REVIEW

Deer antlers: a zoological curiosity or the key to understanding organ regeneration in mammals?

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Abstract

Many organisms are able to regenerate lost or damaged body parts that are structural and functional replicates of the original. Eventually these become fully integrated into pre-existing tissues. However, with the exception of deer, mammals have lost this ability. Each spring deer shed antlers that were used for fighting and display during the previous mating season. Their loss is triggered by a fall in circulating testosterone levels, a hormonal change that is linked to an increase in day length. A complex 'blastema-like' structure or 'antler-bud' then forms; however, unlike the regenerative process in the newt, most evidence (albeit indirect) suggests that this does not involve reversal of the differentiated state but is stem cell based. The subsequent re-growth of antlers during the spring and summer months is spectacular and represents one of the fastest rates of organogenesis in the animal kingdom. Longitudinal growth involves endochondral ossification in the tip of each antler branch and bone growth around the antler shaft is by intramembranous ossification. As androgen concentrations rise in late summer, longitudinal growth stops, the skin (velvet) covering the antler is lost and antlers are 'polished' in preparation for the mating season. Although the timing of the antler growth cycle is clearly closely linked to circulating testosterone, oestrogen may be a key cellular regulator, as it is in the skeleton of other male mammals. We still know very little about the molecular machinery required for antler regeneration, although there is evidence that developmental signalling pathways with pleiotropic functions are important and that novel 'antler-specific' molecules may not exist. Identifying these pathways and factors, deciphering their interactions and how they are regulated by environmental cues could have an important impact on human health if this knowledge is applied to the engineering of new human tissues and organs.

Key words antler; bone; deer; regeneration.

Introduction

Deer antlers are one of the animal kingdom's most dramatic examples of male prowess and thus since ancient times have been held in great regard by humans. However, antlers also provide a model for studying two unique processes: the development of a complete appendage that is delayed until puberty and mammalian organ regeneration. No other mammal can naturally regenerate any lost organ, let alone anything as large and complex as an antler, e.g. the antlers of a 200-kg adult red deer may weigh as much 30 kg but take only 3 months to grow. By contrast, animals that have retained the capacity to regenerate are found in most other phyla and a variety of these are studied by regeneration biologists. These include planaria, hydra, urodele amphibians, *Xenopus* and zebrafish (Brockes, 1997; Fujisawa, 2003; Nye et al. 2003; Poss et al. 2003; Sanchez Alvarado, 2003; Slack et al. 2004). In fact, it has been proposed that only by studying a variety of examples of natural regeneration can we develop our understanding of why some animals regenerate and others do not (Brockes, 2004). However, despite their obvious convenience as experimental

models, these are not mammals and although some mouse strains have been shown to have an increased capacity for repair (Heber-Katz et al. 2004), they are unable to regenerate whole organs. This is why the mechanisms that underlie antler regeneration should continue to be investigated, notwithstanding the limitations of deer as an animal model. This argument made most persuasively by Richard Goss, the regeneration biologist who pioneered antler research in the late twentieth century (Goss, 1995). Antler research can help us understand why regenerative ability has been lost in mammals and take us further towards a 'holy grail' of modern human medicine: the ability to regenerate organs that have been removed through trauma or excision.

The diverse anatomy of antlers

Deer are hoofed, ruminant mammals in the Cervidae family (order Artiodactyl) and are among the most graceful and attractive of animals. This family consists of 17 genera and about 53 species. Deer are native to all parts of the world except Antarctica, Australia, central and southern Africa, Madagascar and New Zealand, and have adapted to virtually every land habitat, from dry deserts to woodlands, prairies, marshes and Arctic regions. Deer are the only animals that grow antlers, which are composed of skin, nerves, blood vessels, fibrous tissue, cartilage and bone, and thus should not be confused with horns, which are a keratinized tissue that grow from their base under the control of underlying mesenchymal cells.

Except for the reindeer (*Rangifer tarandus*), antlers develop only in male deer and in most species this occurs in the spring of the animal's second year of life. As with the developing limb, antlers have three axes: a proximal–distal axis, an anterior–posterior axis and a dorsal–ventral axis (see Fig. 2A; Li & Suttie, 2001). Antlers come in all shapes and sizes from small unbranched antlers of only a few centimetres in length, as seen on the pudu (*Pudu puda*), the smallest of the deer species, to elaborately branched racks or palmate headpieces of impressive proportions in species such as the moose and elk. The moose (*Alces* spp.) is the largest deer (an adult stag's body weight can reach 800 kg) and these animals possess the largest antlers. Elk (*Cervus canadenesis*) (Fig. 1A) also carry impressive sets of antlers that can extend to 1.5 m in length. The antlers of more diminutive adult fallow bucks (*Dama dama*)


Fig. 1 The diverse anatomy of antlers. (A) Moose (*Alces* spp.). (B) Fallow deer (*Dama dama*). (C) Red deer (*Cervus elapus*). B and C are reproduced courtesy of Dr John Fletcher, Reediehill Deer Farm, Auchtermuchty, Fife, UK.

are also very spectacular (Fig. 1B) curving majestically outward and upward. The species studied by us is the red deer (*Cervus elapus*), the antlers of which have a more 'classic' shape, depicted in artworks throughout history (Fig. 1C).

Development of primary antlers

Antlers develop from pedicles, permanent bony protuberances on the frontal bone. As the fawn approaches puberty (at approximately 5–7 months old in the red deer), a collection of determined periosteal cells located in the distal parts of the cristae externae of the frontal bones are activated by rising androgen levels in the blood. This specialized periosteum (Fig. 2B) was originally described as 'antlerogenic periosteum' (AP) by Richard Goss (Goss & Powel, 1985). The AP contains specific binding sites for testosterone (Li & Suttie, 1998) although testosterone does not appear to have direct effects on cultured AP cells whereas IGF-I stimulates their proliferation (Li et al. 1999; Sadighi et al. 2001). Trabecular bone is then formed beneath the periosteum and a pedicle develops (Sempere et al. 1983; Suttie et al. 1984, 1988, 1991). Normally the antlerogenic periosteum of female deer remains quiescent because the hormonal requirements necessary for pedicle formation are not present. However, female deer can be induced to form pedicles if injected with androgens (Kierdorf et al. 1995).

Li & Suttie (1994) have undertaken detailed histological analyses of pedicle formation and have identified four ossification stages. The first is the intramembranous ossification stage (1 in Fig. 2C), and this is where the initial antlerogenic cells start to proliferate and differentiate into osteoblasts. These osteoblasts form trabecular bone in cellular periosteum. The second stage is transitional ossification, which takes place when the pedicle has reached 5–10 mm in height (2 in Fig. 2C). Osseocartilaginous tissue is formed by antlerogenic cells
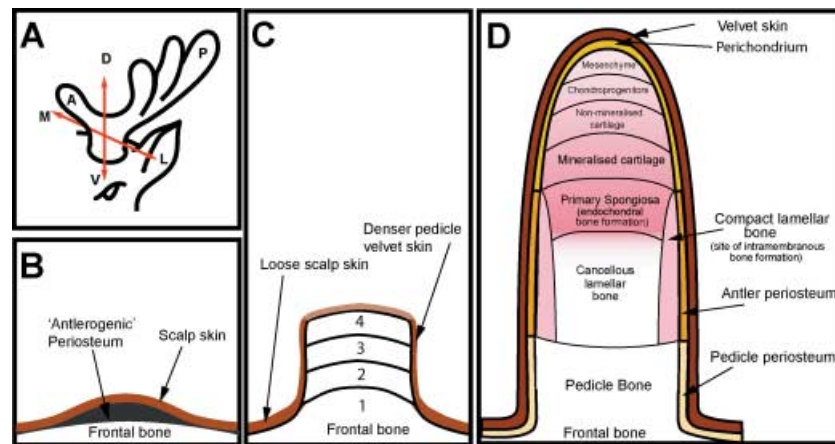
Fig. 2 (A) Schematic diagram to show the three axes of the antler development: A-P, anterior-posterior axis; D-V, dorso-ventral axis; M-L, medio-lateral axis. (B,C) Schematic diagrams illustrating three stages of antler development. (B) Antlerogenic periosteum is present in the embryo and after birth as a localized thickening of the periosteum of the frontal bone. (C) Development of the pedicle occurs through four stages: 1, intramembranous ossification; 2, transitional ossification; 3, pedicle endochondral ossification; 4, antler endochondral ossification and velvet skin formation. (D) Longitudial section through a growing primary antler illustrating the main anatomical regions. Endochondral bone growth occurs at the distal tip while bone forms by intramembranous ossification around the antler shaft.

at the apical surface, which have undergone a change in differentiation pathway to form chondrocytes. Pedicle endochondral ossification is the third stage, when chondrogenesis takes place in the pedicle alone (3 in Fig. 2C). The fourth and final stage is termed antler endochondral ossification. Here the antlerogenic cells maintain their chondrogenic differentiation pathway until the first antler has been fully formed (4 in Fig. 2C). The onset of antler formation is coincident with the appearance of 'shiny' velvet skin covering the distal end of the pedicle. These un-branched antlers (described as 'spikers' in the deer industry) then elongate as a result of an endochondral process in the distal tip (Fig. 2D; Chapman, 1975). Because the cellular anatomy of endochondral ossification is very similar in the primary and regenerating antlers it will be described in a later section.

Growth of the first antler continues until the autumn rutting season approaches when there is another increase in circulating testosterone. This endocrine change is associated with a cessation in longitudinal growth, the antler bone becomes fully mineralized and the overlying velvet is shed exposing bare bone. This leaves the single un-branched antler attached to the pedicle until it is cast the following spring. This inhibition of longitudinal bone growth in response to an increase in sex steroids is similar to that which occurs at puberty in

humans (Riggs et al. 2002). In fact it is now known that in the male skeleton many of the effects of testosterone are indirect, following its conversion to oestrogen by the enzyme aromatase (Riggs et al. 2002). That oestrogen may be an important regulator of antler growth was first demonstrated by Goss (1968), who found that injection of oestrogen inhibited growth of regenerating antlers and promoted premature ossification and shedding of the velvet skin. Bubenik et al. (1975b) subsequently showed that administration of an oestrogen antagonist had an inhibitory effect on antler bone formation. More recently, Bubenick et al. (2005) have provided evidence that oestrogen is actually synthesized by antler tissues in deer stags. It has also been demonstrated that the seasonal antler cycle in female reindeer is regulated by oestradiol, in this case synthesized by the ovary (Lincoln & Tyler, 1999).

Transplantation experiments demonstrated that the tissue from which the first antler develops is antlerogenic periosteum (Fig. 2B). Hartwig (1967) showed that moving this tissue to another region of the frontal bone resulted in an antler forming at the new location but not at the original site. It was subsequently shown that growth of antler structures can be induced by transplantation of this periosteum to a heterotopic location (Fig. 3) overlying the metacarpal bone. Furthermore, these 'mini-antlers' are influenced by the same

Fig. 3 Growth of an 'antler' from transplanted antlerogenic periosteum. Antlerogenic periosteum was transplanted from the frontal bone and grafted onto the metacarpal bone of a young fallow deer. Photograph courtesy of Uwe and Horst Kierdorf, Justus Liebig University of Giessen, Germany.

hormonal cues as antlers on the skull, e.g. they shed their velvet skin at the appropriate time of year (Hartwig & Schrudde, 1974; Goss & Powel, 1985). Structures resembling antlers, or pedicle-antlers, have also been generated when AP was transplanted over the calvariae of nude mice (Li et al. 2001). Studies using a cell lineage tracer described by Li and Suttie in their review of antlerogenic periosteum provide further evidence that pedicles and antlers are derived from this tissue (Li & Suttie, 2001). Taken together, these studies led Li & Suttie (2001) to suggest that, like some embryonic tissues, AP has full self-differentiating capacity. Interestingly, the cellular layer of AP is very rich in glycogen (Li & Suttie, 1998), a characteristic of fetal osteoblasts.

Unfortunately, there is a paucity of information on the local molecular mechanisms involved in antler and pedicle development. *In vitro* studies have shown that insulin-like growth factor I (IGF-I) may be an important systemic regulator of pedicle formation as it stimulates proliferation of antlerogeneic cells from all four ossification stages. Retinoic acid (RA) is also likely to play a role because application of RA to the developing pedicle increased the growth rate of the first antler and this was suggested to occur via an increase in the proliferation of periosteal cells (Kierdorf & Bartos, 1999). Recently, Barling and colleagues (Barling et al. 2004a,b) have undertaken a series of studies aimed at identifying growth factors and their receptors in the skin and underlying bone of primary antlers. Growth factors identified include epidermal growth factor (EGF) and

the EGF receptor, fibroblast growth factor 2 (FGF-2) and the FGF receptors, FGFR1, FGFR2 and FGFR3, bone morphogenetics proteins (BMPs) 2, 4 and 14 and the BMP receptors BMPR1B and ACTRII. They hypothesize that these growth factors signal between the osseocartilagenous and skin compartments of the primary antler (Barling et al. 2004b). The spatial and temporal differences in the localization of these growth factors showed that their distribution in skin of the primary antler resembles that described in adult skin of other species, whereas their distribution in bone and cartilage resembles that in the fetal skeleton.

Identifying the pathways that regulate the prenatal development of antler primordiae should also be a priority for future study. In the embryo, localized thickenings of the periosteum of the frontal bone can be observed at sites of future pedicle/antler development (Fig. 2B; Lincoln, 1973; Li & Suttie, 2001). These primordial pedicles enlarge between 55 and 150 days of gestation but regress at later stages. This tissue specification is likely to involve interactions between mesenchyme and the overlying epithelial cell layer, which Li & Suttie (2001) described as resembling the apical ectodermal ridge in the developing limb. It is noteworthy that development of the mammary gland, which, like antlerogenic periosteum, is regulated by sex steroids, involves epithelial–mesenchymal interactions (Foley et al. 2001). One of the molecules that mediates epithelial–mesenchymal interactions in the developing mammary gland is parathyroid hormone-related peptide (PTHrP). Although the role of PTHrP in the development of antlerogenic periosteum has not been investigated, there is evidence that it plays an important role in both the developing and the regenerating antler. Barling et al. (2004a) have recently identified the PTHrP and the PTH/PTHrP receptor in both velvet skin and underlying mesenchymal tissues of red deer primary antlers. We have also found that PTHrP is widely expressed in tissues of regenerating red deer antlers and that its synthesis is regulated by TGF-β (Faucheux et al. 2004). Clearly a major challenge lies in characterizing the pathways and factors that: (i) define where and how AP forms during fetal life, (ii) enable AP to 'survive' until the systemic environment is appropriate for antler development and (iii) activate expansion of progenitor cells in the perisoteum. The potential role of periosteum in regenerating antlers will be discussed in more detail in the next section.

Systemic regulation of the repeated cycles of antler regeneration

Because the primary function of antlers is to enable stags to protect and retain harems of females (does), their growth is linked to the annual breeding cycle and its associated fluctuations in sex hormone concentrations. In temperate species such as the red deer, it is a change in day length that regulates reproductive activity, although there are endogenous rhythms of antler growth (West & Nordan, 1976). Studies in orchidectomized (castrated) stags and in stags administered exogenous hormones have demonstrated that sex steroids are most important at the start and at the end of the antler growth cycle. Incredibly, Aristotle was the first scientist to describe the effects of castration on antler growth!

Antlers are normally cast in spring when testosterone levels are low. Castration of stags in late winter/early spring will lead to premature casting of antlers (Jaczewski et al. 1976), whereas administration of exogenous sex steroids at this time will prevent casting and regeneration (Fletcher, 1978). The rapid growth of antlers occurs during the early summer when there is a plentiful food supply and stags live sedentary lives while 'testosterone depleted' (Goss, 1983). Because antler regeneration takes place at a time when deer reproductive organs are inactive it was proposed many years ago that there must also be a non-gonadal factor involved and this was named 'antler growth stimulus' (AGS) (Wislocki, 1943). IGF-I synthesized in the liver is the most likely candidate as IGF-I concentrations are high during the period of rapid antler growth (Suttie et al. 1985), there are IGF receptors in the antler's growing tip (Elliott et al. 1992, 1993) and IGFs promote proliferation of antler cells (Price et al. 1994; Sadighi et al. 1994). Changes in concentrations of other hormones are associated with the antler growth cycle, including $1,25(OH)_2D_3$ (Van der Eems et al. 1988; Sempere et al. 1989), thyroid hormones (Shi & Barrell, 1994), cortisol (Bubenik et al. 1975a; Suttie et al. 1995) and prolactin (Sempere et al. 1983; Suttie et al. 1984), although their function remains poorly understood.

Antler growth places an enormous demand for mineral on the skeleton (sufficient mineral is needed to enable a 30-kg bone to grow in ~3 months), and to meet this demand cyclical reversible osteoporosis occurs at other skeletal sites (Bubenik, 1983). This bone loss is greatest in non-weight-bearing bones such as
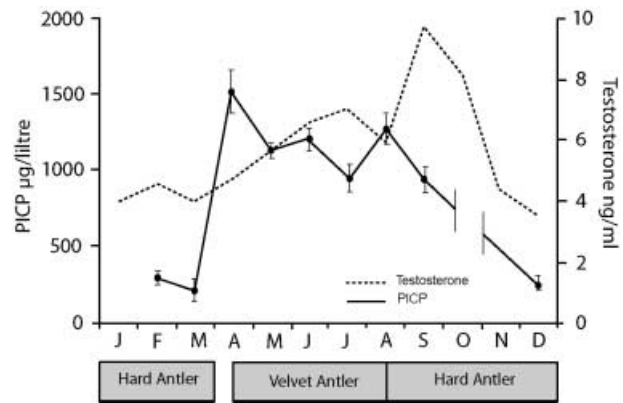
Fig. 4 Changes in circulating concentrations of testosterone and the carboxy-terminal pro-peptide of type I collagen (PICP) during the antler growth cycle in red deer stags. Serum samples were collected at post-mortem from deer stags killed for venison at different times of year. PICP was measured by radioimmunoassay (Orion, Diagnostica, Finland) and the values shown are the mean ± SEM. This illustrates that antler regeneration is associated with significant changes in bone turnover. The testosterone graph is adapted from Muir et al. (1988).

the rib. Measuring biochemical markers of bone cell activity in the circulation has shown how bone turnover increases dramatically at the time of rapid antler growth. Figure 4 shows the results of a study in which we measured changes in circulating levels of the carboxyterminal pro-peptide of type I collagen, a marker of osteoblast activity (Eriksen et al. 1993), at different times of year. This showed that levels were ten times higher during the period of antler regeneration. Baksi & Newbury (1988) have also shown changes in serum osteocalcin and hydroxyproline associated with antler growth.

Atlhough low levels of sex steroids are 'permissive' for regeneration, high levels appear to act as a 'brake', as discussed previously in the context of the developing antlers (Goss, 1968). Therefore, as the breeding season approaches and testosterone levels increase, endochondral growth stops, the antlers become fully calcified, and the velvet skin covering then thins, becomes 'dry' and is then shed (Fig. 5A) to expose the antler as a solid bone that remains firmly attached to the pedicle. The seasonal surge in testosterone also causes a behavioural change in the deer. Even before shedding their velvet, bucks begin to establish positions in the social hierarchy (Fig. 5B). Testosterone levels peak in late autumn when sexual activity is intense (Lincoln, 1971) and then decline again as spring approaches.

Fig. 5 The consequences of the rise in concentrations of circulating testosterone in late summer. (A) Shedding of velvet skin. (B) A pair of boxing stags. (Courtesy of Dr John Fletcher, Reediehill Deer Farm, Auchtermuchty, Fife, UK).

In red deer, castration during the period of antler growth will delay velvet shedding and the antlers do not fully mineralize (Fig. 6B). In some more phylogenetically evolved deer species the growth of castrated antlers is quite aberrant, e.g. fallow deer develop peculiar bony protuberances that Goss (1983) classified as 'antleromas' (Fig. 6C). Recently, Kierdorf et al. (2004) described in detail the structure of the antler of castrated fallow deer and presented evidence to suggest that these protuberances (Fig. 6C) are in fact benign tumour growths with comparable histological changes to osteomas. Roe deer also develop large benign tumours on castrated antlers in which intradermal bone has

been observed. The link between regeneration and cancer was highlighted in a review by Brockes (1998), and led us to suggest previously (Price & Allen, 2004) that castrated antlers develop tumour-like structures because a mechanism has evolved in deer whereby sex steroids (acting directly or indirectly) normally limit cell cycle progression in antler progenitor cells. It is the remarkable capacity for growth and self-renewal of antler progenitor cells that underpins the capacity of antlers to regenerate, but this has a potential downside: an increased risk of transformation. By contrast, lower organisms such as the newt have evolved a system whereby their cells can have an extended lifespan (and thus sustain regeneration) yet remain remarkably resistant to cancer (Tsonis, 1983). Humans and other mammals may have lost the ability to regenerate because the evolutionary benefit is outweighed by the increased risk of developing cancer.

Antler regeneration: casting of the old set of antlers

Casting of the stag's primary antlers marks the onset of an annual regenerative cycle that will continue throughout its life. In a 2-year-old animal the regenerated antlers are relatively unimpressive, but as the stag ages his antlers also become larger. Before the antlers are cast the skin covering the distal pedicle assumes the shiny appearance of antler velvet and the region
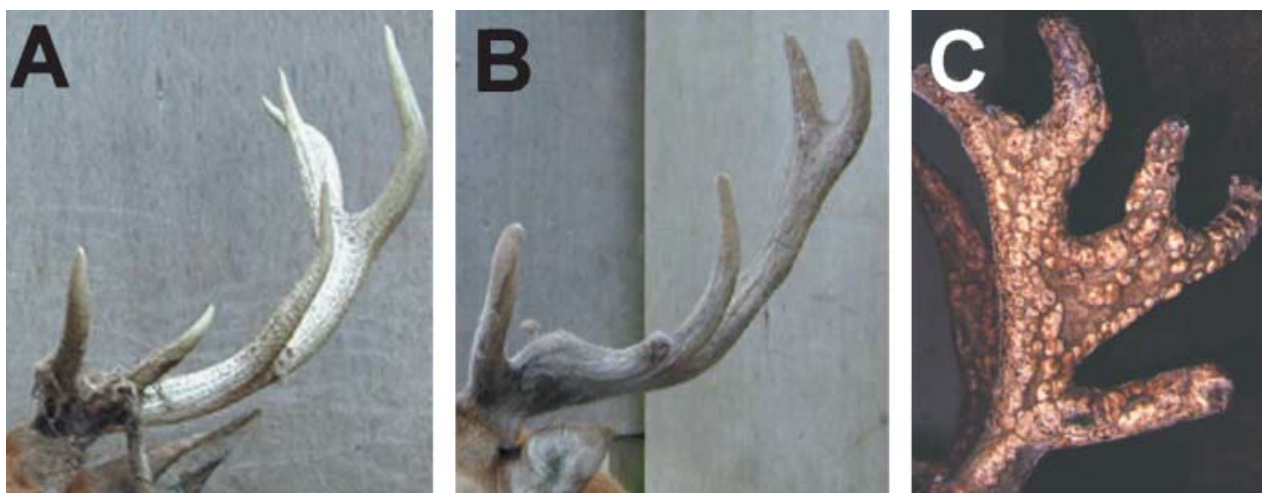
Fig. 6 The effects of castration on antler growth. (A) Antler of an intact red deer stag in early autumn. The velvet skin has been shed following the rise in circulating testosterone levels. (B) Antler of a red deer stag that was orchidectomized during the first month of antler growth; this results in retention of the velvet skin. (C) The antler of a castrated fallow deer. Numerous bony protuberances ('antleromas') can be observed over the antler surface. Photograph courtesy of Uwe and Hans Kierdorf, Justus Liebig University of Giessen, Germany.
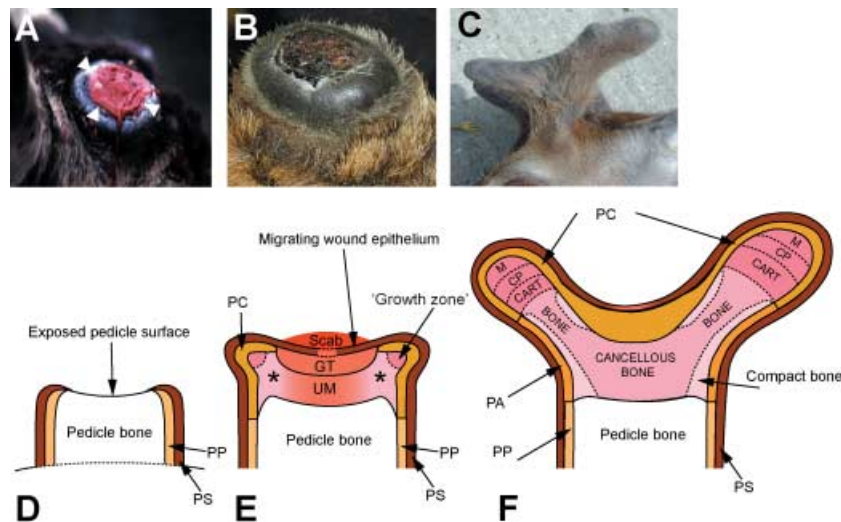
Fig. 7 Different stages of antler regeneration. (A) The pedicle immediately after the old antler has been cast. Note the 'ring' of regenerating tissue around the edge (arrowheads). (Courtesy of Dr John Fletcher, Reediehill Deer Farm, Auchtermuchty, Fife, UK). (B) The early antler bud ~4 days after antler casting. A scab has now formed. Asterisk indicates the position of a future branch. (C) Antlers at ~30 days of growth showing the anterior (brow) and posterior (main beam) tines (arrows). (D–F) Schematic diagrams of sections through the regenerating antler. PP, periosteum of pedicle; PS, pedicle skin; GT, 'granulation' tissue; PC, perichondrium; UM, undifferentiated mesenchymal tissue; PC, perichondrium; CP, chondroprogenitors; CART, cartilage; PA, periosteum of the antler. (D) Day zero. Casting leaves an exposed pedicle bone surface upon which a scab forms (as in B). (E) Antler bud at about days 9–10. The migrating wound epithelium has almost completely covered the pedicle surface. The underlying tissue has features of granulation tissue but also contains undifferentiated mesenchymal cells, so we describe this as 'undifferentiated mesenchyme'. Growth centres have been established at the sites where branches will develop and chondrogeneis is evident beneath them (marked by an asterix). (F) Day 30. Longitudinal growth takes place in the distal tip of each branch. D is adapted from Li et al. (2005).

becomes slightly 'swollen' (Fig. 7A). Antlers are then normally cast within a day or so of each other and this event is effected by osteoclasts resorbing bone in the distal pedicle (Goss et al. 1992). This resorption appears to be very tightly regulated because, unlike teeth, antlers do not start to 'wobble' in the days/hours before they are shed. Casting then leaves an exposed concave pedicle surface that rapidly fills with blood, although within hours a large scab forms to cover it (Fig. 7A). Kierdorf et al. (1993) have described the histological changes in this region and found that after casting osteoclasts continue to resorb bone in the distal pedicle, which creates a smooth surface. This is followed by a phase of bone formation that restores the portion of the pedicle that was lost with the cast antler.

In most species of deer loss of the antler coincides with regeneration of its replacement. However, in reindeer and moose, antlers are cast in late autumn and yet the wound does not heal over the pedicle stump and re-growth does not take place until environmental conditions are appropriate several months later (this delay may have evolved to reduce the energy demands on the animal as a consequence of carrying heavy antlers during harsh winters). Goss also demonstrated experimentally that if the distal end of the pedicle is amputated in winter (a 'non-permissive' time of year) wound healing and regeneration was delayed until spring (Goss, 1972). These observations imply that antler regeneration is not triggered by the tissue repair mechanisms induced by casting, whereas components of the injury response pathway are known to be critical for inducing regeneration in the newt (Brockes et al. 2001). The phenomenon of double-headed antlers also demonstrates that injury is not an absolute requirement for antler regeneration. In this situation, new antlers develop from the base of the previous antlers that are retained on the pedicle (Kierdorf et al. 1994). That is not to say that there is a relationship between antler regeneration and wound repair; by definition the process of epimorphic regeneration requires wound healing to take place (Goss, 1972). For example, Goss (1972) showed that when skin was sutured over the stump following antler removal regeneration could not take place.

The cue for the initiation of deer antler regeneration is therefore most likely to be a systemic factor(s) whose synthesis is regulated by changes in the hypothalamic–

pituitary axis. Regeneration could be initiated either when concentrations of a 'permissive' factor reach a threshold or when concentrations of a 'repressor' fall below a certain threshold. Testosterone is a strong candidate for being the 'repressor' as casting of antlers is known to be associated with a decrease in circulating concentrations (Suttie et al. 1995; Bubenik et al. 1997). One of the consequences of a decline in sex steroid concentrations (and or the number of receptors) could be induction of bone resorption at the antler–pedicle bone interface since sex steroids are known to inhibit osteoclast function in other species (Shevde et al. 2000). However, the role of sex steroids is likely to be more complex and may also involve regulation of stem cell populations in the pedicle tissues (this will be discussed in more detail in a later section).

Antler regeneration: formation of the antler bud

The anatomy of the early stages of antler regeneration has been described in a number of recent publications is illustrated here in Fig. 7 (Kierdoff et al. 2003; Li et al. 2004, 2005; Price, 2005). Immediately after the old antlers have been cast a raised 'swollen' ring of skin surrounds the distal end of the pedicle (Fig. 7A). This has a 'shiny' surface, characteristic of antler velvet skin. Within hours, the epidermis, known as 'wound epithelium', starts to migrate across the exposed surface of

the pedicle bone and covers a mass of cells within loosely packed extracellular matrix tissue (Fig. 8A). These cells have not been well characterized; for example, it is not known whether they express similar markers to cells in the newt blastema. Li et al. (2004) describe the tissue in the centre of the pedicle as granulation tissue. However, we have shown that as early as 4 days after casting a significant proportion of cells stain positive for PTHrP (Faucheux et al. 2004), which leads us to assume that these are of mesenchymal origin.

By days 9–10, epithelialization is complete and a number of zones can be distinguished histologically at anterior and posterior sites on the pedicle that mark the position where future antler branches will develop (Figs 7E and 8). Below the wound epithelium and perichondrium is a zone of proliferating mesenchymal cells, and more proximally chondrogenesis and bone formation takes place (Fig. 8C,D).

The structure which forms on the pedicle after antler casting has traditionally been called a 'blastema' (Fig. 7B) because blastema formation is a fundamental step in the process of epimorphic regeneration. Epimorphic regeneration is defined as 'de novo development of appendages distal to the level of amputation' (Goss, 1980), and is distinct from the processes of cellular regeneration turnover or tissue repair. However, the antler 'blastema' (Fig. 7B,E) is not a morphologically uniform structure like the blastema that forms following amputation of appendages in newts and other
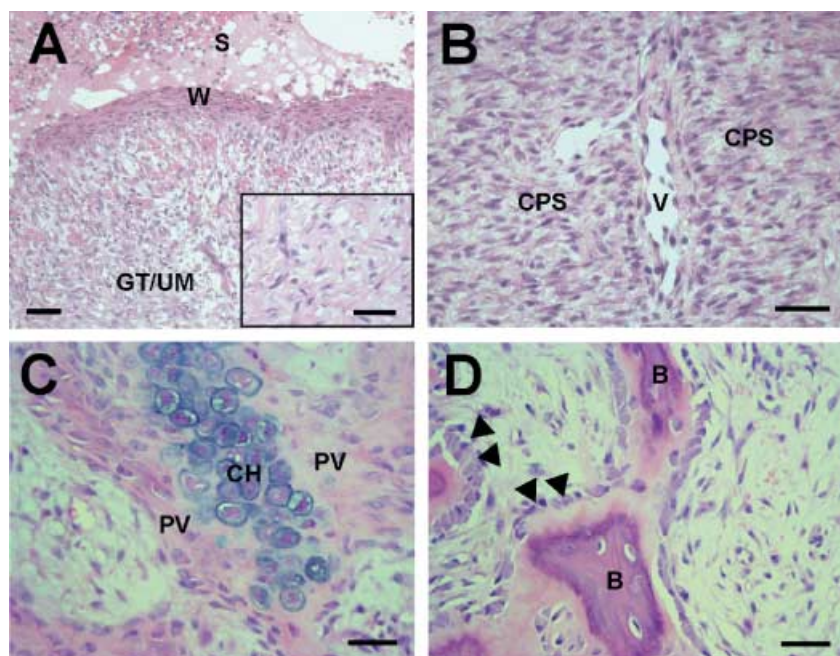
Fig. 8 Histology of the early regenerating antler. Haematoxylin and eosin (H&E)-stained undecalcified paraffin sections. (A) The migratory wound epithelium (W) overlying undifferentiated mesenchyme (UM) and granulation tissue (GT) in the centre of the antler bud at day 4. S, scab. Inset: a higher magnification view of mesenchymal tissue. (B) By day 9 there is a distinct zone of chondroprogenitors (CPs) and longitudinally aligned vascular channels (v). (C) Cartilage formation in the 9-day antler. Chondrocytes (CH) are surrounded by cartilage matrix and pervascular mesencymal tissue (PV). (D) Recent bone (B) formation. Osteoblasts are marked with arrowheads.

lower vertebrates (Brockes, 1997). This, together with evidence that antler regeneration may not involve reversal of the differentiated state, has recently led Li et al. (2004, 2005) to conclude that antler regeneration is not blastema-based. These authors define a blastema as being 'formed from the de-differentiation of all cell lineages on the immediate amputation plane' (Li et al. 2005). However, we would argue that the key issue is whether the undifferentiated cells in a blastema can regenerate a lost part, not whether they are formed by de-differentiation. For example, the regenerating tail of *Xenopus* involves formation of a structure that is generally described as a blastema, although there is no evidence that it forms by de-differentiation (Slack et al. 2004).

Within a few days after casting, distinct 'growth zones' can be identified in the early antler at sites where branches will eventually develop (Fig. 7E) (Price et al. 1994). These can be identified by the presence of proliferating cell nuclear antigen (PCNA)-positive cells (Price, 2005) that also synthesize PTHrP, which we have previously proposed is a marker of antler progenitor cells (Faucheux et al. 2004). We suggest that although the whole structure that forms on the pedicle should not be called a blastema, the individual growth zones do have some of the features that define a blastema, i.e. they consist of 'a mass of undifferentiated cells that will develop into an organ or tissue that is present at a site of regeneration'. Therefore, until cells in the different regions of the early regenerating antler 'blastema' have been better characterized, we suggest that it is somewhat premature to conclude that antler regeneration is not blastema-based.

Because the early stages of antler regeneration have not been studied in any detail at a cellular level, we know little about the signalling pathways that are involved. As discussed above, PTHrP is localized in mesenchymal cells but it is also present in regenerating wound epithelium, suggesting that it may have multiple roles (Faucheux et al. 2004). In the same study we also identified TGF-β in the early antler and found that it up-regulates PTHrP synthesis by cultured blastema cells. There are several lines of evidence to suggest that retinoic acid (RA) is also likely to play a role during the early stages of regeneration: the RA-synthesizing enzyme RALDH2 is synthesized in the early antler and *in situ* hybridization studies have also shown that RARα, RABβ and RXRβ receptors are also expressed, although they are not each present in every cell type. For example, RARβ is specifically expressed in cells at

sites of cartilage/bone formation in more proximal regions (Price & Allen, 2004). This demonstrates that the early antler bud is not a uniform structure and that, like PTHrP, RA may have multiple functions.

Another question in antler biology pertains to the source of cells which give rise to the regenerating antler Wislocki and Goss were of the view that migrating cells from the dermis of the pedicle were the source (Wislocki, 1943; Goss, 1972, 1984, 1995). However, a number of workers in this field currently take the view that regenerating antlers are derived mainly from a population of progenitor cells in the periosteum of the pedicle (Kierdorf & Kierdorf, 1992, 2000, 2001; Li et al. 2005). This tissue is derived from antlerogenic periosteum, which has convincingly been shown to be the origin of the pedicle and primary antlers (Hartwig, 1974; Goss & Powel, 1985; Kierdorf & Kierdorf, 2001). Li et al. (2005) concluded from their histological studies that antler regeneration is stem cell based with the periosteum being the source of these cells. However, before a cell can be defined as a true 'stem' cell it must be shown to have a capacity for self-renewal and to be capable of differentiation into specialized lineages. To date there is no evidence that cells from the pedicle of an adult deer have the capacity for self-renewal or can differentiate into anything other than chondrocytes and osteoblasts (Li et al. 1995). A number of approaches could be taken to address this issue: (1) the identification of stem cell markers in tissues of the pedicle, (2) the demonstration that cells cultured from the periosteum of the pedicle of regenerating antlers are pluripotential *in vitro* and in animal models *in vivo*, and, (3) the demonstration that periosteum cells stably transfected with a genetic marker [e.g. green fluorescent protein (GFP)] differentiate into different antler cell types in regenerating antlers *in vivo*.

Antler regeneration: cartilage and bone growth

In the first month after casting, antlers grow relatively slowly; however, during the next 2 months longitudinal growth is very rapid and this rate of bone formation represents the fastest described in the mammalian kingdom (Goss, 1983). The longitudinal growth of the antler that occurs in the distal end of each branch (shown schematically in Fig. 7F and histologically in Fig. 9) was originally described as a process of modified endochondral ossification by Banks & Newbry (1983). These authors classified the antler tip as consisting of
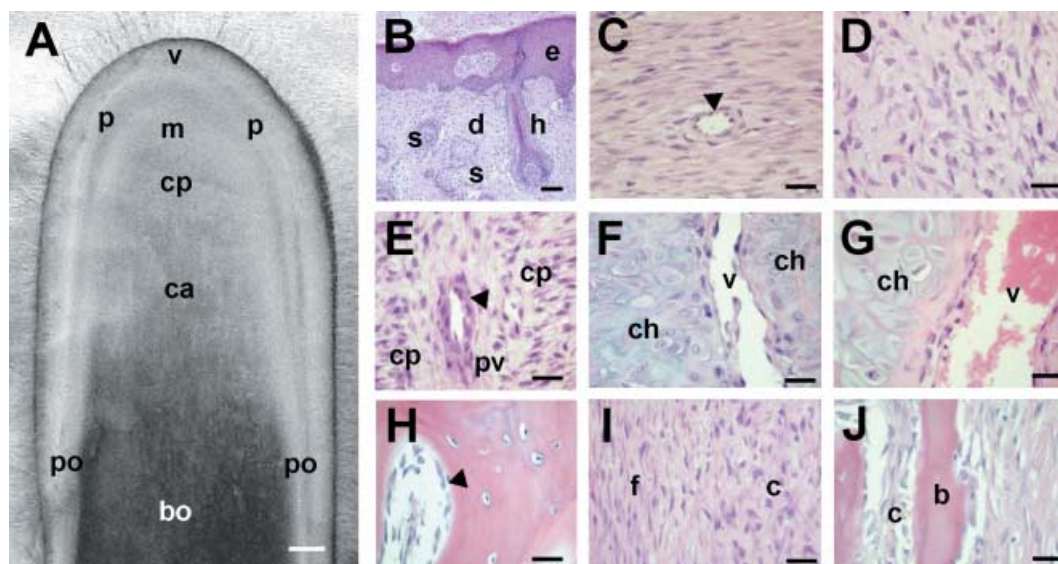
Fig. 9 Histology of the regenerating antler during rapid longitudinal growth. (A) Longitudinal tissue section of antler tip to show macroscopic appearance of regions: v, velvet skin; p, perichondrium; m, mesenchyme; cp, chondroprogenitor region; c, cartilage; bo, bone; po, periosteum. Scale bar = 0.5 cm. (B–J) H&E-stained undecalcified paraffin sections of the tissue regions shown in A. (B) Velvet skin. e, epidermis; d, dermis; h, hair follicle; s, sebaceous gland. (C) Fibrous perichondrium. A blood vessel is marked by an arrowhead. (D) Mesenchymal 'growth zone'. (E) Chondroprogenitor (cp) region. As in the early antler bud, cells start to become aligned in 'columns.' However, the vascular spaces are relatively small (arrowhead). (F) Non-mineralized cartilage. Recently differentiated chondrocytes (ch) are arranged in trabeculae separated by larger vascular channels (v). (G) Mineralized cartilage region. Chondrocytes and the vascular channels (v) increase in size in this region. (H) Spongy bone in the mid shaft of the antler that has formed by endochondral ossification. Osteoblasts are marked with an arrowhead. (I) Fibrous (f) and cellular (c) layers of the antler periosteum. (J) Intramembranous bone formation (b) takes place beneath the cellular periosteum (c). Scale bar (B–J), 100 μm.

four zones, namely the zones of proliferation, maturation, hypertrophy and calcification, representing the spectrum of bone development.

The mesencymal growth zone

Below the dermis of the velvet skin (Fig. 9B) is the perichondrium (Fig. 9C), which is continuous proximally with the periosteum that surrounds the shaft of the antler and is the site of intramembranous bone formation. There is an outer fibrous perichondrium where type I collagen mRNA and protein are highly expressed (Price & Faucheux, 2001) and an inner, more cellular zone (Fig. 9D). This region of the antler has been variously described by different authors (our group is also guilty of this), and the descriptors include 'reserve mesenchyme', 'hyperplastic perichondrium', 'cellular periosteum' and 'mesenchyme'. For consistency we now describe this as 'mesenchyme' or 'mesenchymal growth zone' because cells in this region, like cells in this region of the primary antler, are actively dividing (Matich et al. 2003; Faucheux et al. 2004). In culture these cells

proliferate rapidly as monolayers and synthesize type I but not type II collagen (our unpublished observations), and this reflects their *in vivo* phenotype (Price et al. 1996). However, unlike mesenchymal cells from the developing limb, they cannot be cultured as micromasses; they spread out to form monolayers and chondrogenesis is not initiated. Compared with cells in more proximal regions of the antler, cells in this region express only low levels of alkaline phosphatase and this reflects their undifferentiated state (Price et al. 1994; Price, 2005). However, although these cells will differentiate into chondrocytes in more proximal regions, they do express markers of the early osteoblast lineage (our unpublished observations) and in the presence of dexamethasone alkaline phosphatase expression will increase (Faucheux et al. 2001). This indicates that these cells are at least bi-potential, although we have preliminary evidence that they can also differentiate into adipocyte-like cells under the appropriate culture conditions.

In the mesenchymal growth zone there will be a tightly co-ordinated balance between cell growth, cell

survival and differentiation into chondroprogenitors, with some signalling pathways inducing proliferation and others inducing differentiation. In recent years our group and others have made some progress in identifying the local factors that may play a role. Several years ago it was first shown that their proliferation *in vitro* is stimulated by IGF-I and IGF-II and IGF receptors are present in this region of the antler tip *in vivo* (Price et al. 1994; Sadighi et al. 1994). Furthermore, IGF-I and IGF-II were identified in a screen of antler extracts undertaken some years ago (Mundy et al. 2001). This is consistent with the hypothesis that IGF-I is likely to be the 'antler growth stimulus'. We have found that FGF-2 also stimulates proliferation of mesenchymal cells from regenerating antlers (Price, 2005), although we have not yet tried immunolocalizing it in regenerating antlers. Members of the FGF family and their receptors have also been identified in the primary antler. A proteome analysis of red deer antler has been recently undertaken but surprisingly neither FGFs, IGFs, IGF binding proteins nor IGF receptors were identified (Park et al. 2004).

The BMP and TGF-β signalling pathways appear to induce a more differentiated state in mesenchymal cells, as our preliminary studies have shown that BMP-2 and TGFβ-1 inhibit the proliferation of mesenchymal cells whereas they induce ALP activity (Price, 2005). BMP-2 and BMP-4 have both been cloned from antlers (Feng et al. 1995, 1997) and Barling and collagues have identified BMP-2, BMP-4 and BMP-14 and their receptors in the primary antler (Barling, 2004b). We have recently immunolocalized TGF-β in regenerating antlers and it appears to act upstream of PTHrP, which, together with the PTH/PTHrP receptor, are synthesized by the majority of mesenchymal cells (Faucheux et al. 2004). Interestingly, we have found that PTHrP has no effect on the proliferation of mesenchymal cells (Faucheux & Price, 1999) and may maintain the undifferentiated state, although the functional significance of its abundance in this region requires further study. We have previously presented several lines of evidence that RA also plays a role in controlling mesenchymal cell growth and differentiation: first, the RA-synthesising enzyme RALDH2 can be immunolocalized in mesenchymal cells, and second, retinol, all-*trans*-RA and 9-*cis*-RA were identified in mesenchyme by HLPC and *in vitro* all-*trans*-RA dose dependently increased ALP activity in cultures of mesenchymal cells (Allen et al. 2002). However, the effects of RA in this region are likely to be very

complex, as reflected in the distinct patterns of expression of the RA receptors RARα, RXRβ, RXRβ and RXRγ.

Antler cartilage

With the naked eye it is possible to identify where chondrogenesis starts in a longitudinal antler section; it is where the tissue becomes slightly darker in colour because of an increase in size of the vascular channels (Fig. 9A). It is also possible to distinguish that there is a distal region of non-mineralized cartilage whereas more proximally deposition of mineral takes place (Price et al. 1996). The vascularization of antler cartilage is the most striking difference between its anatomy and that of other hyaline cartilages. This abundant blood supply is required to meet the high metabolic demands imposed by rapid tissue regeneration. That the antler provides a valuable model for the study of angiogenesis was proposed recently by Clark et al. (2004), who have identified VEGF and the VEGF receptor in regenerating antler tissues.

Histologically, the boundary between mesenchyme and the zone of chondroprogenitors is not distinct in longitudinal sections, but is a region where cells start to become arranged into columns between which there are vascular spaces (Fig. 9F). Previously we have described this as a 'zone of transition' because cells at this site are at different stages of differentiation along the chondrocyte lineage (Price et al. 1996). Type IIA collagen mRNA identifies chondroprogenitors in the developing skeleton (Sandell et al. 1994) and this isoform is expressed in the distal antler cartilage whereas type IIB collagen mRNA and protein are expressed throughout the cartilage (Price et al. 1996). Type IIA collagen is not expressed in growth plate cartilage (Sandell et al. 1994), another reason why it is difficult to compare directly the process of endochondral ossification in a long bone with that in antlers. Neither is type I collagen found in the growth plate, whereas it is expressed by antler cells in the 'transition zone'. Yet another difference between the matrix of antler and mammalian growth plate cartilage is the distribution of type X collagen; in the growth plate it is expressed only in proximal hypertrophic chondrocytes (Sandell et al. 1994) whereas in the antler type X collagen can be localized in the majority of chondrocytes.

Non-mineralized cartilage (Fig. 9F) provides us with an abundant source of cells for *in vitro* studies, and culturing them as a micromass helps to maintain the

chondrocyte phenotype (Allen et al. 2002; Price, 2005). This provides a useful model for *in vitro* studies of chondrogenesis; however, as will be described below, we also use the model to study antler osteoclasts. To date we have focused on molecules that we have identified in antler cartilage *in vivo*. PTHrP can be immuno-localized in chondroprogenitors but not in fully differentiated chondrocytes (Faucheux et al. 2004), and in micromass cultures it inhibits differentiation, consistent with its role during limb development (Faucheux, 1999). Indian hedgehog (IHH) and TGFβ-1 are also expressed in antler chondroprogenitors, indicating that these molecules may act with PTHrP in a feedback loop to control differentiation. RA also controls chondrogenesis; both all-*trans*-RA and 9-*cis*-RA are present in antler cartilage, and *in vitro* RA inhibits proteoglycan synthesis by chondrocytes, an effect that requires RAR signalling (Faucheux, 1999; Allen et al. 2002). However, RXRβ may mediate the effects of RA *in vivo* because it is expressed throughout cartilage trabeculae.

Cartilage and bone resorption

The growth of antlers, like that of other rapidly developing bones, requires extensive remodelling of cartilage and bone and thus is dependent on the local formation of osteoclasts from circulating mononuclear progenitors. In addition to columns of chondrocytes and vascular spaces, antler cartilage contains perivascular tissue (Fig. 9F,G) and this has been identified as a site where cells of the osteoclast lineage differentiate (Faucheux, 1999; Faucheux et al. 2002; Szuwart et al. 2002). When digests are obtained from antler cartilage for *in vitro* studies this includes a fraction of osteoclast progenitors because cells with the phenotypic characteristics of mammalian osteoclasts will differentiate in micromass cultures of chondrocytes under the appropriate conditions (Faucheux, 1999). This system is now used routinely in our laboratory to study the mechanisms that regulate antler osteoclast function. Factors that will induce antler osteoclastogenesis *in vitro* include PTHrP, RANKL, M-CSF and RA (Allen et al. 2000; Faucheux et al. 2002). Because sex steroids are important regulators of bone resorption in other species (Riggs et al. 2002), we are currently exploring whether the cessation of antler bone growth induced by high concentrations of testosterone involves direct effects of sex steroids on osteoclast activity.

Antler bone formation

Although osteoclasts start to differentiate in the non-mineralized cartilage, the largest TRAP-positive cells are localized in the mineralized cartilage lower down the antler tip where extensive matrix resorption takes place. This results in the formation of a number of irregular trabeculae that are eventually replaced by woven bone and cancellous lamellar bone in the primary spongiosa (Fig. 9H) at the centre of the antler shaft (Banks & Newbry, 1983). Osteoblasts appear to differentiate from a population of osteoblast progenitors in perivascular tissue in cartilage; these cells synthesize type I collagen mRNA (Price et al. 1996), alkaline phosphatase (Price et al. 1994) and, as they mature, osteocalcin, a marker of the fully differentiated osteoblast phenotype (Allen et al. 2002). At the same time intramembranous ossification takes place around the antler shaft (Fig. 9J) and although we have not studied ALP expression at this site, osteocalcin can be immunolocalized in osteoblasts beneath the periosteum (Allen et al. 2002).

To date, our knowledge of the factors that may specifically regulate osteogenesis in regenerating antlers is derived from descriptive studies, although culturing these cells from explants of antler bone is straightforward and so there is great potential for further work in this area. As discussed recently by Kierdorf & Kierdorf (2004), the rapid rate of antler bone growth makes it an excellent model for investigating osteogenesis. PTHrP, acting through the PPR, is likely to play a role as both are present in osteoblasts at sites of endochondral and intramembranous bone formation. By contrast, we have only found IHH in osteoblasts in the midshaft of the antler, not at the periphery, which suggests a specific role in endochondral bone growth, consistent with the findings of knock-out studies in mice (St-Jacques et al. 1999). IHH mRNA has also been localized in antler cartilage and bone by *in situ* hybridization, following its identification in a cDNA library (Lord et al. 2004). TGFβ-1 is also present in antler osteoblasts (Price et al. 2004). Another molecule that is likely to regulate bone formation in antlers is RA given that it is found at relatively high levels in antler bone and RALDH2 is localized in osteoblast progenitors in antler cartilage as well as in more differentiated osteoblasts. The effects of RA on osteoblast lineage cells may be mediated by the RARα receptor because it is expressed in perivascular tissues where type I collagen mRNA-expressing cells are

also located (Allen et al. 2002). All of the molecules discussed above have been shown to regulate osteoblast function in other species (Grigoriadis et al. 1986; Choong et al. 1993; Oliva et al. 1993; Slootweg et al. 1996; Park et al. 1997; St-Jacques et al. 1999; Kobayashi et al. 2002).

What is clear from the above is that the factors so far identified as being involved in antler regeneration have multiple functions. For example, both RA and PTHrP regulate chondrocyte, osteoclast and osteoblast differentiation. Our knowledge of the pathways involved remains in its infancy, but what is becoming apparent is that nature is conservative and that during antler regeneration a number of developmental pathways are recapitulated. In fact, studies in a diverse range of models are beginning to show pleiotropic functions for known genetic pathways in the context of regeneration (Sanchez Alvarado, 2004). The hunt for novel 'antler factors' has been going on for a number of years; it started in the 1990s with the generation of a cDNA library by Mundy and colleagues (Mundy, 2001) who were trying to identify novel bone anabolic agents. More recently, Lord et al. (2004) generated a large database of genes present in regenerating antlers and the first proteomic analysis of red deer antler has been reported (Park et al. 2004). These studies provide an invaluable resource, inform us of 'what is there' and lead to hypothesis-driven research. However, it is very likely that novel molecular pathways may not be uncovered in antlers. In our view the challenge lies instead in understanding the way that different signalling pathways interact, both with each other and with their local and systemic environment, to regenerate a structure as large and complex as an antler. Dissection of the function of these pathways requires the manipulation of gene expression *in vivo* and we have preliminary data to show that biolistic particle transfer can be used to transfect cells in the blastema (Price & Allen, 2004). However, retroviral/adenoviral approaches may also prove to be useful tools for studying gene function.

Conclusions

Deer antlers are not only spectacular creations of the natural world but also provide us with a unique model for regeneration research (although the limitations of using a large semi-wild animal with a seasonal pattern of regeneration and limited potential for genetic manipulation should not be underestimated). Regeneration of antlers is regulated by environmental and systemic cues, rather than by injury or amputation, and recent evidence suggests that it probably involves stem cells, not de-differentiation of mature cells. Therefore, antler regeneration does not utilize all of the same strategies as regeneration in the urodele amphibian. However, this makes the antler more, not less, relevant as a model for human regeneration because if we can understand how deer have adapted the normal mechanisms of development, cell renewal and repair to regenerate a complete appendage it may be possible to achieve the same outcome in diseased or damaged human tissues.

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