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Antler regrowth as a form of epimorphic regeneration in vertebrates – a comparative view

Uwe Kierdorf, Horst Kierdorf[1]

[1]*Department of Biology, University of Hildesheim, 31141 Hildesheim, Germany*

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1. ABSTRACT

The annual regrowth of deer antlers is a unique case of extensive appendage regeneration in mammals. This review compares basic aspects of antler regeneration with epimorphic regeneration in other vertebrate taxa. The mesenchymal cells that build up the regenerating antler are not derived from dedifferentiated cells in the pedicle stump, but from the proliferation of cells of the pedicle periosteum; and based on different lines of evidence it has more recently been suggested that the pedicle periosteum contains stem cells that are periodically activated to produce a new antler. This constitutes a difference to urodele limb regeneration, where the blastema is (largely) formed from dedifferentiated cells. Antler regeneration involves healing of the large casting wound with no or only minor scarring, making the antler an interesting model for the control of scarring in mammals. Contrary to urodele limb regeneration, antler regrowth does not depend on a functional nerve supply. In our view, a comparative analysis of different regeneration phenomena, including antler regeneration, probably offers the best chance for achieving significant progress in regenerative medicine.

2. INTRODUCTION

Regeneration in a broad sense can be defined as the replacement of worn, damaged or lost parts of the body. The process occurs at different levels of biological organization, ranging from the regular replacement of cells in the epidermis or gut epithelium, over the regrowth of severed appendages like limbs or tails, to the restoration of a complete organism from a small part of the body (1-5). While in the first example, regeneration occurs as a physiological process in order to compensate for everyday wear and tear, the (reparative) regeneration of appendages and whole-body regeneration is triggered by traumatic injury either from an external cause (natural damage or amputation) or due to autotomy, the process of self-amputation. The widespread distribution of the capacity for reparative regeneration across the Metazoa is often taken to indicate that this is an ancestral trait and that the metazoan ancestor was capable of extensive regeneration (4-6). If this notion is correct, it could be assumed that regenerative processes in different taxa use the same or related developmental programs and that in non-regenerating taxa, the ability for reparative regeneration has been lost in the

course of evolution. Recently, however, a different view on the evolution of regenerative capacities in vertebrates has been proposed (7). This view is based on the finding that a specific cell-surface protein (Prod 1) involved in the regeneration of salamander limbs is salamander-specific. This suggest that, in addition to conserved ancestral factors, also "locally" evolved taxon-specific components play a role in regeneration and that in part different mechanisms may be involved in appendage regeneration in vertebrates (7)

The ability to replace lost body parts varies widely, both among different taxa and within a given taxon (2-6). Even within a single species, the capacity for reparative regeneration can vary between different structures. Thus, for example, many species of lizards show tail regeneration following autotomy of the tail as an antipredation measure (2, 8, 9). The regrown tail is not a perfect replica of the lost one; rather the regenerate contains an unsegmented cartilaginous tube instead of a chain of vertebrae, and also the regenerated spinal cord and tail musculature are simplified compared with the corresponding structures in the original tail (9). Contrary to their tails, lizards do not regrow amputated limbs, although occasionally a simple, spike-like structure can grow from the amputation plane (3, 9). Autopodial elements are, however, never regenerated (9).

Among vertebrates, teleosts and amphibians, especially urodeles, possess a high capacity for appendage regeneration (1-3). Compared with these "lower" vertebrates, the regenerative abilities of mammals are generally poor and no mammal is capable of replacing an amputated limb or tail. However, mammals are not completely without the ability for appendage regeneration, as they can replace amputated digit tips. Thus in postnatal humans (both children and adults), fingertip regeneration is possible provided that the amputation occurs through the terminal phalanx and the wound is not covered with full-thickness skin (10, 11). A similar regenerative response has been observed in neonate and adult mice following amputation through the midpoint of the terminal phalanx, whereas no regeneration occurs when the amputation plane is located more proximally and two thirds of the distal phalanx are removed (12, 13). Recently, it was, however, reported that treatment of normally non-regenerating proximal amputation wounds of neonatal mice with bone morphogenetic protein 2 (BMP2) or BMP7 caused a regenerative response, demonstrating that the mammalian digit possesses latent regenerative capabilities that can be induced by growth factors (13).

The antlers of deer (family Cervidae) are a striking exception to the rule that mammals are incapable of full appendage regeneration and have therefore attracted the interest of developmental biologists (14-18). The basic idea underlying their work is that a better understanding of this unparalleled example of appendage regeneration in a postnatal mammal may eventually help to develop ways of stimulating the regrowth of normally non-regenerating appendages in mammals, including humans. This assumes that the process of antler regeneration involves mechanisms that are not specific to this particular developmental system but, at least partly, also operate during appendage regeneration in other vertebrates.

Antlers are periodically replaced, bony cranial appendages that occur in all species of deer, except for the Chinese water deer (*Hydropotes inermis*). It is currently assumed that the lack of antlers in *Hydropotes* represents a secondary reversal (19). Musk deer (with a single living genus, *Moschus*), which were formerly sometimes included in the Cervidae, also lack cranial appendages. However, systematists now exclude musk deer from the Cervidae and regard them as a separate family (Moschidae) within the Pecora (19). Antlers are normally grown only by male deer, with the exception of the reindeer/caribou (*Rangifer tarandus*) in which also females bear antlers (20).

The annual replacement of antlers has been classified as a striking example of physiological regeneration, comparable to the shedding cycle of crustacean exoskeletons, the replacement of the epidermis in snakes, or the annual molting cycle of feathers in birds (4). However, contrary to the above examples, antlers are complex bony organs and not just ectodermal derivatives. Antler regrowth must thus also be classified as an instance of reparative regeneration, following loss of the previous set of antlers and involving a wound healing process.

The present paper first reviews some basic aspects of antler development and then discusses similarities and differences between antler regeneration and other examples of appendage regeneration in vertebrates.

3. PEDICLES AND FIRST ANTLERS

Antlers grow as extensions from permanent bony protuberances of the frontal bones, the pedicles (20). There is evidence that the frontal bones of mammals are formed by cells of neural crest origin (21, 22) and it has therefore been suggested that also pedicles and antlers of deer are neural crest-derived structures (15, 23). Pedicle development starts some weeks or months after birth (Figure 1), and first antler growth commences spontaneously from the tip of the pedicles when these have reached a species-specific threshold size. It is with the detachment of its first antlers that a deer enters into the seasonal cycle of antler loss (casting) and regeneration. Lincoln (24) reported the appearance of "pedicles" already during early fetal life in red deer (*Cervus elaphus*). A later study showed that the conspicuous swellings observable on the heads of male fetuses represent a local thickening of the skin, but that bony pedicles are not formed (25). It has been suggested that the development of these local swellings are triggered by a testosterone surge during a period of increased activity of the fetal testes (24).

As has been demonstrated by deletion and transplantation experiments, pedicles and first antlers are formed by the periosteum overlying the presumptive pedicle sites on the frontal bones of young deer (26, 27). This specialized frontal periosteum was termed antlerogenic periosteum (AP) by Goss (20). AP activation

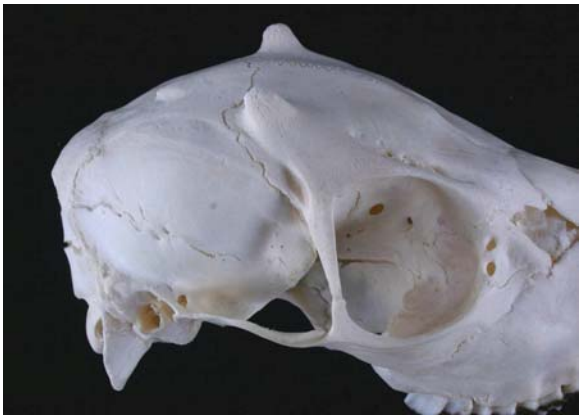

Figure 1. Cranium of a male roe deer (*Capreolus capreolus*) fawn aged about 4 months, showing an early stage of pedicle formation.

Figure 2. Ectopically formed pedicle (asterisk) and (regenerated) antler on the foreleg of a fallow buck (*Dama dama*). Ectopic pedicle and antler formation was caused by autologous transplantation of antlerogenic periosteum.

occurs by an increase in circulating testosterone in young males, and therefore castration prior to this rise in testosterone inhibits cranial outgrowth formation (20). Surgical removal of the AP in prepubertal deer likewise precludes pedicle and antler development, while AP transplantation to other sites of the body causes the formation of ectopic pedicles and antlers (25-29) (Figure 2). The latter are cast and regenerated, following a normal seasonal pattern. As is evidenced by the result of the transplantation studies, AP cells are committed to a certain differentiation pathway. It is currently unknown when during development the AP acquires its self-differentiating ability, but, given the presumed neural crest-derivation of this tissue, this may already occur in pre-migratory or migrating neural crest cells (29). The AP is much thicker than periosteum from other areas of the frontal bones of deer (30, 31), and the cells of the AP's cambial (cellular) layer are rich in glycogen (32). On the basis of more recent findings it has been concluded that the AP contains mesenchymal stem cells that are responsible for pedicle and first antler growth (17). Earlier it was demonstrated that AP cells carry morphogenetic information for the axial orientation of the antlers (33).

In the roe deer (*Capreolus capreolus*), a species with rather small pedicles and antlers, pedicle growth solely involves intramembranous ossification (34). In contrast, in species with larger pedicles and antlers, like fallow deer (*Dama dama*) and red deer, only the initial stages of pedicle formation occur solely by intramembranous ossification, whereas later growth involves both endochondral and intramembranous bone formation (30, 31). The pedicles of deer increase in thickness with age due to periosteal bone apposition and the outer pedicle compacta consists of plexiform (fibrolamellar) bone (35) (Figure 3).

The transition from pedicle to first antler growth is visible externally by a change of the integument that covers the forming cranial outgrowths, namely, from normal scalp skin of the pedicle to a special type of hairy skin (known as velvet) that is only found on growing antlers (20, 23, 25) (Figure 4). Compared with scalp skin, velvet is characterized by a thickened epidermis, larger sebaceous glands, *de novo* formation of hair follicles and the absence of the *arrector pili* muscles normally associated with hair follicles (36-38). Sweat glands are absent in the velvet of some species, e.g., the white-tailed deer (*Odocoileus virginianus*) (36), but present in others, most prominently in red deer and sika deer (*Cervus nippon*) (38). A comparative morphological analysis of the velvet of different deer species was performed by Bubenik (38). Ectopic antler growth caused by AP transplantation demonstrates that the skin from various (but not all) regions of a deer's body is capable of velvet formation and that the competence of the skin to undergo velvet transformation is not restricted to a certain ontogenetic stage (39).

Goss (40) proposed that first antler growth depends on an interaction between AP and overlying epidermis. Experimental evidence in support of this hypothesis came from later studies in which permeable or impermeable membranes were inserted between the AP of either the pedicle site prior to outgrowth formation or the early growing pedicle and its overlying skin. While insertion of an impermeable membrane inhibited antler formation, use of a permeable membrane only caused a slight retardation but no inhibition of first antler growth (41). These findings strongly suggest that the postulated inductive interaction between AP and its overlying skin occurs via diffusible molecules.

Based on a study comparing initiation of antler growth by transplanted AP with normal anatomical orientation (fibrous layer adjacent to skin) and inverted AP (cambial layer adjacent to skin) as well as an experiment using co-transplantation of AP and deer skin onto nude mice, it was further suggested that the cambial layer of the AP is the main source of the presumed inductive molecules

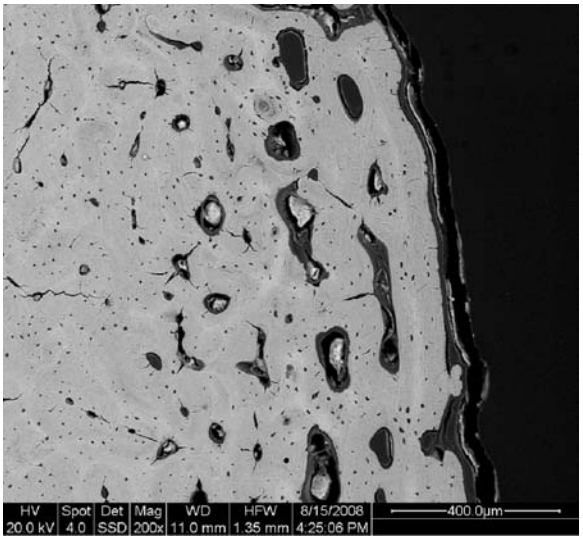

Figure 3. SEM-BSE image of cross-sectioned pedicle of a roe deer (*Capreolus capreolus*) . Plexiform (fibrolamellar) bone with primary osteons in different stages of development in the outer compacta.

Figure 4. Group of red deer (*Cervus elaphus*) stags with almost fully regenerated antlers. The antlers are covered with velvet.

triggering velvet transformation of deer skin (42, 43). In the latter study, a model was proposed in which the signal from the cambial layer of the AP acts on the overlying cells of the dermis that then cause transformation of the epidermis via paracrine and juxtacrine mechanisms. The authors further suggested that the transformed epidermal cells produce a feedback signal that is relayed via the dermis to the periosteal cells to initiate the change from pedicle to first antler growth (43). That transformation of scalp skin to antler velvet is dependent on specific signals, rather than just being a reaction to the formation of a rapidly growing subcutaneous mass of bone or cartilage, is also illustrated by the fact that osseous or osseocartilaginous tumors developing in the forehead region of deer cause an expansion of the scalp skin but not its transformation to velvet (29).

It has been suggested that for an effective interaction between AP and overlying skin a close contact between the two components is required (17). Support for this view was recently provided by an experiment demonstrating that intradermal transplantion of AP was more effective in inducing ectopic antler formation than subcutaneous transplantation. Thus, while intradermal implantation of one-eighth of the original AP mass was sufficient to induce ectopic antler growth, in the case of subcutaneous implantation at least one-fourth of the original AP mass was needed to start the process (44). It may be assumed that in the case of intradermal implantation and the resulting close association between AP and skin, the signal from the cambial layer of the AP could more easily reach the overlying skin. Therefore, compared with subcutaneous transplantation, in the case of an intradermal graft fewer AP cells were needed to produce the amount of inductive molecules needed at the receptor sites to cause transformation of the overlying skin and there was no need for a major expansion of the transplanted AP tissue prior to the induction of ectopic antler growth (44).

Interestingly, it has been observed that under special experimental conditions onset of antler growth from a pedicle can be a localized event (29). In these cases, only certain areas of the skin covering a pedicle undergo velvet transformation (Figure 5). It may be assumed that either only certain groups of AP cells produced the inductive molecules and that therefore only a limited area of the competent skin was affected by the signal or that only in places a tight enough contact between AP and overlying skin had been established that allowed the molecules to affect the skin. Further studies are needed to identify and characterize the molecules thought to be involved in the signaling process between AP and its overlying skin and especially to test the hypothesis of the existence of a feedback mechanism from the epidermis to the periosteum.

4. VELVET SHEDDING, ANTLER DEATH, AND ANTLER CASTING

Growing antlers are complex organs composed of mesenchymal and ordinary connective tissue, cartilage and bone and their enveloping membranes (perichondrium and periosteum), skin, blood vessels and nerve fibers. The growing antler is supplied with blood via several arteries that branch from the superficial temporal artery and are located in the innermost layer of the dermis overlying the periosteum (45). A rise in circulating testosterone of male deer prior to the rutting season causes formation of a dense antler cortex, intense mineralization of antler bone and the death and shedding of the velvet from the antlers, thereby exposing the bare bone of the functional antlers (the so-called hard antlers) that are used in intraspecific fighting

Figure 5. Localized onset of antler growth (asterisks) from the pedicle of a fallow buck (*Dama dama*). The pedicle had belatedly developed as a consequence of partial resection of the antlerogenic periosteum

Figure 6. Velvet shedding in a red deer (*Cervus elaphus*) stag, thereby exposing the bare bone of the antlers.

(Figure 6). In fallow bucks castrated prior to the onset of antler regeneration, the antlers that were produced after casting of the previous set stopped growing at around the normal time for the species, indicating that cessation of antler growth did not depend on a rise in circulating testosterone (46). It is therefore assumed that local mechanisms are involved in the control of the length of the antler growth phase. This may include the action of molecular clocks or counters in the cells of the mesenchymal growth zone.

Prior to being shed, the velvet undergoes necrotic changes that are due to a progressively diminished blood flow, which itself is attributed to the increasingly dense ossification of the antler cortex (34, 45). Pieces of velvet transplanted to other parts of the body do not undergo necrosis at the time when the antlers' velvet dies off and is shed, but remain viable for extended periods of time (36, 47). This has led Goss (20) to conclude that velvet death is clearly a case of "murder", not "suicide".

It is generally believed that the bony antlers die at or shortly after velvet shedding due to insufficient blood supply (ischemic necrosis) (20, 34, 48, 49). Wislocki (49) observed focal necrosis in antlers of white-tailed deer even shortly before the end of the antler growth period, and ascribed these necrotic changes to the then already markedly impaired circulation in the velvet antlers. From time to time, however, the view that hard antlers are dead bone is challenged by authors who claim that the antlers survive velvet shedding and that hard antlers must therefore be considered living, not dead structures. The most recent of these claims was by Rolf and Enderle (50), who reported the occurrence of living osteocytes, active osteoblasts and signs of ongoing bone formation in hard antlers of fallow deer. These authors proposed that a sufficient blood supply of the antler core to keep it alive is maintained almost until the time of antler casting by vessels connecting the pedicle with the antler (50). Contrary to this view, a recent study in red deer demonstrated that antlers cut about a month after velvet shedding do not lose weight after cutting and are as dry as the atmosphere allows, which led the authors to conclude that it is extremely unlikely that the hard antlers carried by the stags had a functioning blood supply sufficient to keep even the antler core wet (51). Moreover it was demonstrated that dry antler bone is mechanically better suited than wet bone for its function during fighting between stags, which means that possession of dry instead of wet antlers is actually advantageous for deer (51).

Antler casting has been viewed as a case of autotomy, differing from other examples of self-amputation in that a dead, not a living, structure is lost (20). Given the fact that the body does not tolerate a dead bone, detachment of the antlers is an inevitable event. The process of antler casting has been characterized as an "abacterial sequestration" by Gruber (52), i.e., as a form of self-amputation without signs of osteomyelitis, a process that has occasionally been observed in humans following ischemic necrosis of a limb (52).

Antler casting is preceded by intense osteoclastic activity in the distal pedicle, both in the interior of the pedicle and along its periphery (Figure 7) (34, 53). Bone resorption occurs proximal to the dead antler, not in the dead antler bone itself (34, 53). Normally, the antler plus a small portion of the distal pedicle breaks away from the remaining pedicle before all osseous connections have been resorbed, resulting in the rough appearance of the separation plane of the pedicle (Figure 7) and the basal surface of the cast antler (54). Since the loss of pedicle bone at each casting event is not fully compensated post-casting, pedicle height tends to decrease with age (16). In the red deer, the shortening and thickening of the pedicles with age is even used for age estimation (55).

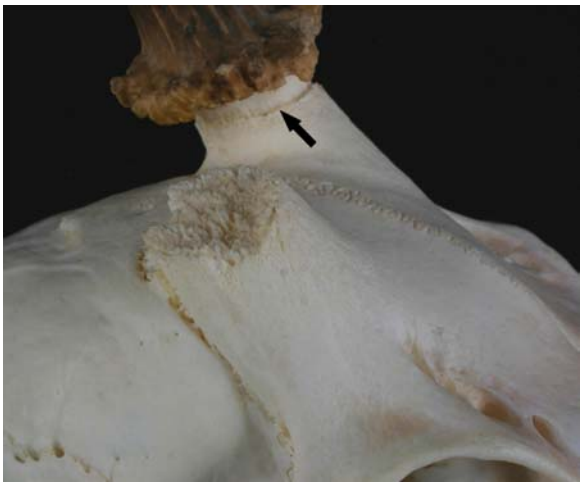

Figure 7. Cranium of a roe buck (*Capreolus capreolus*) that had already cast the right antler but still retained the left antler when it was killed. Note rough structure of the separation plane of the right pedicle and zone of bone resorption at the surface of the left pedicle (arrow).

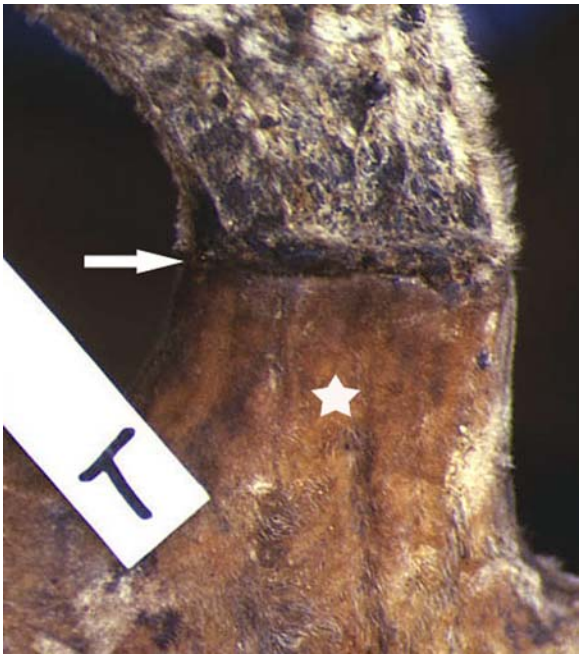

Figure 8. Demarcation line (arrow) between distally located necrotic portion and proximally located viable portion (asterisk) of the antler of a castrated fallow buck (*Dama dama*). The upper portion had become necrotic due to frostbite and was subsequently detached along the demarcation line.

Contrary to the situation in lizards, in which autotomy of the tail primarily occurs along predetermined sites of weakness, referred to as autotomy (or fracture) planes (8, 9), there exists no such predetermined plane in deer. Instead, casting always occurs proximal to the border between living and dead bone, regardless of where this border is situated. This is demonstrated for instance by the casting of parts of the antlers of castrated deer. Antlers of castrates retain their velvet and remain permanently alive, because due to the lack of testosterone they do not develop a dense cortex and are therefore able to maintain a reduced but sufficient blood circulation (46, 56, 57). However, when such "castrate antlers" are exposed to freezing temperatures for several days, a further reduction of the already diminished blood flow in the antlers will cause frostbite on and necrosis of their distal portions (46, 56). A study in fallow deer showed that the necrotic antler parts became demarcated from the proximally adjacent living portions and were detached in a way corresponding to the regular casting process (46) (Figure 8).

Several studies have demonstrated that antler casting is inhibited by high levels of circulating sex steroids and that premature casting of hard antlers can be induced by androgen withdrawal and/or androgen receptor blockade (46, 56, 58-60). In red deer stags that were prevented from casting their antlers at the normal time by treatment with exogenous testosterone or estradiol-17β, the border between dead and living bone tissue was reported to have gradually shifted proximally into the pedicle and then further into the skull, a process that was referred to as "die-back" (61). When the antlers were belatedly cast following the cessation of sex steroid application, a larger than normal portion of the pedicle was therefore detached together with the antler. This observation suggests that the deer's body is unable to keep the junction between dead and living bone stable (61). The die-back phenomenon has not received much attention in recent years, but clearly warrants further study.

It has been shown that the mechanical properties of dry antlers are superior to those of wet bone (51), and it is thus the possession of dry antlers that is an adaptive trait. This and the fact that the body does not normally tolerate dead structures and is apparently unable to maintain a stable position of the junction between living and dead bone tissue, is in our view a convincing explanation for the evolution of the regular replacement of the antlers of deer.

5. THE EPIMORPHIC REGENERATIVE RESPONSE – SIMILARITIES AND DIFFERENCES AMONG VERTEBRATES

5.1. Tissue origin of the regenerating antler

The nature of antler regeneration has long been a matter of controversy, regarding both the source of the cells responsible for antler regrowth and the classification of the regeneration process in comparison with other examples of appendage regeneration in vertebrates (15).

Based on histological studies, some authors believed that the cells forming the regenerating antler are derived from the pedicle dermis (49, 62). However, it was later demonstrated that antler regeneration is not inhibited by preventing the pedicle skin from participating in the regeneration process. In these cases, the formation of stunted "skinless antlers" was observed (63). That antler regeneration can occur without skin participation does,

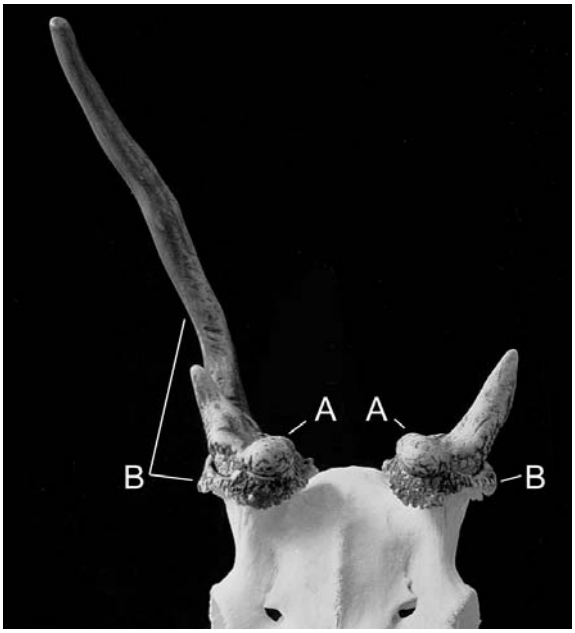

Figure 9. Bilateral double-head antler formation in a fallow buck (*Dama dama*). A – undetached primary antlers, B – regenerated antlers. From (15).

Figure 10. Malformed antler of a red deer (*Cervus elaphus*) stag caused by belated antler casting. The regenerating antler tissue had formed a rim (asterisks) around the distal pedicle and two upwards projecting outgrowths from this rim. The casting plane of the pedicle formed by belated detachment of the previous hard antler shows an uneven surface of somewhat lighter color than the newly formed antler bone that emanates from the pedicle below the casting plane.

however, not necessarily mean that the skin plays no role in the process. Thus, it has been proposed that a transient interaction between pedicle bone [according to our view more likely the pedicle periosteum] and pedicle skin is a necessary prerequisite for the regeneration process (63).

Within the last two decades, different studies have indicated that the actual source of the cells that build up the regenerating antler is the pedicle periosteum (PP). Evidence for this view originally came from the analysis of a special type of antler malformation known as double-head antler (Figure 9). A double-head antler is formed when the old antler is not cast and the regenerating antler grows below and around the undetached antler (54, 64). The hunting literature even contains descriptions of triple-head antlers, i.e., of three successive antler generations simultaneously originating from a pedicle (54). The phenomenon of double-head antler formation clearly illustrates that the popular belief that antler regeneration is triggered by the casting of the previous set of antlers is wrong. Rather, it has been suggested that it is the death of the antlers occurring some months prior to casting that triggers regeneration (54). Onset of the regeneration process and antler casting are then, however, both suppressed by high levels of circulating androgens, and only after androgen levels have dropped below specific thresholds, the two processes can start (54).

Morphological and histological studies furthermore indicated that the regenerated antler structure forming the second generation of a double-head antler develops as a periosteal exostosis from the distal pedicle (54, 64, 65). As can be seen in the case of "former" double-head antlers, in which the antler of the first generation has been belatedly cast, the antler of the second generation emanates from the pedicle well below the (belatedly formed) casting plane, which demonstrates that no cells originating from the marrow spaces of the pedicle stump participated in the regeneration process (54) (Figure 10).

Histological studies of normal antler regeneration later showed that early after antler casting, the distal PP is markedly thickened, which is indicative of a high proliferative activity in this area (66, 67). It was further observed that the growth zones for the main beam and the brow tine of the red deer antler form very early during the regeneration process by local thickening of the PP (67). The view that the PP is the tissue responsible for antler regeneration is also supported by experimental evidence. Thus, surgical removal of the PP either inhibited antler regeneration during the following antler growth season or delayed the process until after the PP had regenerated (68).

It has recently been concluded that PP is restricted developmentally to the process of antler regeneration and, contrary to AP, is not able to induce first antler growth (44). This conclusion was based on the finding that transplantation of a mass of PP that was two to three times the minimum mass of AP needed to induce ectopic antler growth, failed to cause the development of an ectopic antler. Compared with AP, which rapidly increased in mass in the ectopic location, the expansion of the transplanted PP tissue was much reduced. Histological examination suggested that this difference in tissue growth rate between AP and PP was due to a less efficient vascularization of the latter (44). There are, however, occasional observations of wild deer with extra antlers growing from the shaft of the pedicles (23, 29). This

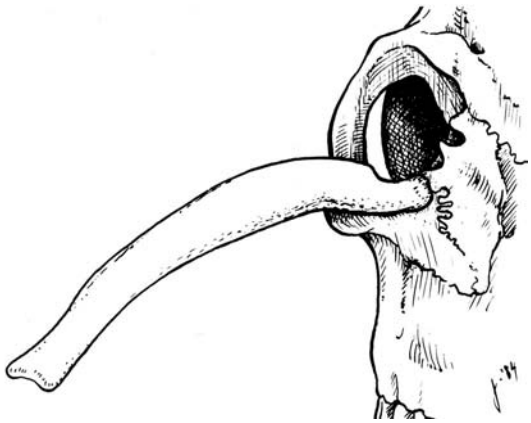

Figure 11. Ectopic antler that had grown from the zygomatic bone of a white-tailed buck (*Odocoileus virginianus*). Drawn after photo in Nellis (70). The buck's age was estimated at 6.5 years (70). The orthotopic antlers had five (right side) and four points (left side), respectively. Note that a pedicle structure is not discernable in the ectopic outgrowth and that, judging from the nature of its attachment, the ectopic antler has never undergone replacement.

suggests that under special circumstances yet to be defined, PP can initiate antler growth from an existing pedicle. Thus it might be better to characterize the PP as normally being unable to induce full pedicle formation and thereby, secondarily, also first antler growth.

Naturally occurring ectopic antlers have been reported from frontal as well as extra-frontal sites of the cranium, namely, the parietal, interparietal, nasal, and zygomatic bones (23, 69, 70) (Figure 11). This has led Wislocki (69) to conclude that all membrane bones of the top of the cervid skull are apparently endowed with the potential for antler growth, although the capacity for this is much stronger in the frontals than in the other bones. The fact that the "antler territory", i.e., the skull area potentially capable of producing an antler, extends beyond the pedicle anlage area, should, however, not be taken to indicate that the periosteum of this area is specifically committed to antler growth (23, 29). Thus, contrary to the grafting of AP, transplantation of skull periosteum from outside the presumptive pedicle site to other body regions did not cause the growth of ectopic antlers (26, 27). Evocation of antler growth outside the pedicle anlage area requires strong unphysiologic stimulation of the periosteum, such as severe trauma to the tissue (23, 29), and also in the case of naturally occurring extra antlers from the pedicle shaft such unphysiologic stimuli can be assumed to have triggered their formation. Often, the ectopic antlers found on the skulls of deer do not grow from a distinct pedicle, but directly from the skull (Figure 11), indicating that in the case of a severe trauma to the periosteum and simultaneous damage to the overlying tissues, antler growth can be initiated without the previous formation of a pedicle.

5.2. Dedifferentiation-based and stem cell-based epimorphic regeneration

The term "epimorphosis" was coined by Morgan (71) in order to characterize a regenerative process in which cell proliferation precedes the replacement of the lost part. Some later authors refined Morgan's (simple) definition of epimorphic regeneration to include the fact that a mass of dedifferentiated, homogeneously appearing cells, the regeneration blastema, accumulates at the site of damage underneath a wound epidermis (1, 3, 72). Antler regeneration can certainly be classified as an example of epimorphosis in the original sense defined by Morgan (71), which is also used by several modern regeneration biologists (73). There has, however, recently been a controversy about whether or not antler regeneration involves the formation of a blastema (14, 15, 17, 67). The answer to this question depends on the definition of the term blastema (15). If, for instance, we follow Brockes and Kumar (4) and use the term blastema in a very general sense for any accumulation of regenerative cells without reference to a specific origin of these cells or other special properties of this cell accumulation, antler regeneration can clearly be classified as a case of blastema-based regeneration.

There exist different possible sources for the cells forming the regeneration blastema (74). They could derive from the dedifferentiation of cells in the stump or from the proliferation of reserve (stem) cells, either present in the stump or circulating in the organism; however, also a mixed derivation of the blastema is possible. The classic example of epimorphosis involving dedifferentiation of cells in the amputation stump is limb regeneration in urodeles (2, 3). The extent of dedifferentiation in the urodele limb stump as well as the question of whether the blastema cells are able to redifferentiate into cells types different from those of their respective tissue of origin (transdifferentiaton) have long been matters of controversy (3). Some earlier authors believed that the blastema cells reverted to an embryonic state and became pluripotent (74). A recent investigation in the axolotl (*Ambystoma mexicanum*) applying tissue-specific labeling with an integrated green fluorescent protein (GFP), however, demonstrated that limb regeneration involves only limited dedifferentiation of the blastema cells (75). The study confirmed the known fact (1, 74) that the epidermis remains separate and does not contribute to the blastema. In line with earlier results (74, 76), the majority of the cells forming the blastema were shown to be derived from the dermis. The dermis-derived blastema cells contributed to dermis, tendon and cartilage, but did not enter the myogenic lineage. The vast majority of cartilage-derived blastema cells differentiated back to chondrocytes, while others contributed to tendons, perichondrium and perhaps dermis. GFP-labeled muscle cells entering the blastema redifferentiated only to muscle, and GFP-labeled Schwann cells only contributed to new nerve tracts (75). In summarizing their findings, Kragl and coworkers (75) concluded that the blastema formed during limb regeneration in the axolotl must be characterized as a heterogeneous assemblage of progenitor cells with

restricted developmental potential according to their respective tissue of origin.

There is evidence that urodele limb regeneration may also involve the activation of resident stem cells in the stump. Thus $Pax7^+$ satellite cells were observed in limb muscles and, following amputation, also in the regeneration blastema of the red-spotted newt (*Notophthalmus viridescens*) (77). In conclusion, the blastema formed during limb regeneration in urodeles can no longer be considered a homogeneous mass of pluripotent cells, but should be viewed as an accumulation of dedifferentiated cells with limited developmental potential and probably some admixture of stem cell progeny.

A previous study on the African clawed toad (*Xenopus laevis*), using GFP-labeling for cell lineage tracing, concluded that, in contrast to urodele limb regeneration, the use of the term blastema for the regeneration bud of the *Xenopus* tail is not appropriate (78). This conclusion was based on the finding that the spinal cord and the notochord of the tail regenerated exclusively from the same tissue types in the amputation stump, i.e., regeneration of these two structures occurred within self-contained compartments without exchange of cells with other regions and with no dedifferentiation or transdifferentiation. Of the analyzed axial structures in the tail, only the regenerated myofibers originated from cells located within a mass of undifferentiated cells (which could be termed a blastema) that presumably derived from dermis and fin mesenchyme as well as $Pax7^+$ muscle satellite cells. Lineage tracing suggested that muscle regeneration occurred by multiplication, differentiation and fusion of these satellite cells, not from pre-existing myofibers (78). Thus, also in the case of tail regeneration in *Xenopus* the cells forming the different tissues of the regenerate were clearly not pluripotent. A study of limb and tail regeneration in *Xenopus laevis* tadpoles and caudal fin regeneration in the zebrafish (*Danio rerio*) also found no indication for the participation of pluripotent cells in these processes (79). In both species, the cells forming the regenerates neither upregulated key transcription factors needed for induction of pluripotency in differentiated cells nor significantly upregulated transcription factors involved in the self-renewal of such induced pluripotent cells (79). Recently it was reported that in zebrafish fin regeneration, dedifferentiated osteoblasts contribute to the blastema, whose derivation is otherwise presently unclear. The cells downregulated expression of bone differentiation markers, expressed genes known from bone progenitors, proliferated in an FGF-dependent manner, and remained lineage restricted during the regenerative process in that the dedifferentiated cells only gave rise to new osteoblasts in the regenerate (80). Osteoblast dedifferentiation was thus not accompanied with attainment of multipotency by the cells (80), thus corroborating the view of the blastema as a heterogeneous assemblage of lineage restricted cells.

In the case of tail regeneration in lizards, formation of a blastema has been described, but the contribution of the different tissues in the stump to this structure has not yet been determined (9). However, also for this system there are findings suggesting that most cells in the blastema redifferentiate into the same type from which they were derived and that there is no or only limited transdifferentiation (9). Moreover there is some evidence from studies utilizing thymidine autoradiography and 5-BrdU immunodetection suggesting the presence of stem cells in the autotomy planes of the lizard tail (9).

Histological studies in different deer species revealed a lack of any obvious dedifferentiation processes in the pedicle stump after antler casting (34, 66, 67). The mesenchymal (blastemal) tissue that forms the proliferative zones of the antlers was shown to be derived from a specific local source, viz., the cambial layer of the periosteum of the distal pedicle (66, 67). In red deer, it has furthermore been demonstrated that the growth centers for the main beam and the brow tine form already very early during the regeneration process due to locally intensified proliferative activity within the periosteum (67). The pedicle periosteal cells themselves are descendants of the AP that is responsible for the formation of the pedicle and first antler (64).

The derivation of the antler regenerative tissue from a specific local source resembles the situation previously described for tail regeneration in *Xenopus laevis* (78). The periosteal origin of the antler regenerative tissue and the fact that the distal pedicle periosteum provides the cell material for regeneration annually throughout an animal's life led to the view that antler regeneration may be a stem cell-based epimorphic process (15-17, 67, 81, 82). It has been hypothesized that adult stem cells responsible for antler regeneration are located in a stem cell niche in the pedicle periosteum, where they are periodically activated to provide the pool of mesenchymal progenitor cells that build up the new antler (17, 81). To allow periodic antler regeneration to take place, the presumptive stem cell pool in the PP must be maintained by lifelong self-renewal (18).

In support of the above hypothesis it has been reported that cells from both AP and PP express different stem cell markers. Thus, Rolf and coworkers (81) located cells positive for the cell surface antigen Stro-1 (a marker of mesenchymal stem cells/progenitor cells) in the cambial layer of the PP of fallow deer. Li and coworkers (17) reported that *in vitro* cells from AP and PP express considerable levels of the cell surface antigen CD9 and also show gene expression for the transcription factors Oct4 and Nanog. Moreover these cells were reported to show marked telomerase and nucleostemin activity (17). It has further been demonstrated that *in vitro* the presumed stem cells from AP and PP can be stimulated to differentiate along the chondrogenic, osteogenic, and adipogenic lineages (17, 81). In addition, a lineage-tracing study using AP cell labeling with the *lacZ* gene at the time of pedicle initiation observed ß-galactosidase-positive cells in mesenchyme, precartilage, cartilage and bone of the growing cranial appendage (17). Thus there is evidence for multipotency of the periosteal cells within the mesenchymal lineage (17, 81). According to Li and coworkers (17), AP can even be induced to differentiate into multinucleated muscle precursors and into cells resembling neurons upon cultivation in appropriate

media. Details of this study are, however, not yet available, and further evidence is needed to substantiate this conclusion. Previously it was reported that PP cells of red deer also express neural crest markers (83), which is in line with the presumed neural crest-derivation of AP and PP.

That certain cells from AP and PP can be induced to differentiate along various lineages *in vitro* does, however, not mean that all of these differentiation pathways are also followed *in vivo*. Thus, for example, cells from AP and PP can be induced to differentiate into adipocytes, but fat cells are not normally present in antlers. This suggests that the developmental potential of the presumed AP and PP stem cells is greater than what is actually realized *in vivo* or, in classical embryological terminology, that the cells' prospective potency is greater than their prospective fate.

In the study by Rolf and coworkers (81), Stro-1^+ cells were also found in different locations within the pedicle skin and antler velvet of fallow deer. This suggests that stem cells could also play a role in the rapid growth of antler velvet, including the neogenesis of hair follicles, during antler regeneration.

In conclusion, there is some evidence to suggest that both AP and PP harbor a population of adult stem cells. These cells are capable of differentiating along different (mesenchymal) lineages *in vitro*, but may be largely restricted to the formation of chondro- and osteoprogenitors *in vivo*. Going a considerable step further, Li and coworkers (17) recently suggested that the cells responsible for antler generation and regeneration may actually be pluripotent adult stem cells. This might be the case; however, more evidence would be needed to substantiate such a view. There is thus a demand for further studies addressing the developmental potential of the AP and PP cells, the way they are maintained, and the factors controlling their periodic activation. The current concept of stem cell-based antler regeneration holds that the regenerative tissue derives completely from the PP. However, a minor contribution of circulating stem cells to the "antler blastema" that could be attracted to the casting wound could occur by chemotactic signals (84), can at present not be ruled out with certainty.

In adult lizards, amputation of the tail at its base, i.e., proximal to the (predetermined) autotomy planes, is not followed by tail regeneration (9). This regenerative failure was traditionally interpreted as being caused by the loss of the 'tail regeneration territory'. However, recently it has been suggested that the lack of regeneration could be due to the fact that the (presumed) stem cells located in the autotomy planes of the tail are removed by such basal amputations (9). Interestingly, removal of the distal part of the pedicle or of the complete pedicle along with its periosteum and skin cover does not inhibit antler regeneration (85, 86). Even when the pedicle along with the underlying outer table (lamina externa) and diploë of the frontal bone was chiseled out, leaving only the inner table (lamina interna) in place, wound healing was eventually followed by the regeneration of an antler (87). In these cases, the distal PP, which is thought to harbor the stem cells responsible for regeneration, was removed; however, this did not prevent antler regeneration. As was already discussed, it has been suggested that the periosteum of all dermal skull bones has the potential of producing antlers, but that activation of this potential in non-AP cells requires strong unphysiologic stimuli and is always associated with an extensive wound healing process (23). At present, the specific mechanisms allowing antler growth under such experimental conditions are, however, unclear.

5.3. Wound healing mode and regeneration

Appendage regeneration in vertebrates always starts with wound healing, and migration of epidermal cells over the wound surface (epithelialization) is the first step in this process (2, 3). There are characteristic differences in the mode of the wound healing process between regenerating and non-regenerating appendages, in that in the latter the wound heals by scarring while in the former the formation of scar tissue is (largely) avoided (88-90). Scar-free wound healing and scarring can occur simultaneously in the same organism, suggesting that local conditions play a decisive role in the regulation of the healing process (89).

Scar-free wound healing and regeneration as it occurs for example after limb amputation in urodeles involves close contact and intense signaling between the wound epidermis, which thickens to form the so-called apical epidermal cap (AEC), and the underlying mesenchymal tissue. This close epidermal-mesenchymal contact is necessary for establishing and maintaining the blastema and, thereby, for successful regeneration (2-4, 91). Thus, removal of the wound epidermis or the AEC inhibits limb regeneration, and also covering of the amputation surface with full-thickness mature skin causes regenerative failure, as it inhibits the formation of a wound epidermis (2-4, 91, 92). Similarly, destruction of the wound epidermis at the tip of the regenerating lizard tail or covering of the wound surface of the tail stump with full-thickness skin results in failure of tail regeneration (9).

The nature of the signaling between wound epidermis/AEC and underlying blastema is not yet fully understood; however, expression patterns of genes for fibroblast growth factors (FGFs) in regenerating amphibian limbs suggest an important role of FGFs for blastema survival and growth (2, 91). FGF-1 and FGF-2 are also very likely involved in the control of tail regeneration in lizards, the pattern of FGF distribution in the regenerating lizard tail being similar to that in regenerating tails and limbs of urodeles (9), and FGF-depended proliferation of dedifferentiated osteoblasts was observed in the regeneration blastema of zebrafish fins (80). It was recently further shown that insulin-like growth factor (IGF)-signaling between blastema and wound epidermis is a crucial step in zebrafish fin regeneration (93). There is evidence that FGFs and IGF-1 are also involved in the control of antler regeneration. Thus, FGF-2 increases the proliferation rate of cultured cells from the mesenchymal growth zone(s) of the regenerating antler (14) and a microarray study indicated that FGF-signaling is activated

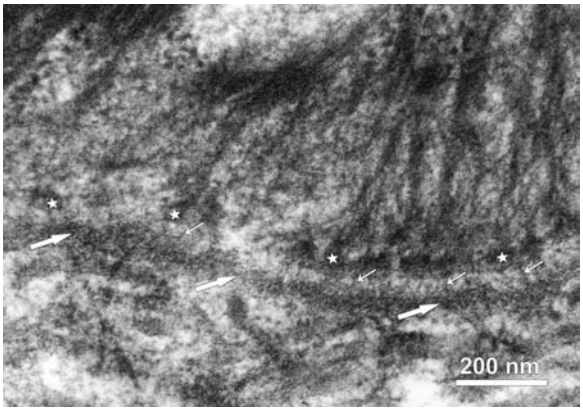

Figure 12. Transmission electron micrograph of the epidermal-dermal interface in the skin covering the peripheral portions of the pedicle stump of a fallow buck (*Dama dama*), two days after antler casting. Wound healing in the central part of the wound area had not yet been completed. A continuous, electron-dense layer, the *lamina densa* or basal lamina *sensu stricto* (large arrows) is overlain by an electron-lucent layer, the *lamina lucida*. Asterisks: hemidesmosomes at the undersurface of the basal keratinocytes, small arrows: anchoring filaments that traverse the lamina lucida and connect the hemidesmosomes with the lamina densa. From (15).

in the antler mesenchyme (94). Based on *in vivo* and *in vitro* studies it has further been suggested that IGF-1 plays a major role in the control of antler growth (95-97), although this has been disputed by other authors (98). However, none of these studies addressed the early stages of antler regeneration, and, thus, the possible role of growth-promoting factors during these stages is still unknown.

The most obvious difference between scar-free wound healing and the healing process leading to scar formation is the degree of inflammation involved. Scarring is always preceded by an intense inflammatory reaction, as is for instance the case in the (non-regenerating) limb stump of lizards (9). Epithelialization of the limb stump in lizards takes about twice as long as that of the tail stump, and the wound epidermis over the limb stump is rapidly turned into a typical epidermis (9, 99). Mesenchymal cells are reported to be present initially under the wound epidermis of both, the tail and the limb stumps of lizards. However, only in the tail stump the mesenchyme replaces the granulation tissue and develops into a blastema. Compared with the tail stump, the inflammatory reaction in the limb stump is more intense and longer-lasting, and the granulation tissue is rapidly transformed into a fibrotic scar (9). It has been demonstrated that cauterization of the tail stump in lizards causes an intense inflammatory response and cicatrization instead of regeneration. Cauterization strongly stimulates the migration of macrophages into the wound area. Production of cytokines, especially transforming growth factor ß1 (TGF-ß1) and platelet-derived growth factor (PDGF), by these macrophages then attracts numerous fibroblasts to the wound which subsequently produce scar tissue (9).

Formation of a new basal lamina between the wound epidermis/AEC and the underlying mesenchymal tissue occurs late during limb regeneration in urodeles, thereby permitting a prolonged close contact and signaling between the tissues, which are crucial for the formation and growth of the blastema (3, 100). Similarly, in the early regenerating lizard tail, the basal lamina, especially its lamina densa, has been shown to be discontinuous, at least in the more central parts, allowing interactions between epidermis and subepidermal tissue to take place. By contrast, a continuous basal lamina is present in the healing lizard limb (9, 99). It is thought that formation of a continuous basal lamina inhibits the communication between wound epidermis and underlying tissue that is required for successful regeneration. Electron microscopic and immunohistochemical studies have demonstrated that also during antler regeneration a continuous basal lamina is formed very early at the undersurface of the wound epidermis, the process starting in peripheral areas of the healing casting wound at a time when epithelialization of the more central wound areas is not yet completed (Figure 12) (15, 101). This suggests that already early during antler regeneration signaling between epidermal and subepidermal tissues is not or no longer required. This may be related to the fact that the mesenchymal tissue (blastema) responsible for antler regeneration is not derived from dedifferentiation and subsequent proliferation of cells in the pedicle stump but from the activation of precursor cells in the PP that are supposed to be stem cells and therefore do not need to undergo dedifferentiation prior to proliferation.

Typically, wound healing in adult mammals occurs by scar formation, in which the missing normal tissue is replaced by an extracellular matrix composed largely of collagen (types I and III) and fibronectin (89). This happens in all tissues and organs, but because the skin, being the barrier between an organism and its environment, is the most frequently injured organ of the body, it is also the one that has been most intensively studied in recent years. Especially the difference between the healing of cutaneous wounds in adult mammals and in mammalian embryos/early fetuses has received attention, because the latter are able to heal wounds without scarring and to regenerate a normal skin structure (89, 90).

The findings in the case of mammalian wound healing confirm the insights derived from studies in other vertebrate taxa, namely, that a decisive difference between scar-free healing and healing by scarring is the degree of inflammation involved in the process. Thus, skin wound healing in adult mammals typically involves a strong inflammatory response, while there is very little inflammation during the scar-free healing of skin wounds in mammalian embryos and fetuses (89, 90). It has been shown that expression profiles of several signaling molecules differ between skin wounds of fetal and adult mammals, indicating that it is the local control of the inflammatory response that permits scar-free healing. For example, production of the pro-inflammatory cytokine interleukin-6 (IL-6) in wounded fetal human skin is much

Figure 13. Casting wound of a red deer (*Cervus elaphus*) stag. Some bleeding has occurred after casting, but a blood clot is already being formed. Note rim of velvet skin around the wound.

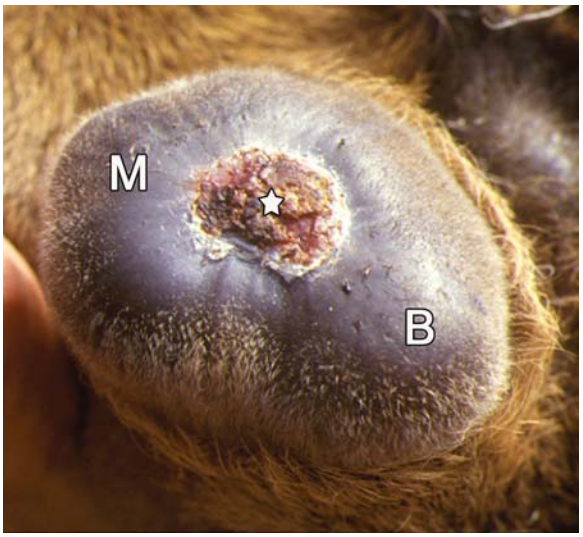

Figure 14. 'Bud' stage of antler regeneration in a fallow buck (*Dama dama*). Wound healing is almost complete, with a scab still being present in the typical location, namely the central depressed area of the antler bud between the growth centers for the brow tine (B) and the main beam (M). Note formation of new hairs in the velvet.

lower and occurs over a shorter period than in adult skin (102). Skin wounds in fetal knockout mice deficient in IL-10 (a potent anti-inflammatory cytokine) exhibit a marked inflammatory response and heal by scarring (103), while overexpression of IL-10 in adult skin wounds causes a decrease in the quantities of pro-inflammatory mediators, including IL-6, and a reduced inflammatory response (104). Regarding growth factors, fetal skin wounds show low levels of TGF-β1 and TGF-β2 and of PDGF, but high levels of TGF-β3. Application of exogenous TGF-β1 to fetal wounds induced scar formation, while experimental reduction of TGF-β1, TGF-β2 or PDGF levels in adult wounds or exogenous application of TGF-β3 to such wounds resulted in scar-free wound healing (89).

Given the facts that in adult mammals wounds heal by scarring and that (extensive) scarring is incompatible with regeneration, the prospects for appendage regeneration in mammals, humans in particular, appear bleak on first sight. However, there are some notable exceptions to the rule that wound healing in adult mammals always leads to the formation of a scar. Thus, following amputation of fingertips in humans and digit tips in mice, the resulting wounds heal with only minimal formation of scar tissue (12). Another example, in which a normal skin structure is reestablished, is antler regeneration (105). Antler regeneration differs from other examples of epimorphic appendage regeneration in vertebrates in that the creation of a bone wound (at antler casting) occurs several months later than that of a skin wound (at velvet shedding). This means that for a considerable period of time the hard antlers penetrate the body's skin cover, which may constitute a potential route for the entry of infectious agents. It has, however, been demonstrated that during the hard antler phase a very tight interface exists between the pedicle skin and the underlying pedicle bone/periosteum, thereby providing an efficient sealing (106).

Following antler casting, there is some bleeding from the pedicle's casting plane due to the rupture of blood vessels. This bleeding, however, soon stops and the casting plane is covered with a blood clot (Figure 13) that upon drying forms a scab (Figure 14). Already shortly before antler casting, the distal pedicle skin becomes tumescent and attains the appearance of velvet, while the skin covering more proximal portions of the pedicle remains unchanged. Antler regeneration starts from the distal pedicle even if the old antler is not yet detached (Figure 15), a process that will lead to the formation of a double-head antler if the previous antler remains fixed to the pedicle (Figure 9) (54, 65). Following antler casting, the epidermis migrates centripetally over the wound area, moving underneath the scab. Histological studies showed that the wound area of the pedicle stump is, however, not just overlain by the newly formed epidermis, but also by ingrowth from the dermis (53, 66, 67), i.e., the casting wound is covered with a layer of (velvet) skin.

During wound healing in adult mammals, a capillary-rich granulation tissue is initially formed in the wound area and later transformed into scar tissue (88, 107). A tissue resembling granulation tissue has also been reported to overlie the bone of the pedicle stump (48, 53, 66, 67). Goss and coworkers (53) suggested that this tissue later contributes to the developing antler bud, which would be in contrast to the transformation into fibrous scar tissue observed in other cases of wound healing. We, therefore consider it more likely that the granulation tissue initially present in the casting wound is later largely replaced by mesenchymal tissue. Depending on local conditions in the wound area, some of this granulation tissue could, however, be transformed into fibrous tissue, causing the formation of a small dermal scar. At present, the views whether such a scar is normally formed in the course of antler regeneration remain divided. Some authors regard antler regeneration as a process involving scar-free wound

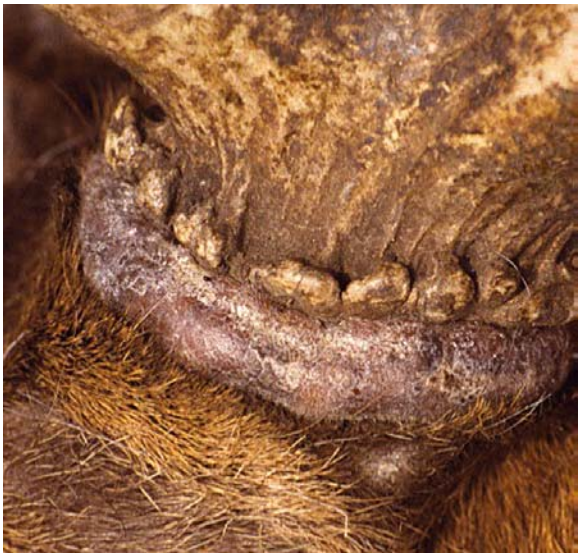

Figure 15. Pronounced rim of velvet-covered new antler tissue underneath an undetached antler of a fallow buck (*Dama dama*). Shortly after the photo was taken, the antler was cast and antler regeneration proceeded in a normal fashion. If the antler had not been cast, a double-head antler would have developed.

Figure 16. More advanced stage of antler regeneration in a fallow buck (*Dama dama*). Note that on the left side a small dermal scar is present in the furcation area between brow tine and main beam (arrow), while this is not the case in the corresponding location of the right antler.

healing (88, 105), which would be in line with the established view that scar-free wound healing is the first step in successful appendage regeneration. Other authors, however, report that antler regeneration involves the formation of a dermal scar that is mostly of a small size (17, 45, 67, 108). According to our experience, in some velvet antlers a small dermal scar can indeed be found in the area where the scab has persisted longest. In fallow deer and red deer, this is the bifurcation area between the main beam and the brow tine. The fibrous scar tissue is probably derived from the granulation tissue reported to occur in the casting wound. Interestingly, occasionally scar formation is observed only unilaterally (Figure 16), corroborating the statement by Ferguson and O'Kane (89) that scarring and scar-free healing can occur within the same animal and the same tissue. It may be speculated that rather small differences in the intensity of local inflammation and the amount of granulation tissue formed over the pedicle stump decide about the presence or absence of a scar.

Skin wound healing in mammals frequently involves pronounced wound contraction, which is brought about by myofibroblasts (109). Due to this contraction, the wound area to be healed over and the size of the resulting scar are reduced. In the case of antler regeneration, wound contraction does not occur and, in line with this, myofibroblasts have not been observed during healing of the casting wound (110). The lack of wound contraction in antler regeneration can be linked to the fact that the pedicle skin is tightly attached to the underlying structures (106, 108). That antler regeneration involves no or only minor scarring, despite the large size of the casting wound, testifies to the effectiveness of the control of the inflammatory reaction in the wound area. Studying expression profiles of pro- and anti-inflammatory cytokines in healing casting wounds therefore appears as a promising area for future research. The prediction would be that the expression profile seen in casting wounds is similar to that observed during wound healing in mammalian embryos and early fetuses and distinctly different from that occurring in the case of wound healing with scar formation.

Suturing of full-thickness skin over the wound area inhibits post-amputational digit tip regeneration in mammals (2, 10). A corresponding result has also been reported for antlers (47). In this experiment, the distal half of the pedicle was surgically removed and the skin that had been peeled off the pedicle sutured over the pedicle stump. This caused a complete lack of antler growth (in one case) or led to the growth of small abortive antlers growing from the corner of the pedicles where the skin had not healed completely (in the remaining four cases). The outcome of the experiment was interpreted as indicating that a healing skin wound is a necessary prerequisite for regeneration (47). This would be in line with other examples of appendage regeneration, where dependence of the process on the presence of a wound epidermis/AEC was demonstrated. However, it was later shown that antler regeneration from pedicle stumps can occur when the skin is prevented from participating in the process, leading to the formation of (stunted) "skinless" antlers (63). Moreover, when interpreting the experiment of Goss (47) it must be considered that by amputating the distal pedicle most or all of the PP cells that according to our current view are responsible for antler regrowth were removed.

If the antler regeneration process, like other cases of appendage regeneration depends on the interaction between epidermis and underlying tissue, it may be hypothesized that any crucial interaction takes place already before casting of the old antlers. This would be in accordance with the observation that already prior to antler casting the skin of the distal pedicle attains a velvet character. Basically, the above reasoning is in line with the view expressed by Li and coworkers (63) that an interaction between pedicle bone (in our view, more likely the PP) and overlying skin is necessary for antler regeneration, but that this required interaction is only of a transient nature.

5.4. The role of nerves in regeneration

It is known for long that amphibian limb regeneration depends on a quantitatively sufficient supply of nerves, i.e., a certain amount rather than a special type of innervation is required for successful regeneration (1, 3, 111). More recently, our understanding of how nerves control limb regeneration in urodeles has been much improved by the identification of a mitogen (nAG) that binds to a surface protein (Prod1) of blastemal cells which itself is a critical determinant of the cells proximodistal identity (112). Expression of nAG is very low in the intact limb, but highly upregulated in the Schwann cells that surround regenerating nerve fibers. Later, nAG expression occurs in glandular cells of the wound epidermis. *In vitro*, recombinant nAG increased cell cycle entry of blastemal cells. However, as has been discussed before, this mechanism is regarded to be salamander-specific and can thus not serve as a general model for the influence of nerves in appendage regeneration of vertebrates (7). Exceptions to the nerve dependence of amphibian limb regeneration are the so-called aneurogenic limbs, i.e., limbs that developed in the absence of nerves. Such limbs can regenerate despite the lack of a nerve supply (1, 3).

Regenerating antlers are richly supplied by sensory fibers that regenerate from the nerves present in the pedicle (20). The innervation of the growing antler is by parasympathetic fibers that are derived predominantly from branches of the trigeminal nerve (113, 114). In red deer, in addition parasympathetic fibers from a branch of the facial nerve were sometimes found to innervate the medial aspect of the pedicle and antler (114). The sympathetic supply of the pedicle is derived from the second cervical nerve, while the antler normally lacks a sympathetic innervation, except for (long-lived) antlers of castrates (114). There is evidence that nerve growth factor (NGF) promotes the outgrowth of the nerve fibers of the regenerating antler (115, 116) and it has been suggested that NGF produced by smooth muscles of arteries and arterioles provides a guidance cue defining the track followed by the outgrowing nerve fibers (115). However, also other as yet unidentified molecules appear to be involved in promoting nerve fiber growth (116).

Sectioning of the sensory nerve fibers supplying the antlerogenic region of young red deer stags did prevent neither pedicle development nor the growth of primary antlers. These antlers were cleaned of velvet, cast, and regenerated in a normal fashion, but were smaller than controls (117). Also unilateral denervation of antlers (operations performed before the onset of regrowth) did not prevent antler regeneration. Compared with the control antlers from the un-operated side, the denervated antlers were stunted and deformed. This was attributed to the repeated trauma to the insensitive antlers that had occurred during the growth period, not to the loss of a specific growth stimulus exercised by the nerves (115). Also in this case, velvet shedding and antler casting were unaffected by the denervation. These findings were essentially confirmed in a later study comparing the regrowth of amputated velvet antlers with and without innervations (118). The fact that the denervated antlers were smaller and of somewhat different shape than the controls was interpreted as indicative of a general stimulating effect of nerves on antler growth by the authors (118). It has been suggested that the nerve-independence of antler regeneration is linked to the fact that, contrary to limbs and tails, antlers are amotile structures (16). Moreover, it may be speculated that the independence of the formation and growth of the "antler blastema" from nerve-derived factors could be related to its derivation from the PP rather than from dedifferentiated pedicle cells.

Although antler growth per se does not require a functional nerve supply, it has been hypothesized that the nervous system exerts an influence on antler morphogenesis (119). Thus, it has been reported that certain growth deviations caused by trauma to the growing antlers of a (non-anesthetized) deer in one year tend to re-appear during subsequent antler cycles. The phenomenon has been referred to as "trophic memory" and was interpreted as being suggestive of the existence of specific "antler growth centers" in the brain that are involved in the control of antler growth (119). Thus far, this hypothesis has not been unanimously accepted and clearly needs further study. However, in recent years the focus of research has been on the local control of antler growth and morphogenesis (16).

6. CONCLUSIONS AND PERSPECTIVES

Antler regeneration is the most impressive example of appendage regeneration in mammals. As has been discussed above, the process of antler regrowth differs in several aspects from the regeneration of urodele limbs. On the other hand, there are certain similarities between antler regeneration and other examples of epimorphic regeneration in vertebrates, such as tail regeneration in *Xenopus.* On the basis of our current understanding, antler regeneration may be characterized as a dedifferentiation-independent and nerve-independent epimorphic process that is thought to involve the periodic activation of PP stem cells.

A more thorough understanding of the process of antler regeneration as well as other examples of appendage regeneration in vertebrates, be it limb regeneration in urodeles, tail regeneration in lizards or *Xenopus*, fin regeneration in zebrafish, or digit tip regeneration in mice, may provide valuable insights for regenerative medicine in achieving its ultimate goal of limb regeneration in humans. The view that if such regeneration could be triggered it would use mechanisms known to operate in urodele limb regeneration, such as formation of a blastema from dedifferentiated cells (10), is certainly a feasible starting point for further research. However, given our recent insights into the heterogeneity of regeneration phenomena among vertebrates and the possibility of taxon-specific mechanisms operating in regeneration, in our opinion, a broad comparative analysis of a wide range of regeneration phenomena, including antler regeneration, offers the best chance of eventually finding a way to induce the regrowth of severed human limbs.

An impressive aspect of antler regeneration is the healing of the large casting wound with no or only minor scarring. To understand how this is achieved may provide

clues as to how control inflammation and thereby reduce scarring during wound healing in adult humans. This alone would justify future studies on antler regeneration, despite the many problems associated with the use of deer as experimental animals.

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Send correspondence to: Uwe Kierdorf, Department of Biology, University of Hildesheim, Marienburger Platz 22, 31141 Hildesheim, Germany, Tel: 49-5121-883-917, Fax: 49-5121-883-916, E-mail: uwe.kierdorf@uni-hildesheim.de

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