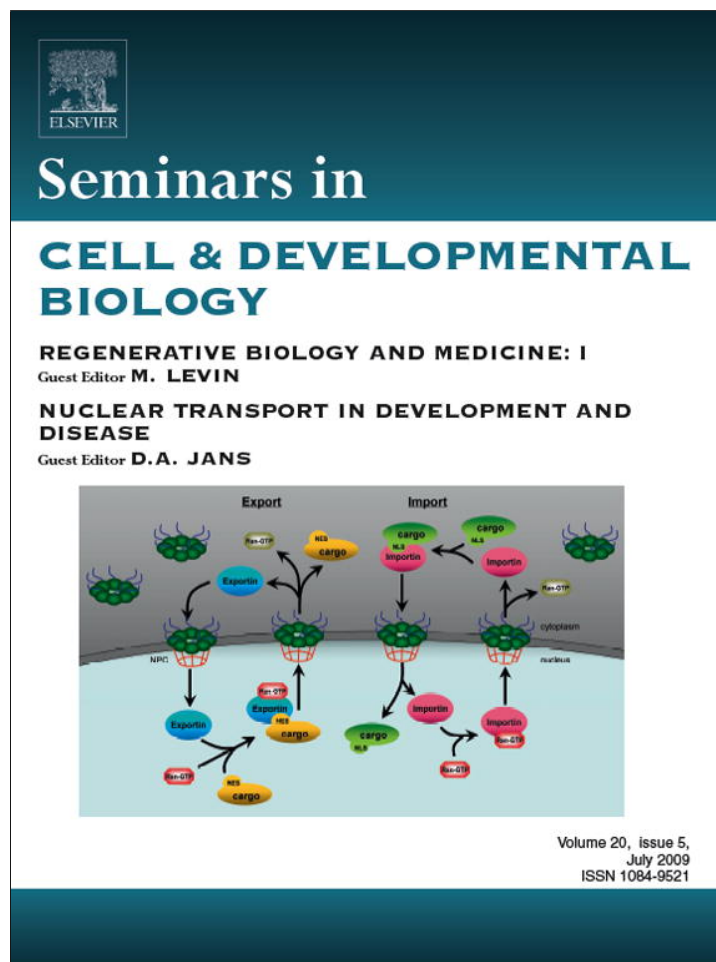




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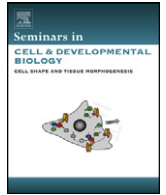
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Review

Improbable appendages: Deer antler renewal as a unique case of mammalian regeneration

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ABSTRACT

Deer antlers are periodically replaced cranial appendages that develop from permanent outgrowths of the frontal bones known as pedicles. Antler re-growth is a unique regenerative event in mammals which in general are unable to replace bony appendages. Recent evidence suggests that antler regeneration is a stem cell-based process that depends on the activation of stem cells located in the pedicle periosteum which are presumed to be neural crest-derived. It has been demonstrated that several developmental pathways are involved in antler regeneration that are also known to play a role in the control of skeletal development and regeneration in other vertebrates. However, in contrast to most other natural examples of regeneration of complete body structures, antler regeneration apparently neither depends on a functional nerve supply nor involves a direct contact between wound epithelium and mesenchymal tissue. Antlers thus demonstrate that regeneration of a large bony appendage in a mammal can be achieved by a process that differs in certain aspects from epimorphic regeneration in lower vertebrates.

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1. Introduction

“The antlers of deer are so improbable that if they had not evolved in the first place they would never have been conceived even in the wildest fantasies of the most imaginative biologists” [1]. This statement by Richard J. Goss in his monograph on antlers prompted the title of the present review. Antlers are remarkable organs, not least because they have an exceptional rate of growth and play a major role in the social life of deer [1]. To regeneration biologists antlers are particularly important because their

annual replacement is a unique case of complete regeneration of a bony appendage in an adult mammal [1–6]. The horns of bovids and antilocaprids are developmentally and morphologically very different from antlers and horns and antlers are considered non-homologous structures [1,7].

The capacity to regenerate missing body parts is widespread, although not ubiquitous, in metazoans [5,8,9]. Among vertebrates, urodele amphibians possess the greatest capacity for regeneration [10]. Thus, for instance, following limb amputation a salamander will regenerate a faithful replica of the lost appendage. In contrast, the ability for limb regeneration in mammals is extremely poor, being restricted to the re-growth of digit tips, and even this limited capacity for regeneration declines with age [5,11,12]. Nevertheless, re-growth of digit extremities in mammals indicates that

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a modest ability for appendage regeneration has been maintained in a specific context. This suggests that appropriate (re)activation of retained developmental programs would allow the regeneration of an entire mammalian limb [11,12].

Antler regeneration demonstrates that a large bony organ can be fully regenerated by an adult mammal that, like mammals in general, is unable to regenerate a limb. Exploring the mechanisms underlying antler regeneration and comparing this process with appendage regeneration in other vertebrates may therefore provide crucial insights into why mammals are unable to regenerate limbs and might even provide clues on how to overcome this inability [2,3,4,6,13].

2. The antler cycle

Except for the reindeer (*Rangifer tarandus*), antlers are grown only by male deer with the annual antler cycle being closely linked to seasonal variation in circulating androgen levels [14–17]. During antler growth, testosterone concentrations in blood are low and although a growth-promoting role of low androgen levels has been proposed [15,18], there is evidence from *in vivo* [19,20] and *in vitro* studies [21–24] that insulin-like growth factor-I (IGF-I) is the main hormone with a stimulatory effect on antler growth. It has been shown that IGF-I is not only produced by the liver but also by the growing antlers themselves [25,26].

A gradual rise in testosterone levels prior to the rutting season is associated with a slowing and eventual cessation of longitudinal growth, full mineralization of antler bone and the shedding of the specialized type of skin (velvet) that covers the growing antlers [14,15,27]. Velvet shedding exposes the bare bone of the antlers, which are then referred to as 'hard antlers'. A decline in circulating testosterone after the rut causes casting of the hard antlers, which is followed by the re-growth of a new set of antlers. There is evidence to suggest that the effects of testosterone on the antler cycle are at least partly of an indirect nature, occurring after local conversion to estrogens [28–33].

3. Development of pedicles and first antlers

Antlers grow from pedicles, which are permanent outgrowths of the frontal bones covered by normal scalp skin. Pedicle growth is initiated by a rise in circulating androgen levels during the first year of life [34], and pedicle development can be inhibited by castration of a male deer prior to pedicle initiation [1]. When the pedicle has reached a threshold size, the growth of the first antler starts from its apex. The transition from pedicle to first antler growth is externally visible by the change of the integument covering the cranial outgrowth from normal scalp skin to velvet [35]. It is with the casting of its first antlers that a deer enters into the yearly cycle of antler regeneration and loss.

The sites where the pedicles will develop postnatally can be identified as conspicuous swellings on the heads of male deer fetuses [36,37]. It has been suggested that these swellings, which regress during later fetal life, form as the result of a surge in testosterone secretion following gonadal differentiation [36]. A histological investigation has shown that the swellings represent a local thickening of the skin (epidermis and dermis), whereas the periosteum and underlying bone in these areas do not differ histologically from those in adjacent head areas [37]. According to Li and Suttie [37], the thickened epidermis at the prospective pedicle site resembles the apical ectodermal ridge (AER) in the developing vertebrate limb.

The question of which tissue is responsible for pedicle and first antler formation has been addressed by deletion and transplantation experiments. Excision of the skin from the prospective pedicle

site did not prevent pedicle and antler development [38,39], and autologous transplantation of skin or subcutaneous tissue from this site to other body areas did not cause ectopic antler growth [39,40]. In contrast, resection of the periosteum from the prospective pedicle site prevented pedicle and antler development [39]. Autologous transplantation of this 'antlerogenic periosteum' (AP) [1] to other areas of the cranium, or to more remote body regions (forelimb, hind limb, hip, tail), led to ectopic pedicle and antler growth [37,40–43]. The small ectopic antlers that developed as a result of AP transplantation were covered with typical velvet during growth and, if the animal lived long enough, underwent repeated rounds of casting and regeneration. These experiments show that the capacity for pedicle and first antler growth resides in the AP. It is presently unknown when the AP acquires its self-differentiating ability. There is, however, evidence that the AP carries morphogenetic information for axial orientation of the antler. Thus, when excised discs of AP were put back to their original site after rotation by 180°, reversal of the anterior–posterior axis was evident in all antlers that were subsequently formed and whose polarity could be determined with certainty [44].

The embryological origin of pedicles and antlers remains unclear; however, since in the mouse (*Mus musculus*) the frontal bones are formed by cells of neural crest origin [45], it is likely that AP as well as pedicles and antlers of deer are also neural crest derivatives [6,46,47], as was originally proposed by Hall [48].

Histological studies have demonstrated that the AP is much thicker than the periosteum from other cranial sites [49,50]. The cells of the cellular (cambium) layer of the AP contain abundant glycogen, thereby resembling embryonic cells [51]. Based on a study using *in vivo*-labeling of AP cells with the *lacZ* gene, Li and Suttie [37] concluded that all cell types in the interior (subdermal) component of the pedicle and first antler are progeny of AP cells. Some authors hold that the AP, especially its cellular layer, contains adult stem cells [37,46,52]. In line with this view, it was recently demonstrated that *in vivo* AP cells can differentiate into osteoblasts and adipocytes, the latter cell type not being present in antlers [52]. Expression of stem cell markers by AP cells has, however, not been studied so far.

In vitro studies on cultured AP cells have demonstrated that their proliferation is stimulated by IGF-I. In contrast, a wide range of concentrations of sex steroids (testosterone, dihydrotestosterone, estradiol), alone or in combination with IGF-I, had no mitogenic effects on cultured AP cells, even though the cells possess specific binding sites for testosterone [53]. This finding was unexpected given that *in vivo* pedicle growth depends on stimulation by elevated androgen levels. It has therefore been suggested that the role of androgens may be to sensitize the cells of the AP to the mitogenic effects of growth factors [53].

Another significant implication of the AP transplantation studies is that they demonstrate that competence for velvet differentiation is widespread, although not ubiquitous, in the skin [40,42]. It has been hypothesized that induction of velvet transformation in competent skin is induced by diffusible molecules produced by the subdermal antlerogenic tissue [46,54–56]. Potential candidates that may act as the producers of these instructive messenger molecules are cells of the periosteum or perichondrium covering the growing cranial appendage [46,55,56].

The above hypothesis was recently supported experimentally [57]. Thus, while insertion of an impermeable membrane between subcutaneously transplanted AP and overlying skin on the forehead region of male sika deer (*Cervus nippon*) fawns prevented velvet transformation of the skin, insertion of a permeable membrane did not. Insertion of the impermeable membrane also resulted in a growth arrest of the pedicle, i.e., an antler was not formed. In contrast, insertion of an impermeable latex sheath between skin and periosteum/perichondrium in red deer (*Cervus elaphus*) after first

antler transformation had occurred did not prevent further antler growth. On the basis of these findings it has been suggested that while pedicle growth involves a process of self-differentiation of the AP without a need for significant tissue interaction, velvet transformation of the skin and initiation of first antler development from the pedicle are processes that require transient (reciprocal) signaling between the subdermal antlerogenic tissue and the overlying skin [57].

4. Antler casting

Antler casting is initiated by osteoclastic activity in the distal pedicle. Resorption occurs both in the interior of the bony pedicle as well as along its periphery and is most intense in a narrow zone corresponding to the future separation plane [58,59]. The wound surface in the distal pedicle that forms as a result of antler casting is initially covered with blood, but a scab is rapidly formed. Ongoing osteoclastic activity in the distal pedicle leads to a smoothing of the initially rough casting surface [60,61]. This is followed by the formation of bone that partially restores the portion of the pedicle that was lost with the cast antler. The newly formed slender bony trabeculae that extend from the much thicker trabeculae of the pedicle stump form by intramembranous ossification [60].

5. Cellular origin of the regenerating antler

There are two questions that pertain to the cells which give rise to the regenerating antler. Firstly, there is the question of the locality of origin of these cells (topographic aspect), while the second question relates to the characteristics of these cells; viz. whether they form by dedifferentiation of mature cells or represent stem cells.

Growing antlers possess mesenchymal growth zones located at the tips of the main beam and the branches (tines). Regarding the topographic origin of the cells forming the initial growth zone(s) of the early regenerating antler, it was originally believed that the dermis of the pedicle was the source of these cells [62,63]. However, it was later shown that antler regeneration can occur when the skin was mechanically prevented from participating in the process [64]. In this case, the structures that grew out from the pedicles lacked a covering of skin, but instead were covered by a scab. One of the outgrowths even developed a rudimentary branch. This led the authors to conclude that the regenerated structures were actually 'skinless' antlers and not just extensions of the pedicle bone [64].

Other authors have suggested that the cells responsible for antler regeneration are derived from the pedicle periosteum (PP) [37,55,65]. This view was initially based on an analysis of an antler malformation known as 'double-head' antler [65,66]. A double-head antler is formed when a hard antler fails to be cast and the regenerating antler forms below and around the undetached previous antler. In this case, the regenerating antler develops as a laterally oriented periosteal exostosis from the distal pedicle [66].

In support of the suggestion that PP cells are responsible for antler regeneration, histological studies of normal antler regeneration subsequently showed that soon after antler casting the periosteum of the distal pedicle is markedly thickened, which is suggestive of an increased proliferative activity in this tissue [60,61]. Moreover, it was shown that independent growth centers for the main beam and brow tine of the re-growing antlers of red deer are formed *in situ* by a thickening of the local PP very early during regeneration, even before the completion of wound healing in the pedicle stump [61]. The presence of parathyroid hormone-related peptide (PTHrP) in both the periosteum and the mesenchymal cells of the growth zone of the early regenerating

antler [67] is consistent with the view that the mesenchymal cells are derived from the periosteum.

The hypothesis that antler regeneration depends on the PP gains further support from tissue deletion experiments which showed that surgical removal of the PP either completely inhibited or significantly delayed antler regeneration [68]. Histological investigation indicated that the bone tissue of the belatedly regenerated antlers was derived from the newly formed PP that itself had regenerated from the frontal periosteum around the pedicle base. Since in the experiment each pedicle had been sawn of 10 mm proximal to the antler/pedicle junction, and the cut surface covered with bone wax, participation of cells from the pedicle bone in the regeneration process could largely be excluded [68].

The observation that following PP removal antler regeneration was performed by cells derived from frontal periosteum [68], corroborates earlier results indicating that the 'antler regeneration territory' is extensive. Thus, amputation of the distal pedicle [69], the complete pedicle [69,70] or the complete pedicle along with an attached portion of the frontal bone, leaving only the inner table (lamina interna) of the frontal bone in place [71], did not prevent antler regeneration following wound healing. Rarely, growth of ectopic antlers has been observed from membrane bones of the skull other than the frontal bones [46,72]. This has led to the suggestion that the entire periosteum overlying the bones of the skull vault is potentially capable of forming an antler, but that with increasing distance from the center of the regeneration territory the propensity for antler growth gradually decreases [55].

In urodele limb regeneration, the subepidermal tissues in the amputation stump undergo large-scale dedifferentiation, and the dedifferentiated mesenchymal cells then aggregate into a blastema from which the regenerate develops [5]. There is, however, accumulating evidence that also stem cells are involved in the regeneration of limbs and other body structures in amphibians [73–75].

Histological observations suggest that no large-scale dedifferentiation occurs in the pedicle following antler casting [60,61], indicating that the mesenchymal cells of the growth zone do not (or at least not to any significant extent) constitute dedifferentiated progeny of cells of the pedicle stump. Rather, it has been suggested that the mesenchymal progenitor cells are derived from stem cells in the PP which themselves are progeny of stem cells (probably neural crest-derived) originally present in the AP [6,37,60,61]. In line with this view, a recent study demonstrated the presence of cells expressing stem cell markers, including the mesenchymal stem cell marker STRO-1, in the PP of fallow deer (*Dama dama*) [76]. We also have data to suggest that cells in the PP of red deer express stem cell [77] and neural crest cell markers [78]. It has further been reported that *in vitro* these cells can be induced to differentiate into osteoblasts, chondrocytes and adipocytes, thereby demonstrating their multipotency [77,78].

Taken together, these data have led to the suggestion that stem cells are located in a niche in the cellular layer of the PP, where they are periodically activated to regenerate a new antler [6,76]. Further studies are needed to test this concept and to provide information on the precise localization and characteristics of these stem cell, their differentiation potential, the way they are maintained in the putative PP stem cell niche and what controls their periodic activation.

6. Antler bud formation and the regeneration process

The exposed casting surface of the pedicle is rapidly covered by a migrating epidermis (wound epithelium) originating from a tumescent skin rim that forms already prior to antler casting in the distal pedicle [4,60,61]. Wound epithelialization is accompanied by the formation of a dermal layer, so that the pedicle stump is overlain by full-thickness skin rather than just the wound epithelium [59–61].

A basal lamina starts to form at the epidermal–dermal interface already before complete closure of the casting wound [2,6]. This observation and the finding that the prevention of skin participation does not inhibit antler re-growth [64] indicates that, unlike the situation in urodele limb regeneration, direct contact between wound epithelium and mesenchymal tissue is not of crucial importance to antler regeneration.

The growing tips of regenerating antlers can be subdivided into different zones in a distal-to-proximal direction. Beneath the velvet and the fibrous layer of the perichondrium lies a mesenchymal zone, also known as the reserve mesenchyme [79,80]. This zone is characterized by intense proliferation of mesenchymal progenitor cells, as was demonstrated by BrdU-staining [81] and PCNA-staining [82], and a high level of apoptosis, as demonstrated by TUNEL-staining [82]. It has been hypothesized that the high rate of apoptosis in the mesenchymal growth zone may be required to prevent transformation of the rapidly growing tissue or may be a crucial step in delineating the position of antler branches [82]. There is consensus that the reserve mesenchyme is the antler growth zone, i.e., progenitor cell proliferation in this zone is largely responsible for growth in length [3,6,80,82]. Like embryonic cells, these mesenchymal progenitor cells have an extended life span *in vitro*; they can be grown for over 80 passages and for up to 10 months in culture before they stop dividing [83].

Proximally, the mesenchymal growth zone is followed by a prechondroblastic/chondroblastic or precartilaginous zone, the boundary between the two zones being indistinct histologically [6,79–81,84,85]. In the precartilaginous zone, the cells start to become arranged into longitudinal columns that are separated by vascular spaces. The precartilaginous and cartilage zones of the antler are richly vascularized, this being a major difference to growth plate cartilage. The arteries supplying the antler are branches of the superficial temporal artery and are located at the base of the dermal component of the velvet [86,87]. Angiogenesis in the antler tip is most intense in the precartilaginous zone [87].

Further proximally, the chondrocytes undergo maturation and hypertrophy and the cartilage matrix is mineralized. The mineralized cartilage is then resorbed by chondroclasts and bone is laid down on the scaffold of the remaining cartilaginous trabeculae. Eventually, the mineralized cartilage is completely replaced by bone [3,4,79,80,88]. This mode of bone formation has been characterized as a form of endochondral ossification [79,80,84,88]. The same sequence of histological events as in antler regeneration also occurs during the growth of the first antler [89]. Also (subperiosteal) intramembranous ossification occurs in the growing antler, leading to the formation of a sleeve of bone around the cartilage [79,80,88].

The growing antler is richly innervated by sensory fibers derived from branches of the trigeminal nerve. The growth of these nerve fibers is very rapid, keeping pace with antler elongation [90–92]. It was recently shown that the regenerating axons follow the route of the major blood vessels and that nerve growth factor (NGF) mRNA is expressed in the smooth muscle cells of arteries and arterioles of the growing antler tip [92]. This led the authors to conclude that NGF may attract sensory axons into the growing tip and provide a guidance cue to define their track [92].

While a rich nerve supply is vital if deer are not to injure their rapidly growing antlers, there is experimental evidence suggesting that antler regeneration does not depend on a functional nerve supply because antler re-growth can take place despite pedicle denervation [90,93]. In contrast, in lower organisms regeneration of a lost or damaged body structure has generally been shown to depend on the concomitant regeneration of the nerve supply [5,9]. A particularly exciting recent discovery is that of a new growth factor (nAG) for blastemal cells that is sequentially expressed in the Schwann cells of the regenerating nerve and in the wound epithelium after limb amputation in adult salamanders [94]. Expression of nAG in

both locations and proliferation of the blastema are abrogated by denervation. nAG interacts with Prod 1, a surface protein of the blastemal cells that is a critical determinant of their proximodistal identity [94]. The apparent lack of dependence of antler regeneration on an intact nerve supply highlights a potentially important difference between the mechanisms that underlie antler re-growth and appendage regeneration in lower vertebrates. It also is not consistent with the suggestion that the main difference between development and regeneration is the nerve dependence of the latter process [9]. An intriguing possibility is that this unusual feature of antler regeneration may relate to the fact that, contrary to limbs or tails, antlers are amotile structures.

7. Local control of antler growth

Identifying the mechanisms that control the proliferation and differentiation of the mesenchymal progenitor cells in the growing antler is crucial for developing a deeper understanding of the antler regeneration process [47]. Another vital issue is the identification of the factors and processes involved in the control of antler shape. Some authors suggest that 'antler growth centers' located in the brain are involved in the control of antler morphogenesis [15,95]. However, addressing this hypothesis experimentally would be very difficult. Therefore, current research has largely focused on local mechanisms of growth control, particularly on molecules that are known to play a role in the control of skeletal developmental and/or regenerative processes in other organisms [4].

There is evidence that retinoic acid (RA), a molecule critical for both limb development and regeneration in lower vertebrates, is involved in the antler growth process. In regenerating antlers all-*trans*-RA, which is also present in regenerating amphibian limbs, has been found in the skin, the fibrous layer of perichondrium, mesenchyme, cartilage, bone and periosteum, and 9-*cis*-RA was also found in the fibrous layer of perichondrium, mesenchyme, mineralized cartilage and bone. The RA synthesizing enzyme RALDH2 was localized in different antler tissues as were receptors of the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families [96]. *In vitro*, all-*trans* RA inhibited the expression of the chondrocyte phenotype, this effect being dependent on RAR signaling. The expression pattern of RAR α suggests that RA may inhibit the differentiation of chondroprogenitors, thus maintaining the mesenchymal cells of the antler growth zone in the proliferation phase [96]. There also exists some *in vivo* evidence suggesting that administration of exogenous RA influences antler growth. Thus, injection of a high dose of all-*trans*-RA into the incipient pedicle of a fallow deer caused alteration in pedicle and first antler shape [97], and injection of all-*trans*-RA into the growing pedicle was shown to increase first antler size in fallow deer [98].

There is also evidence that both the PTHrP-Indian Hedgehog (IHH) pathway and the canonical Wnt signaling pathway are involved in the regulation of antler regeneration [67,99]. In a study on red deer antlers during the period of most intense growth (weeks 4–8 of regeneration), PTHrP, the PTH/PTHrP receptor (PPR) and transforming growth factor β 1 (TGF β 1) were localized in the skin, fibrous layer of perichondrium, mesenchyme, recently differentiated chondrocytes and perivascular cells in cartilage, but not in hypertrophic chondrocytes. It has been suggested that in the regenerating antler PTHrP plays a role in the control of skin growth, stimulates proliferation of mesenchymal progenitor cells, inhibits chondrocyte differentiation and may also be involved in the regulation of osteoblast differentiation. *In vitro*, TGF β 1 stimulated PTHrP synthesis by mesenchymal cells from the growth zone, cells from the perichondrium, and chondrocytes suggesting that TGF β 1 may be involved in the regulation of chondrogenesis by inducing PTHrP [67]. However, the widespread distribution of TGF β 1 in the growing antler suggests that this growth factor probably has multiple

functions [4]. It has also been shown that PTHrP regulates the differentiation of antler osteoclasts and that antler osteoclasts are unusual among mammalian osteoclasts in expressing PPRs [100]. A pattern of PTHrP and PPR expression similar to that in the regenerating antler was also observed in the developing first antler of red deer [101], which indicates that during antler regeneration, as in appendage regeneration in lower vertebrates, signaling pathways operating during development are recapitulated. In contrast to the fairly promiscuous pattern of expression of PTHrP in the regenerating red deer antler, IHH was localized only in recently differentiated chondrocytes and in perivascular cells in cartilage [67] indicating that IHH is involved in the control of chondrocyte differentiation [4].

The role of the canonical Wnt signaling pathway in antler regeneration was studied by localizing the activated form of β -catenin (β -CAT) in antler tissues during early antler re-growth and in the phase of rapid elongation. The most intense β -CAT staining was found in the dividing mesenchymal cells of the growth zone, suggesting that the canonical Wnt pathway may be involved in the control of the lineage specification of these progenitor cells [99]. Strong staining for β -CAT was also found in the cellular layer of the periosteum and in osteoblasts at sites of intramembranous bone formation (antler periphery), whereas only weak staining was seen in chondrocytes and osteoblasts at sites of endochondral ossification, indicating that the canonical Wnt pathway was not activated at the latter sites [99].

Bone morphogenetic proteins (BMPs) have been localized in growing antlers by different groups [4,102,103]. Barling et al. [103] reported that BMP-2, BMP-4, BMP-14 and the BMP receptors BMPRII and ACTRII are present in skin, mesenchyme, cartilage and bone of developing first antlers of red deer. *In vitro*, BMP-2 decreased the proliferation of mesenchymal cells and increased alkaline phosphatase activity, suggesting that BMP-2 induces the differentiation of the mesenchymal progenitor cells [4].

Both IGF-I and IGF-II are expressed in different tissues of growing antlers (skin, mesenchyme, precartilage, cartilage) [25] leading to the suggestion that, while the main growth signal from IGF-I is endocrine, both IGFs, but especially IGF-II, also stimulate antler growth in an autocrine or paracrine fashion [25]. Recent studies have also demonstrated that epidermal growth factor (EGF) and its receptor (EGFR), as well as fibroblast growth factor 2 (FGF 2) and the FGF receptors FGFR1, FGFR2, and FGFR3 are widely expressed in the skin, mesenchyme, cartilage and bone of growing first antlers of red deer [104,105]. The same applies to the expression of vascular endothelial growth factor (VEGF) and the VEGF receptor VEGFR-2 (KDR) [105,106]. It has been suggested that FGF-2 induces expression of VEGF in the developing/regenerating antler to stimulate the rapid angiogenesis necessary for meeting the high metabolic demands of the growing antler [105].

Clearly progress has been made in recent years in identifying some key molecules involved in the local control of antler regeneration. Notwithstanding these achievements, our understanding of the inevitably complex mechanisms that regulate the renewal of these unique structures remains quite rudimentary. In particular, we know very little about the mechanisms that control the early stages of antler regeneration, the activation of the stem cells in the PP and their proliferation and differentiation to form an antler bud.

Techniques used in antler research so far include conventional light and electron microscopy, immunohisto- and cytochemistry, *in vivo* tissue deletion and transplantation experiments as well as a variety of cell and tissue culture approaches. A study labeling AP cells *in vivo* using *lacZ* gene injection was successful in cell lineage tracing [37]. An *in vivo* transfection experiment employing a biolistic particle-delivery system has yielded promising results and offers interesting possibilities for future *in vivo* loss- or gain-of-function experiments [47]. Another potentially useful tool for studying the

mechanisms of antlerogenesis is a xenograft approach, in which deer tissues or cells are transplanted into nude mice. In this way it is possible to study different *in vivo* aspects of antler development and regeneration in a system that is much easier to handle than a deer [56]. In addition, genomics and proteomics approaches offer an opportunity to identify many more potential regulatory molecules and signaling pathways involved in antler growth [85,107,108]. The challenge for antler biologists will be to establish their functional roles; however, techniques such as siRNA and viral transfection could be used for such studies in the future.

The exceptional growth rate of antlers raises the intriguing question of whether they are susceptible to neoplastic transformation [109]. Our recent interpretation of antler growth as a process depending on stem cell/progenitor cell proliferation makes this question even more important. It has already been mentioned that the high rate of apoptosis in the mesenchymal growth zone may be a mechanism for exercising strict control of the growth process, thereby minimizing the risk of neoplastic transformation [82]. Moreover, it has been argued that it is the short life span of antlers that precludes tumor formation, because most tumors require longer periods of development before becoming overt [109]. Tumor formation in antlers in fact appears to be a very rare event. To the best of our knowledge, only one case of bone tumor has been reported in the scientific literature. This tumor, which was diagnosed as a solitary osteochondroma, was present in the first antler of a fallow deer [110].

Interestingly, castrated deer develop tumor-like outgrowths on their permanently velvet-covered antlers. The growth intensity of these amorphous outgrowths differs between species, being highest in the roe deer (*Capreolus capreolus*), in which the massive overgrowth leads to the formation of so-called 'peruke antlers' [1,95]. The abnormal growth processes in the antlers of the castrates affects both the bony component and the skin of their velvet antlers, which do not elongate further because they no longer possess growth zones at the tips of the main beam and the tines. The fallow deer is the only species in which the histological structure of the tumor-like outgrowths has been studied in more detail. The skin outgrowths, which were termed 'antleromas' by Goss [1], contain copious amounts of collagen fibrils interspersed with scattered fibroblasts and blood vessels and were therefore interpreted to represent a hypertrophic growth of the dermis [111]. The bony protuberances present on the antlers of the fallow deer castrates were demonstrated to develop by intramembranous ossification (appositional growth) from the periosteum [112]. Both the skin and bone outgrowths may be classified as benign tumors [111,112]. Malignant transformation of these structures has not been reported. Why such a fast growth system as the velvet antler appears to be resistant to malignant cell transformation even after significantly prolonging its life span by castration and why the antlers of different deer species develop benign tumors in the absence of gonadal sex steroids are important questions, although they will not be straightforward to address. Unveiling the mechanisms of antler growth regulation may have important implications for cancer biology.

8. Antler regeneration—a model for mammalian appendage regeneration?

There has recently been some debate about how to classify antler regeneration in comparison with appendage regeneration in lower vertebrates, urodele limb regeneration in particular [6]. Without doubt, antler regeneration is a case of periodic physiological regeneration [2,5,6], although whether antler regeneration can be classified as an example of 'true' epimorphic regeneration depends on how exactly one defines this process [5,6]. For this reason we agree with the recent statement made by Carlson [5] which is that one should avoid to become wedded to terminology, since

the labels that we use may be inadequate to cover the full spectrum of naturally occurring regenerative phenomena. However, what is clear is that the process of antler regeneration resembles epimorphic regeneration in lower vertebrates, and there is evidence that in both cases the regenerating structures use genetic programs that have already been active during initial development.

There are, however, also some major differences between urodele limb regeneration and antler re-growth [2,3,6]. Thus, while the formation of the regeneration blastema of a urodele limb involves the large-scale dedifferentiation of stump cells and is nerve dependent, the process of antler regeneration apparently does not depend on a functional nerve supply and also does not appear to involve major dedifferentiation processes in the pedicle tissues. Available evidence suggests instead that the early mesenchymal growth zone is populated by cells derived from stem cells that reside in a niche in the pedicle periosteum. In urodeles, no basal lamina is present between the wound epithelium covering the blastema and the underlying blastemal tissue. In contrast, the casting wound on top of the pedicle is covered by skin (epidermis + dermis) and a basal lamina starts to form underneath the epithelium well before wound closure is complete.

It has been argued that the fact that antler regeneration partly utilizes different mechanisms than regenerative processes in urodeles makes the antler more, not less, relevant as a potential model for mammalian (particularly human) appendage regeneration [3]. Gardiner [11] claimed that the challenge for inducing limb regeneration in humans is to discover how to induce fibroblast dedifferentiation in an amputation stump. This assumes that if it could be induced, successful limb regeneration in mammals would occur in the same way as in amphibians. While this view is certainly a reasonable starting point for further research into mammalian regeneration, it may be rather narrow as antlers demonstrate that complete regeneration of a large bony appendage can be achieved by a process that differs in certain aspects from epimorphic regeneration in lower vertebrates. Let antler regeneration serve as a reminder that we must consider a range of strategies when searching for ways of how to overcome the obstacles that prevent limb regeneration in mammals.

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