction and mechanical stimulation are involved in the regeneration of velvet skin. The former alters the skin type from scalp to velvet, and the later drives the rapid elongation of the velvet skin (19,26). Further evidence is provided by our membrane insertion experiment (22), in which the two events, chemical induction and mechanical stimulation, were effectively torn apart. Without mechanical stimulation, the chemically transformed antler velvet did not grow. Clinically, it is the general practice to produce new skin through mechanical stretching (29-31). Therefore, it is not surprising that mechanical stimulation is involved in antler velvet regeneration and elongation.

3.3. Blood vessels

Compared to the counterparts in pedicles, antler arteries are unique in that they have a very thickened wall and small constricted lumen (1,32,33). The major blood vessels supplying pedicles and antlers are mainly located in the space between the periosteum and the enveloping skin, although in a pedicle these vessels are more closely associated with the dermis of the skin (10,32). Therefore, the major blood vessels would have stayed with the pedicle skin when the skin was separated from the PP by inserting an impermeable membrane (22). Hence, failure of these major antler blood vessels to regenerate must be, like pedicle skin, also caused by the separation from the PP. Likely, regeneration of antler blood vessels is also realized through both the PP cell-derived chemical induction and mechanical stimulation. The former claim is supported by our chorioallantoic membrane assay, in which some of the passing blood vessels being bending toward the PP tissue were observed. There is no direct experimental evidence for the later claim. However, studies do show that mechanical stretch can make blood vessels to elongate considerably without suffering structural damage (34). If the lengthening of blood vessels due to mechanical stretch is a common phenomenon, it would be conceivable that rapid elongation of antler blood vessels is achieved under substantial mechanical forces. However, the elongation rate of antler arteries is extraordinary as it is at least 20 times the optimal lengthening rate of somatic blood vessels (35).

3.4. Nerves

The nerves of antlers regenerate from the stumps of pedicle sensory counterparts (32,36-39). After velvet skin shedding, the nerve stumps remain dormant in the vascular

layer of the pedicle, awaiting the signal to regenerate. The nerves follow the same route of blood vessels in antlers and pedicles (22,39). Therefore, major pedicle nerves would have stayed with the pedicle skin (like blood vessels) in the membrane insertion experiment, when the skin was separated from the associated PP (22). Likely, regeneration of antler nerves is achieved also through both PP cell-derived chemical induction and mechanical stimulation. The former claim is supported by our in vitro experiment (10), in which the PP extracts were added in the culture medium for SK-N-SH cells (human nerve progenitor cells) and subsequently numerous neurites were observed to grew out from the differentiated SK-N-SH cells. Experimental evidence is thus far lacking for the later claim. However, it is reported that mechanical stretch represents the most effective means for rapid and long-term axon growth and axon tracts can remain structurally and functionally intact under extreme stretch-growth conditions (40). If mechanical stretch-growth is the most effective means for nerve lengthening, it would be conceivable to assume that mechanical stretch, derived from the fast expansion of PP cell-derived tissue mass, has played a critical role in rapid antler nerve growth and regeneration.

Overall, we would like to draw the tentative conclusion that antler regeneration is a single cell type-based (PP cell) process: multiplication of the PP cells directly give rise to cartilage and bone (the internal tissue components) of a regenerating antler, and close association with PP or PP-derived tissue is the prerequisite for regeneration of antler skin, blood vessels and nerves (the external components). This close association would facilitate whatever the signal is from the PP to reach these external components and to transform them into the antler counterparts respectively (chemical induction); at the same time fast expansion of the PP-derived tissue mass would drive the external components to rapidly elongate through mechanical stretch (mechanical stimulation).

4. STEM CELLS FOR ANTLER REGENERATION

A capacity for extensive self-renewal and the latent capability to differentiate are hallmarks of stem cell populations. Here, we apply criteria generally accepted and used for the characterization of putative stem cell lines, to assess the characteristics of isolated PP cell populations.

4.1. Self-renewal

The PP cells display an astonishing potential for population expansion. Although deer pedicles are called permanent bony protuberances, they do become shorter with each passing season, with the first year's pedicle being the longest (1,6). We calculate that in red deer around 3.3. million PP cells within the distal part of a pedicle participate in each round of antler regeneration, giving rise to up to 10 kg of antler tissue mass over about 60 days (9). The PP cell populations are therefore clearly capable of self-renewal. Hence, to qualify them as adult stem cells is a matter of demonstration as to whether they express key stem cell markers and possess multipotency.

4.2. Stem cell marker

The expression of particular antigens, genes and enzymes has been widely used to define stem cell populations (41). Embryonic stem cells express the cell surface antigen CD9. We have demonstrated that PP cells express high levels of CD9 antigen. Principal amongst the so-called 'pluripotency genes' for embryonic stem cells are the POU domain family member Oct4, and Sox2 and Nanog (42). Critically, we (9) have found all these genes to be present in the PP cells. Recently, Rolf et al (43) confirmed the expression of Oct4 in antler stem cells. Additionally, we have shown elevated telomerase enzyme activity and nucleostemin in both cell types. Telomerase activity has been linked to enhanced self-renewal in cells (44), which might explain the phenomenon of why so few antler stem cells (3.3. million PP cells) can form such an impressive amount of antler tissue mass within a very limited period. Expression of nucleostemin has been linked to control of the proliferation of stem cells (44) and newt limb regeneration (45). The range and nature of markers that we have demonstrated in PP cells strongly suggest that these cell populations not only function as tissue-specific 'stem' cell populations in the adult organism, but that they retain characteristics of an embryonic origin throughout the life-time of the animal.

4.3. Multipotency

Stem cell populations, by definition, must also be capable of differentiation into a number of specialized cell types. The potency of PP cells has been investigated by several laboratories (9,11,46). Clearly, both populations in vitro can give rise to chondrocytes and osteoblasts (46,47) respectively. Interestingly, PP cells can also be induced to differentiate into adipocytes (9,46). We, therefore, conclude that the PP cell populations are the "stem cells" which underpin the regeneration of deer antlers.

5. ANTLER BUD VS CLASSICALLY-DEFINED BLASTEMA

Whether a blastema, equivalent to the one that forms during the regeneration of the appendages of some lower vertebrates, is also present during antler regeneration is a matter of controversy (48). The apparent resemblance between regeneration of antlers and newt limbs, a gold standard for the classical blastema-based epimorphic regeneration, has prompted some biologists, such as Goss (1,14,49,50) to suggest that regeneration of antlers is realized through the same mechanism as that operating in lower vertebrates. Because the formation of a blastema is the hallmark of epimorphic regeneration, this mode of regeneration is also referred to as a "blastema-based" process. A blastema has been classically defined

Table 1. Comparisons between a newt limb blastema and a regenerating antler bud

Limb blastema	Antler bud
Regeneration activated by the accidental loss of distal part of a limb	Regeneration activated by the natural loss of dead antlers, induced by changes in sex hormones
Round/cone shape	Flat/concave shape
All dedifferentiation, transdifferentiation and differentiation of diverse origin limb stump cells involved in the process	Proliferation and differentiation of the PP cells involved in the process
Epithelium heals the initial wound	Full thickness of skin heals the wound
Absence of basal lamina	Presence of basal lamina
Avascular	Richly vascularised
Nerve-dependent	Nerve-independent
Wound healing-dependent	Wound healing-independent
Scar-less wound healing	Healing with a scar
Dividing cells evenly distributed	Dividing cells regionally localised
G2/M accumulation in cell cycle	G1 accumulation in cell cycle

as the cone-shaped mass of de-differentiated cells from diverse origins on the stump remaining after amputation of an appendage (1,51,52).

Based on the double-head phenomenon (53) and histological findings (6,18), some researchers have questioned whether an early antler bud should be considered as a classically-defined blastema. Further experiments, such as deletion of the partial or total PP prior to antler regeneration (17) and the membrane insertion between the PP and pedicle skin (22) functionally demonstrated that the PP is the very tissue type that gives rise to antler regeneration. Consequently, an early regenerating antler bud is not, or at least is not mainly, derived from de-differentiated cells of diverse origins on the pedicle stump, but rather from the proliferation and differentiation of PP cells. Hence it does not meet the criteria of a classically-defined blastema. In order to demonstrate whether early regenerating antler buds are really fundamentally different from a classically-defined blastema, some comprehensive comparisons have been made between antler regeneration and newt limb regeneration (9,47,48). These comparisons are further refined and some new findings are added in Table 1.

Morphologically, antler regenerating bud is different from the blastema of a newt limb, the former is flat or concave (6), whereas the latter round or cone (54). Regeneration of newt limbs depends on process of wound healing (55); whereas, regeneration of antlers can take place even if the wound skin is physically prevented from participating in the healing process (22). The formation of a newt limb blastema requires nerve supply (52); whereas, formation of antler buds is not affected by transection to the supplying nerves (37,39,56). The formation of a classically-defined blastema requires all cell types in the amputation plane of a stump to participate in the growth of a newt limb (52); whereas, the regeneration of antlers is realized through the proliferation and differentiation of the PP cells (17). Wound healing over a newt limb stump for blastema formation is a scar-less process (54); whereas, regeneration of antlers leaves a scar from the wound healing process albeit not an obvious one (9).

The blastema of a newt limb stump is avascular (52); whereas, early regenerating antler buds are richly vascularised (47). Proliferating cells are evenly distributed throughout the blastema of a newt limb stump (54); whereas, the dividing cells are located mainly in the mesenchymal layer and in the vascular walls of the precartilage zone in the early regenerating antlers (47). The basal lamina, a thin layer located between the dermis and the epidermis, is absent during the blastema formation of a newt limb stump; whereas, a well-developed basal lamina is detectable throughout the healing skin over the pedicle stump (47,48).

Recently, we found that majority (over 95%) of PP cells show a G1 arrest when investigating cell cycle phenotype of antler stem cells (Guo et al in submission). Previous studies showed that G2/M accumulation is the distinct phenotype for the cells capable of epimorphic regeneration, such as hydra (57), amphibian (58), mammalian liver (59) and MRL mouse (60). An advantage of the G2/M accumulation over G1 is the enhancement in cell proliferation rate, which is obviously needed for regeneration. Nonetheless, it also involves the risk becoming uncontrolled growth as the G2/M phase is the last checkpoint of a cell cycle. Notably, the PP cells that possess the full potential of epimorphic regeneration (10) and at the same time exhibit normal phenotype of cell cycles. Therefore, deer antlers not only provide a single model for mammalian epimorphic regeneration, but also a unique model for risk-free epimorphic regeneration.

Clearly, all these comparisons (Table 1) have set the classically-defined blastema of lower vertebrates and the regenerating antler bud apart. Nonetheless, findings from recent studies have made some researchers to re-define a regenerating blastema to include the contribution from resident stem cells for the formation of a blastema, rather than solely from the de-differentiation of differentiated cells (61-65). For example, Bely et al (61) have stated “The blastema arises through proliferation of undifferentiated cells, either dedifferentiated cells or stem cells.” Based on this new definition, the early regenerating antler bud could be well qualified as a “blastema”.

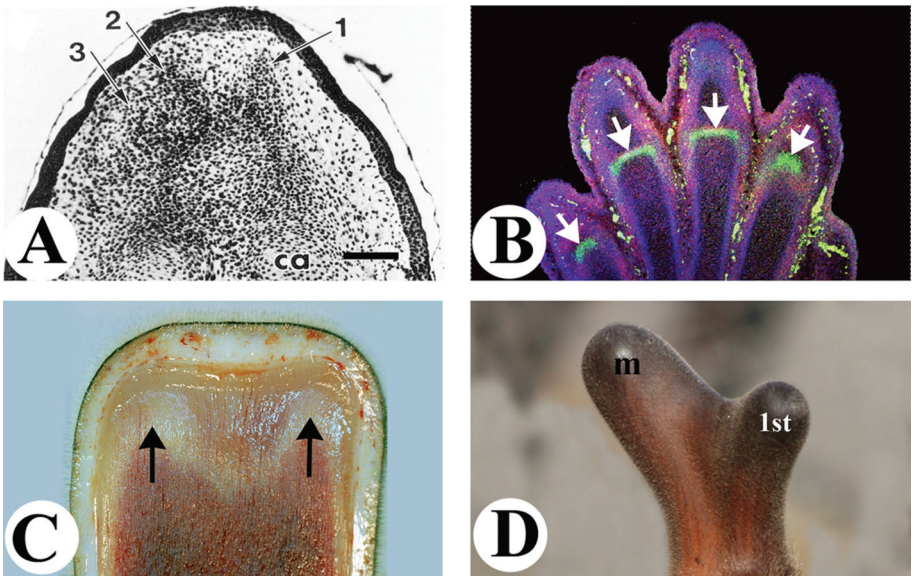

Figure 3. Dedifferentiation-based vs stem cell-based epimorphic regeneration. A. Dedifferentiation-based limb regeneration over a newt limb stump (reproduced with permission from Neufeld, 1982; Devel Biol. 93:36–42). Note that at the early stage (notch) the miniature organ has already taken shape. B. Joint formation in a hand through a dedifferentiation-based process (arrows). C. Stem cell-based antler regeneration. The species-specific shape is gradually unfolding during antler regeneration. Note that a new growth center has formed for the first tine and is separated from the growth center of the main beam of the antler before the tine is visible externally (arrows).

6. DEDIFFERENTIATION-BASED VS STEM CELL-BASED REGENERATION

Although "blastema" has been redefined (from previously solely a de-differentiation derivative; (52,54,55,66); to now the resident undifferentiated "stem cells" are also included; (61). However, whether the dedifferentiation-based and the stem cell-based blastemas have similar capacity to regenerate the lost appendages has not been addressed so far. An overview of the relevant literature suggests that there is a tendency for stem cell or stem cell-based processes to operate more in the regeneration of simple structured systems, and that the dedifferentiation-based process is associated more with the regeneration of complex structures like organs and/or appendages.

Turnover of cells to counteract wear and tear (e.g. renewal of blood cells or epidermis), and compensatory growth in response to increased functional load (e.g. removal of one kidney or hepatectomy) are typical examples of stem cell or progenitor-based regeneration (1,67). Regeneration of limbs and other structurally complex body parts in urodele amphibians is mainly a dedifferentiation-based process (2,3,52). A plausible explanation for this is that a dedifferentiation-based process allows for a miniature prototype-structure of a lost part to be formed. This process complements that of ontogeny, wherein a mini-organ, including joints, is developed at the initial stage (Figure 3A and 3B), and then development progresses to match the size of the organ that was lost. In contrast, a stem cell-based process builds up the missing tissue mass through proliferation and differentiation of the resident stem cells, and as such, it may not be compatible with the formation of morphologically and structurally complex organs and/or appendages.

However at odds with that dogma, the regeneration of deer antlers, which are morphologically complex mammalian appendages, is a stem cell-based process. The encoded morphogenetic blueprint of species-specific antlers is unfolded as the appendages elongate (Figure 3C and 3D) and is driven by the multiplication of stem cell-derived mesenchymal cells in the apex of the growing antler. Despite the impressive regenerative capabilities of antlers, it remains unclear as to whether such a process can cope with the regeneration of joints and muscles, as these are absent from antlers. Perhaps this reflects a limitation of this type of regeneration.

7. LOCAL FACTORS AND TISSUE INTERACTIONS

Since the late last century, the focus of antler research has shifted from the study of endocrine control to that of local signaling factors and tissue interactions. Comprehensive reviews about endocrine controls of antler development have published (1,68-70). It has generally reached consensus in the antler research community that androgen hormones, such as testosterone, control antler growth cycles; whereas growth factors, such as IGF1, stimulate antler growth. In this review, we chose to highlight the potential significance of local factors and tissue interactions between antler stem cells and adjacent cell populations and associated extracellular matrices.

7.1. Local factors

Our group was the first to study the differential expression of molecules of the PP over deer facial periosteum through 2-dimensional electrophoresis, and the signaling pathways through Ingenuity Pathway Analysis (IPA), including both PP tissues and cells collected from the pedicle stumps just prior to antler regeneration (71). In the study, 98 proteins were detected to be overexpressed in the PP. Amongst these proteins, Galectin-1, gelsolin-1 and COL6A1 were upregulated 20-, 10- and 10-fold respectively. Galectin-1 is a carbohydrate binding protein with a variety of structure dependant functions in the cell and the extracellular space: modulates the immune response (72), regulates myotube growth in regenerating skeletal muscle (73), and promotes the growth of various nerve tissues (74). Our IPA analyses point to that Galectin-1 interacts with Nanog, MYC and MYCN in the PP cells. Gelsolin-1 binds, cleaves and caps the barbed end of actin filaments in a calcium dependant manner (75), and hence mainly involves in cell motility, which fits with the notion that the PP cells migrate to form the apical mesenchymal layer during the development of the early antler blastema and provide a pool of progenitor cells for subsequent antler growth. COL6A1 is an extracellular protein, which is upregulated by TGFB1 via SMAD3 (76), suggesting a role in ECM remodeling but the relevance of this to antler regeneration is unknown as yet.

Results of overall IPA analysis in our study (71) showed that two pathways, PI3K/AKT and MAPK, dominate in the PP cells that are ready to regenerate. These two pathways have been shown to be important for maintaining self-renewal and pluripotency of stem cells (77). Mount et al (78) investigated the early regenerating antler buds/blastema (growing for over two weeks) and found that the most intense staining of activated β-catenin, the key molecule of canonical Wnt signaling pathway, was in dividing cells in the mesenchymal growth zone, suggesting that canonical Wnt signaling pathway is involved in the early regeneration of antlers. This is consistent with previous findings that canonical Wnt signaling pathway plays an important role in both blastema formation and out-growth in amphibian limb regeneration (79). Therefore, different stages of antler regeneration are likely to be regulated by different signaling pathways.

To further confirm the true involvement of the PI3K/AKT pathway in antler stem cells and antler regeneration, we recently carried out an experiment to inhibit this pathway in the PP cells in vitro using a specific inhibitor LY294002. The results showed that the effective inhibition of the pathway caused a significant decrease in proliferation rate ($P<0.0.1$) and a significant increase in adhesion ($P<0.0.1$) of the PP cells (Liu et al, in submission). Therefore, the PI3K/AKT pathway may regulate initial antler regeneration by stimulating proliferation of the PP cells and at the same time facilitating these cells migration through decreasing cell-cell adhesion.

7.2. Tissue interactions

The importance of heterotypic tissue interactions during annual antler regeneration was first proposed by Li and Suttie (80). These authors noticed that there was a difference in the degree of association between the enveloping skin and the PP along a pedicle shaft. The skin of the proximal two thirds of the pedicle is loosely attached to PP; whereas on the distal third of the pedicle, the skin is tightly bound to PP (80). These observations indicate that antler regeneration requires the PP and the enveloping skin to come together for interplay. Therefore, Li and Suttie (80) hypothesized that the distal closely associated region of a pedicle stump is in a primed state for antler regeneration compared to the proximal loosely attached region; they termed the distal 1/3 the "potentiated region", and the proximal 2/3 the "dormant region".

This hypothesis has been tested using membrane insertion experiments subsequently (22). In the study, two types of pedicle stumps were created by removing the distal parts of the antler at two different levels: Type 1 stump at the junction between a pedicle and an antler; and Type 2 stump at the junction between the potentiated and the dormant regions. An impermeable membrane was then inserted into the space between the enveloping skin and PP in each type of resulting pedicle stumps. The operation did not stop Type 1 pedicles to regenerate antlers, albeit the skin-less ones (Figure 2D), with one such antler even developing a rudimentary branch. In contrast, Type 2 pedicle stumps failed to give rise to regenerating antlers. Therefore, these experiments clearly demonstrate that interactions between PP and the enveloping skin are indispensable for antler regeneration.

Recently our laboratory has carried out a series of studies to try to reveal the mechanism underlying this PP potentiation from the dormant state. At the transcriptional level, genes (such as Notch 1, Enodmucin and Lymphoid enhancer-binding factor 1) related to embryonic appendage/limb morphogenesis, blood vessel and nerve development, response to wounding and negative regulation of cell differentiation were up-regulated, whereas those (such as CD86, Complement receptor 2 and C4b-binding protein) related to immunity significantly down-regulated in the potentiated PP (Ba et al. in submission). This change in gene expression may have created a suitable milieu for the regeneration of deer antlers to take place. At the post-transcriptional level, amongst the highly expressed miRNAs in the potentiated PP over the dormant PP, two are worth mentioning: miR-296 and let-7 series (let-7a/b/c/f/i/j). The miR-296, which is solely expressed in the embryonic stem cells, is reported targeting

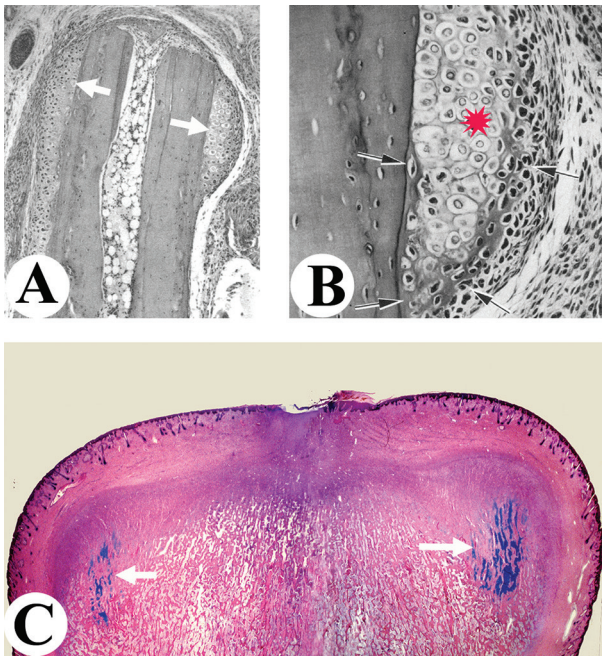

Figure 4. Wound healing over a mouse limb stump (reproduced with permission from Neufeld, 1982; Devel Biol. 93:36–42). A. A week after amputation, periosteum became thickened and substantial amount of hyaline cartilage was formed surrounding the distal end of the stump bone. B. A similar area of 4A to show the cartilage mass (asterisk) formed surround the distal mouse stump at a higher magnification. C. Sagittally cut histological section of an early antler bud at late wound healing stage, note the two established growth centres (arrows).

the transcription factor Nanog (81,82), expression of which had been previously detected in the PP through 2 dimensional electrophoresis (71). Therefore, miR-296 may play an important role in activating proliferation of PP cells for the initiation of antler regeneration. The let-7a and let-7f have been reported to regulate the expression of IGF-1R (83), IGF1 is the most potent factor for stimulating antler growth (84). Therefore, let-7 series may be positively involved in regulation of initial antler growth (Ba et al. in submission). At the epigenetic level, we found that the genome-wide DNA methylation extent of the potentiated PP in both the cells and tissues were significantly lower than that of the dormant PP ($p<0.0.5$). These findings provide the first evidence for a strong correlation between DNA methylation level and appendages regeneration (Yang et al. in submission).

Overall, activation of antler stem cells (PP cells) for the initiation of antler regeneration is a complicated process and requires multiple levels of regulation. Decrease in the concentration of testosterone and increase in insulin-like growth factor 1 trigger the initial antler regeneration. These endocrine factors exert their functions via direct or indirect pathways to mediate local factors, which in turn activate the potentiated the PP cells to proliferate to form antler blastema (Figure 5).

8. THE ANTLER IS A UNIQUE MODEL FOR REGENERATION OF MAMMALIAN APPENDAGES

The ultimate goal of studying regeneration of antlers is to learn whether it can be used as a suitable model for regenerative medicine. During evolution, vertebrates have lost the ability to replace their missing appendages (54). The wound healing over the stump of lost limbs have been studied histologically in mice (85). Surprisingly, the processes in the early antler regeneration are very similar to those in the wound healing over the amputated mouse limb stump. In this respect in both cases: 1) the wounds over the stumps are healed with the full thickness of skin and with the formation of a scar (6); 2) distal periosteal cells of the stumps of both the pedicle and the mouse limb are activated to enter a mode of fast proliferation and differentiation to form cartilage; and 3) substantial amounts of cartilage are formed, which surrounds the distal ends of the stumps, with a very limited amount of cartilage formed on the amputation/cast plane (Figure 4A, 4B and 4C). The most notable difference between these two processes is the potential of the periosteal cells to proliferate. In the case of mouse limbs, proliferation ceases as soon as the newly formed cartilage seals the open end of the amputated long bone, and subsequently the nascent cartilage is remodeled to bone. In the case of deer antler, the PP cells continue to produce cartilage tissue until the entire antler is fully regenerated.

Full regeneration of antlers is solely dependent on the presence of the PP. Regeneration of cartilage and bone (internal components) is directly achieved through the proliferation and differentiation of the PP cells (17,18), and the skin, blood vessels and nerves (external antler components) through the chemical induction and mechanical stimulation from the PP cell-derived progeny (20). Through these comparisons, we can see that the limb stumps of mammals, as observed in mice, cannot grow beyond the wound healing phase to even partially replace the lost organ because of limited potential of periosteal cells in long bones to proliferate. If we can impart a greater proliferation potential to these periosteal cells to a similar extent to that of the PP cells through a means of dedifferentiation/reprogramming (86) or transdifferentiation (87), we might be able to realize the dream of regenerating limbs in humans. Indeed, an overgrowth of bone over the limb stump sometimes occurs in subjects after an amputation transects the long bone (88). This phenomenon occurs most commonly in children under 12 years of age, but never after a person reaches skeletal maturity, which indicates that so long as the periosteal cells of the long bone possess the ability to proliferate, they would be able to extend a stump further. If we can properly control and manage this partial regeneration, we might be able to further enhance the functionality of amputees and achieve a better outcome beyond that of wound healing alone. Overall, a better

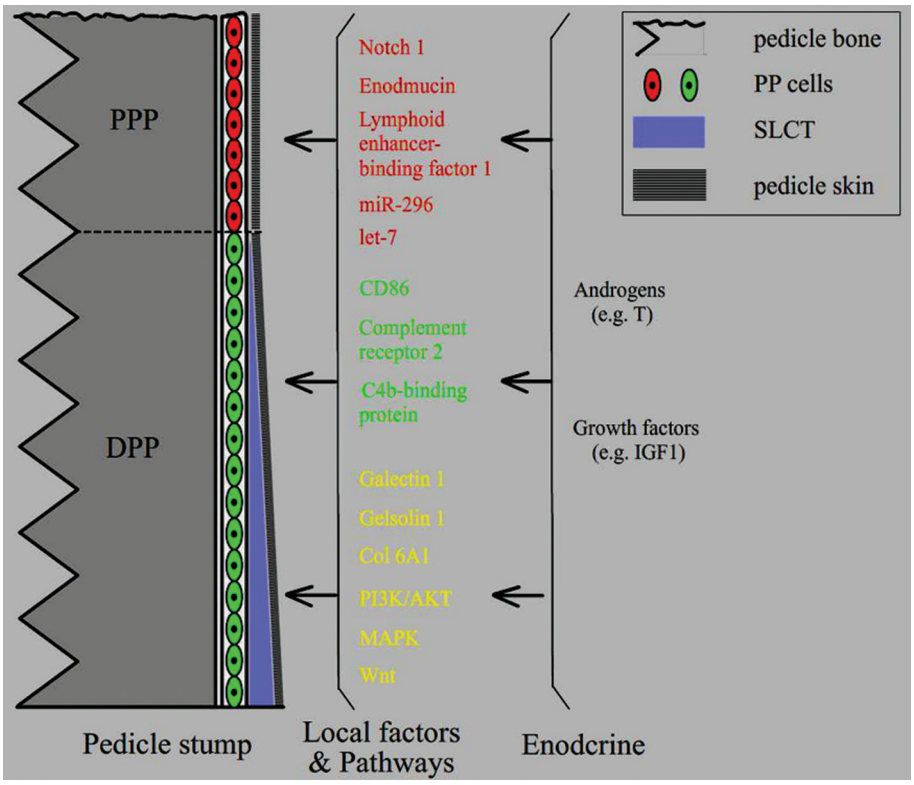

Figure 5. Schematic drawing of regulations in antler regeneration (see text for the explanation). Local factors and pathways in red mean upregulated in the PPP, in green mean upregulated in the DPP, and in yellow mean upregulated in the PP. PP, pedicle periosteum; PPP, potentiated pedicle periosteum; DPP, dormant pedicle periosteum; SLCT, subcutaneous loose connective tissue; T, testosterone; IGF1, insulin-like growth factor 1.

understanding of the mechanisms that regulate the regeneration of antlers, the only mammalian organ that can fully regenerate, may provide valuable insights in the development of future treatment options in the rapidly developing field of regenerative medicine.

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