Adult Stem Cells and Mammalian Epimorphic Regeneration-Insights from Studying Annual Renewal of Deer Antlers

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Abstract: Mammalian organ regeneration is the "Holy Grail" of modern regenerative biology and medicine. The most dramatic organ replacement is known as epimorphic regeneration. To date our knowledge of epimorphic regeneration has come from studies of amphibians. Notably, these animals have the ability to reprogram phenotypically committed cells at the amputation plane toward an embryonic-like cell phenotype (dedifferentiation). The capability of mammals to initiate analogous regeneration, and whether similar mechanisms would be involved if it were to occur, remain unclear. Deer antlers are the only mammalian appendages capable of full renewal, and therefore offer a unique opportunity to explore how nature has solved the problem of mammalian epimorphic regeneration. Following casting of old hard antlers, new antlers regenerate from permanent bony protuberances, known as pedicles. Studies through morphological and histological examinations, tissue deletion and transplantation, and cellular and molecular techniques have demonstrated that antler renewal is markedly different from that of amphibian limb regeneration (dedifferentiation-based), being a stem cell-based epimorphic process. Antler stem cells reside in the pedicle periosteum. We envisage that epimorphic regeneration of mammalian appendages, other than antler, could be made possible by recreating comparable milieu to that which supports the elaboration of that structure from the pedicle periosteum.

INTRODUCTION

Advances in regenerative medicine offer the hope of restoring and/or replacing damaged tissue/organ by recapitulating part or all of its embryonic development. An essential prerequisite for realizing this goal, however, is to first understand the biology of regeneration [1, 2]. Regeneration in animals has been classified into four categories: 1) Physiological/homeostatic regeneration, which counteracts every day's wear and tear, and occurs in those tissue types subject to high cellular turnover or mechanical abrasion, such as blood and epithelium. 2) Wound healing, also referred to as tissue regeneration, which is a stopgap measure to restore continuity of the interrupted tissue. 3) Compensatory growth, which occurs in some organs (i.e. kidney) as a response to an increase in functional load. 4) Epimorphic regeneration, which is the phenomenon of *de novo* development of appendages distal to the level of amputation [3]. A typical example for epimorphic regeneration is the growing back of missing limbs by the newt, an urodele amphibian. Nearly all animals possess the capability to undertake the first three categories of regeneration; only relatively few species, however, are capable of epimorphic regeneration.

Our current knowledge of epimorphic regeneration is largely gained from the studies on lower vertebrates, particularly on amphibians. Deer antlers (Fig. **1**) are the only mammalian appendages capable of full renewal, and therefore offer a unique opportunity to explore how nature has solved the problem of mammalian epimorphic regeneration.

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ANTLER REGENERATION

Deer antlers are renewed in yearly cycles (Fig. **2**). In spring, the nascent antler regenerates from a permanent cranial bony protuberance, known as a pedicle, following casting of the previous hard antler (Fig. **2A**). Rapid antler elongation and bifurcation occur in summer (Fig. **2B**) with growth rates reaching up to 2 cm a day in large deer species [4]. A regenerating antler is enveloped with a special type of soft pelage, called velvet skin. In autumn, the regenerating antler attains its full size and becomes totally calcified, resulting in the shedding of the velvet skin (Fig. **2C**). In winter, a bare bony antler is firmly attached to its living pedicle (Fig. **2D**) and is not "cast" until the following spring, which triggers another round of antler regeneration.

MORPHOGENESIS AND HISTOGENESIS

The morphogenesis [5] and histogenesis [6, 7] of antler regeneration have recently been studied in detail. Immediately after a hard antler falls off, bleeding occurs on the rough cast surface of the pedicle stump and the centre of depressed bony tissue is surrounded by a rim of shiny and hair-sparsely-populated skin (Fig. **3A**). Histologically, this rim of skin has already acquired the peculiar features of velvet skin [5, 8], specifically a thicker epidermis, the *de novo* formation of hair follicles, and larger sebaceous glands (Fig. **3B** and **3H**), which distinguishes it from the more proximal pedicle skin, typical of the scalp. Within days after the hard antler casting, wound healing by centripetal growth of velvet skin over the cast plane of a pedicle nears completion (Fig. **3C**). At the same time, pedicle periosteum (PP), a tissue that is closely attached to the shiny skin rim, becomes thickened through the active division of cells resident within it (Fig. **3D** and **3G**). Subsequently, at the late wound healing stage two crescent-shaped growth centres are formed directly from the

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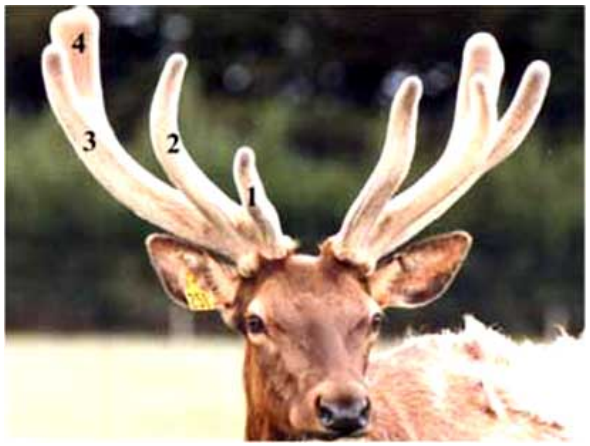

Fig. (1). A pair of 60-day-growth antlers. 1, brow tine; 2, bez tine; 3, trez tine; 4, main beam.

Fig. (2). Annual antler growth cycle. In spring, hard antlers drop off from the pedicles (arrow), and antler regeneration immediately follows. Rapid antler growth occurs in summer. Growing antlers are enveloped with velvet skin (asterisk). In autumn, antlers become fully calcified and velvet skin starts to shed. In winter, hard antlers are attached to their pedicles and subsequently cast in the next spring, which triggers a new round of antler regeneration.

thickening distal PP, one located anteriorly and the other posteriorly. Each centre is made up of cartilaginous clusters that are capped by a layer of hyperplastic pedicle periosteum/perichondrium (Fig. **3D**). Further augmentation of each growth centre raises anterior and posterior portions of the pedicle stump and leaving the central scab region behind (Fig. **3E**). These posterior and anterior growth centres are the centres for the formation of the antler "main beam" and "brow tine" (Fig. **3F**; for the terminology of antler morphology, refer to Fig. **1**).

These studies clearly indicate that the growth centres of a regenerating antler are formed exclusively from the proliferation and differentiation of distal PP cells of a pedicle stump; whereas pedicle skin only plays a role in wound healing to seal the cast plane of the pedicle stump. There is a considerable temporal overlap between the late stage of wound healing and establishment of the early periosteal growth centres for the formation of main beam and brow tine (Fig. **3I**). This overlap seems to rule out the possibility that the antler regeneration bud is derived from the healing pedicle skin, as previously suggested [9-12]. These histological observations support the notion that annual antler renewal

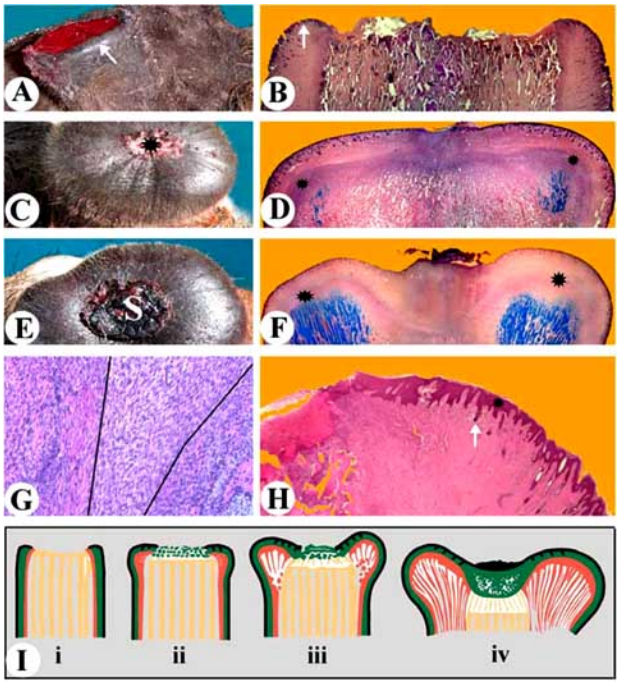

Fig. (3). Morphological and histological examinations of antler regeneration. **A**. Pedicle stump with a fresh cast surface. Note the rim that has shiny appearance and sparsely populated hairs (arrow). **B**. Sagittally cut histological section of a pedicle stump at a stage similar to **3A**. Note that the distal end of pedicle skin had acquired some velvet skin features: thickened epithelium and *de novo* formation of hair follicles (arrow, also see **3H**). **C**. Early regenerating antler bud at late wound healing stage. **D**. Sagittally cut histological section of an early antler bud at the regenerating stage similar to **3C**. Note that the newly formed cartilage and the hyperplastic pedicle periosteum/perichondrium constitute two clear growth centres (asterisks). There was a considerable overlap between the completion of wound healing and the establishment of these growth centres. **E**. More advanced regenerating antler bud. Note that differential growth had left the central scab region (S) behind. **F**. Sagittally cut histological section of the regenerating antler bud in **3E**. At this stage, it becomes clear that the posterior and anterior growth centres (asterisks) are the centres for the antler main beam and brow tine formation. **G**. Higher magnification of an area similar to that in the anterior corner in Fig. **3D** to show the thickened pedicle periosteum (within the solid lines). **H**. Higher magnification of an area of the distal pedicle skin in Fig. **3B** to show that at the time of hard antler casting, the distal pedicle skin already acquired some velvet skin features, such as thickened epidermis (asterisk) and *de novo* formation of hair follicles (arrow). **I**. Schematic drawing of histogenesis of antler regeneration. i, casting; ii, early wound healing; iii, late wound healing and establishment of the two growth centres; iv, main beam and brow tine formation. Black, epidermis; green, dermis; brown, pedicle periosteum; and yellow, pedicle bone. (for color, refer to the e-version) (**3A**, **3C** and **3E**: reproduced with permission from [5]. **3B**, **3D**, **3F**, **3G** and **3I**: reproduced with permission from [7]).

represents stem cell-based epimorphic regeneration, and that the stem cells reside in the PP of the pedicle stump [5, 13, 14].

PEDICLE PERIOSTEUM AND ANTLEROGENIC PERIOSTEUM

1. Pedicle Periosteum and Pedicle Periosteal Cells

The foregoing morphological and histological results, while indispensable in linking PP with antler renewal, and in advancing the stem cell-based regeneration hypothesis, do not allow us to conclude that regenerating antler is derived exclusively from PP. To confirm this, we have conducted a number of *in vivo* functional studies [14]. In the first of these experiments, the PP tissue was completely removed from a pedicle stump (Fig. **4A**) and subsequent antler regeneration assessed in its absence. Significantly, when PP deletion was carried out within a critical time window, the PP depleted pedicles failed to give rise to a regenerating antler, in marked contrast to sham-control pedicles which formed multi-branched antlers (Fig. **4B**). Further experiments involved only partial PP deletion (Fig. **4C**) to determine whether antler regeneration could occur at a point along a pedicle shaft that is markedly distant from the original antler regeneration site, i.e. the cast plane of a pedicle stump. Convincingly, early regenerating antler buds did indeed form on the pedicle shafts where the distal ends of PP and its enveloping skin met (Fig. **4D**). In these cases, the pedicle bone was effectively precluded from participating in the process of antler regeneration. These experiments provided strong evidence that PP is the key tissue type that gives rise to regenerating antlers.

Although deer pedicles have been called permanent bony protuberances, they do become shorter and thicker with each passing season, with the first year's pedicle being the longest and thinnest [5, 15]. We calculate that in red deer around 3.3 million PP cells within a pedicle participate in each round of antler regeneration, giving rise to up to 10 kg of antler tissue within just 60 days. Our calculation is based on the following measured parameters: average decreased pedicle length (5.5 mm/year), average increased pedicle diameter (2 mm/year), PP thickness (1.5 mm) and cell density (by stereological counting, approximately 10,000 cells/mm^3), and suggests that PP cells display an astonishing potential for proliferation and self-renewal.

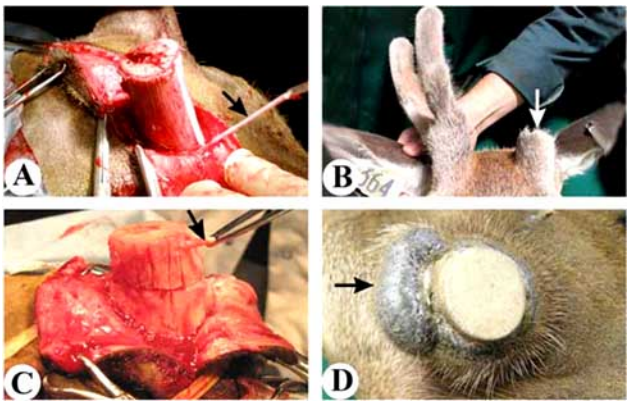

Fig. (4). Pedicle periosteum (PP) deletion in yearling red deer stags (reproduced with permission from [14]). Following the exposure, PP (arrows) was peeled off the pedicle bone either totally (**A**) or partially (**C**). The PP-less-pedicle in total deletion group failed to regenerate antler (arrow), although the sham-control pedicle regenerated a normal branched antler (**B**). Interestingly, the partial-PP-deleted pedicle regenerated an antler bud (arrow) on the pedicle shaft at the site where PP and the distal pedicle skin met (**D**), which was markedly distant from the normal regeneration surface.

Collectively, our *in vivo* experiments confirm the suggestions arising from histological observations and unequivocally demonstrate that PP is the key tissue type that gives rise to regenerating antlers, and that antler regeneration is derived from a finite number of spatially restricted cells resident within the PP. We, therefore, conclude that PP cell populations are the "stem cells" which underpin antler regeneration, a stem cell-based epimorphic process.

2. Antlerogenic Periosteum and Antlerogenic Periosteal Cells

The remarkable ability of PP cells to support the full regeneration of a complex mammalian appendage such as the deer antler, is not shared by any of the cell populations remaining in the stump of a lost deer leg, which at best can only seal the open end of the long bone. We suggest that the unique attributes of PP cells result from their developmental origin, as direct derivatives of the antlerogenic periosteum (AP), a tissue that overlies each frontal crest in prepubertal deer (Fig. **5A**; [16]).

The initial discovery of AP [17] has been hailed as a "hallmark" event in antler research history [15]. Surgical removal of AP from the future growth region abolishes both pedicle and subsequent antler formation, while subcutaneous transplantation of AP elsewhere on the deer body, such as to the forehead (Fig. **5B**) or a foreleg (Fig. **5C**), induces ectopic antler growth [18]. Interestingly, co-transplantation of AP and deer skin onto a nude mouse can cause an antler-like protuberance to form (Fig. **5D**). When a disaggregated single suspension of AP cells is cultured in a defined medium for an extended period, large cylindrical bony nodules (Fig, **5E** and **5F**) can form [19]. Histologically, these nodules have a well-organized structure, reminiscent of the bony trabeculae within growing pedicles or antlers. Specifically, more differentiated cells are located in the centre and actively forming extracellular matrices, whereas the less differentiated spindle-shaped cells are found peripherally (Fig. **5G**). Notably, AP cells are rich in glycogen (Fig. **5H**) [20], a property shared with embryonic osteoblasts [21].

To further confirm that the entire pedicle and first antler (except for the enveloping skin) are derived from AP cells, we have used lineage tracing methods to follow the fate of a small population of AP cells *in vivo* [22]. After introducing the genetic marker LacZ at the time of pedicle initiation, the fate of the labelled AP cells was assessed by subsequent X-gal staining of the resulting pedicle and antler tissue. Not unexpectedly, LacZ positive cells were detected in every tissue type (except for covering skin) of the appendage (Fig. **6A**) including mesenchyme (Fig. **6B**), precartilage (Fig. **6C**), cartilage (Fig. **6D**) and cortical bone (Fig. **6E**). Consequently, the 'embryological' potential to generate a pedicle (and thus PP cells) and first antler is exclusively held in AP tissue.

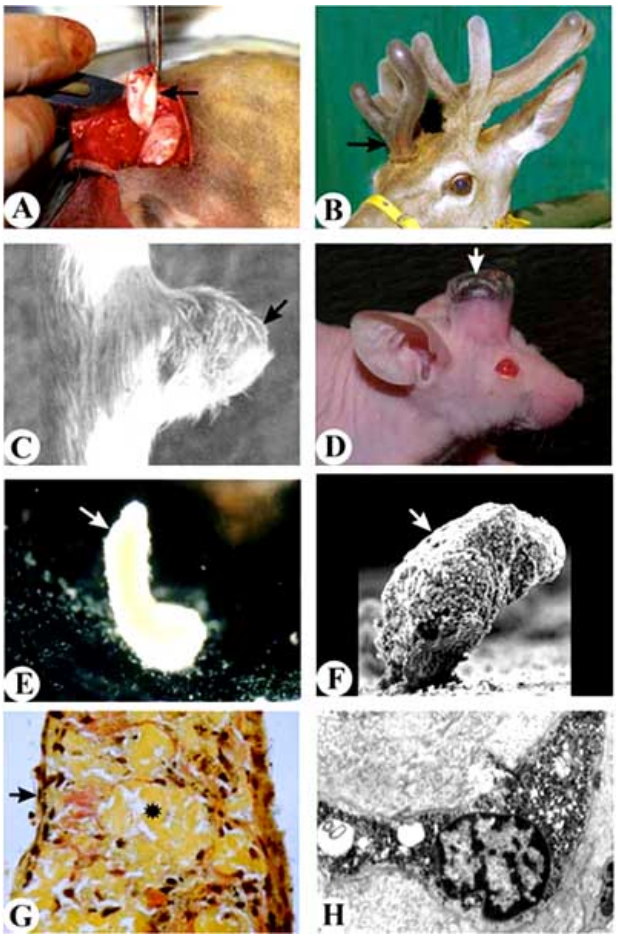

Fig. (5). Antlerogenic periosteum (AP) and antlerogenic cells. **A**. AP (arrow) was peeled off a frontal crest. **B**. Two branched-ectopic-antler (arrow) formed from the grafted AP on the forehead region of a 3-year-old red deer stag. **C**. Ectopic antler (arrow) formed from the grafted AP on the foreleg of a fallow buck. **D**. Antler-like bony protuberance (arrow) formed from the co-transplantation of AP and deer skin on a nude mouse. **E**. Bony nodule formed in a culture dish from the singularly cultured AP cells. **F**. Bony nodule similar to the one in 5E and photographed using a scanning electron microscope. **G**. Longitudinal histological section of a bony nodule formed from AP cells in a culture dish. Note that more differentiated cells are located in the centre producing ample extracellular matrix (asterisk), whereas the less differentiated cells mainly found peripherally with a spindle-shaped morphology (arrow). **H**. Transmission electron micrograph of an AP cell. Note that the cytoplasm of the cell was densely occupied with glycogen granules. (**5A**, **5E**, **5F**, **5G** and **5H**: reproduced with permission from [19]. 3C: reproduced with permission from [15] p129).

Although the importance of AP in antlerogenesis has been fully appreciated, the true embryonic origin of AP tissue remains unclear. Since the remarkable capability of AP tissue for self-differentiation (perhaps unique to adult mammalian tissues) is more reminiscent of transient embryonic tissue anlage, such as lateral plate mesoderm, which predicate organogenesis during development, Li and Suttie considered that AP may represent "a piece of postnatally retained embryonic tissue" [19]. Recently, Mount *et al.* provided experimental evidence for an embryological link with the neural crest [23]. Irrespective of its embryological heritage, the progenitor cell population responsible for antler generation and regeneration exhibits a substantial developmental multipotency, if not pluripotency. Interestingly, some biologists believe [24] that cells with pluripotency also reside in postnatal organisms, and these pluripotent cells might be some type of neural crest cells, as the neural crest cells is an embryonic cell population that does seem to undergo a more stochastic type of differentiation than other embryonic progenitor cells; and these cells might represent some kind of "embryonic remnant" comprising pluripotent cells left over from the early embryo. The collective body of work in antler research (also see the following "antler stem cell" section) apparently supports this "pluripotent adult stem cell" view.

ANTLER STEM CELLS AND STEM CELL NICHE

A capacity for extensive self-renewal *in vitro* and the latent capability to differentiate into multiple diverse cell lineages are hallmark features associated with stem cell populations. The greatest developmental potential (pluripotency) is exhibited by embryonic stem cells (ESCs), which are derived from the inner cell mass population of a nascent embryo at the blastocyst stage of development. As embryogenesis progresses and lineage commitment proceeds, the potency of cell populations becomes increasingly restricted. While it is now generally accepted that populations of somatic stem and precursor cells are retained in the adult organism, they are considered relatively few and of notably restricted potential, often reflecting pre-determination toward lineages with a common germ layer heritage. Based on histological detail alone, expansion and differentiation of mesenchymal progenitor cells in the tip of an antler might account for antler growth. If however AP represents a postnatally retained embryonic tissue, the cells within it might display features typically associated with more potent stem cell populations. We have sought to resolve this by applying criteria generally accepted and used for the characterization of putative ESC cell lines, to assess the 'stem cell' characteristics of isolated AP and derivative cell populations.

As suggested above, both AP and PP cells display an astonishing potential for population expansion. The antlerogenic periosteum, a tissue of around 2.5 cm in diameter and 2.5-3 mm in thickness, contains around five million AP cells which sustain the seasonal renewal of the entire antler for the extent of the deer's life. During the 60 days of the antler growth phase of each annual regeneration, this AP population will provide the roughly three million PP cells, from which approximately 10 kg of antler tissue mass is generated. Both the AP and PP cell populations are therefore clearly capable of self-renewal. Therefore, to qualify AP and PP cells as adult stem cells is a matter of demonstration whether they express key stem cell markers and possess multipotency.

1. Stem Cell Markers

The expression of particular antigens, genes and enzymes has been widely used to define stem cell populations [25]. Embryonic stem cells, as derivatives of the inner cell mass of the embryo, express the cell surface antigen CD9. We have demonstrated that both AP and PP cells express considerable levels of CD9 antigen (Fig. **7A**). The phenotypic fidelity of established stem cell lines is monitored by the characteristic

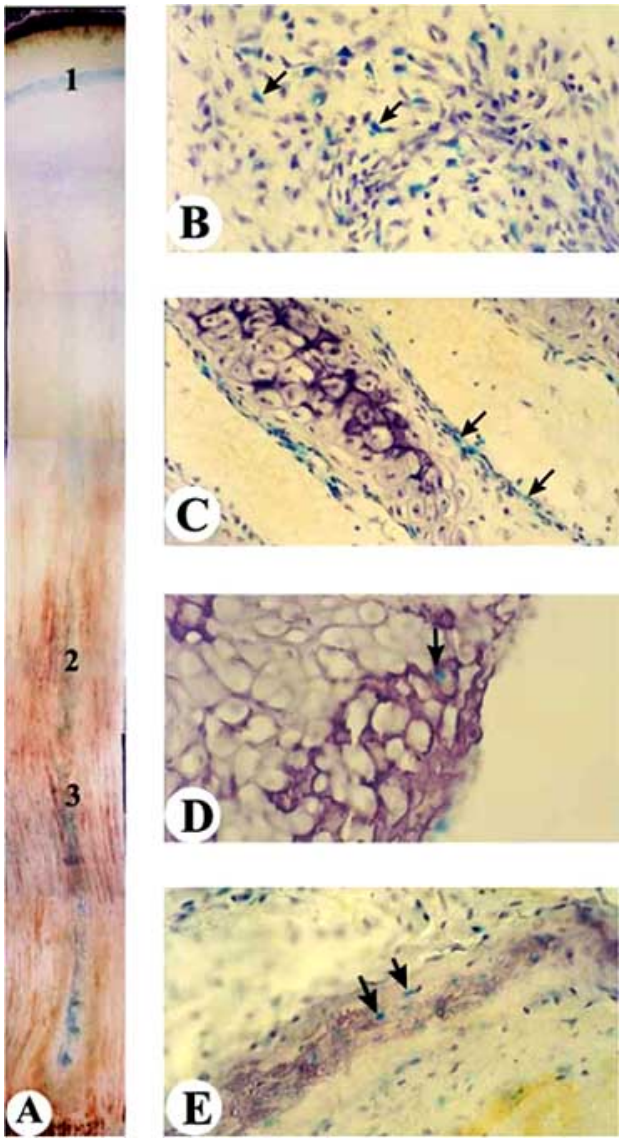

Fig. (6). AP cell lineage tracing using a genetic marker, LacZ gene, *in vivo*. The histological sections were stained with X-gal. **A**. Vertical section cut through the pedicle and the growing antler. Note that longitudinal blue strip located in the centre of the section is made up of LacZ gene expressing cells. **B**. Cells from the area labelled 1 in 6A. Note that LacZ gene expressing cells (arrows). **C**. Cartilaginous column from the area labelled 2 in 6A. Note that the blue cells (for color, refer to the e-version) are mainly less-differentiated chondroblasts (arrows). **D**. Part of a cartilaginous column from the area labelled 3 in 6A. Note that a LacZ gene expressing chondrocyte (arrow). E. Lamellar bone from the cortical layer at the base of the antler outside 6A. Note that most of the cells (arrows) in the bone were expressing the LacZ gene.

expression of a defined set of transcription factor genes which are thought to underpin the genetic hierarchy that maintains this unique phenotype [26]. Principal amongst these so-called 'pluripotency genes' are the POU domain family member Oct4, and Nanog. Critically, we have found both of these genes to be present in both AP and PP cells (Fig. **7B**). Additionally, we have shown elevated telomerase enzyme activity (Fig. **7C**) and nucleostemin (Fig. **7D**) in both cell types. Telomerase activity has been linked to enhanced self renewal in cells [27], which might have explained the phenomenon why so few antler stem cells (3.3 million PP cells) can form such an impressive amount of antler tissue mass (around 10 kg) within a very limited period (55-60 days). Expression of nucleostemin has been linked to controlling proliferation of stem cells [28] and newt limb regeneration [29]. Recently, PP cells have been shown to express stro-1, a recognized marker of mesenchymal precursor populations [30]. The range and nature of markers that we have demonstrated in both AP and PP cells strongly suggests that these cell populations not only function as tissue specific 'stem' cell populations in the adult organism, but that they retain characteristics of an embryonic origin throughout the life-time of the animal.

2. Multipotency

Stem cell populations by definition must also be capable of differentiation into a number of specialized cell types. The potency of AP and PP cells has been investigated by several laboratories [30-33]. Clearly, both populations *in vitro* can give rise to chondrocytes (Fig. **8A**) and osteoblasts when in micromass culture [34] and in media containing dexamethasone and ascorbate [31] respectively. As essential cell types

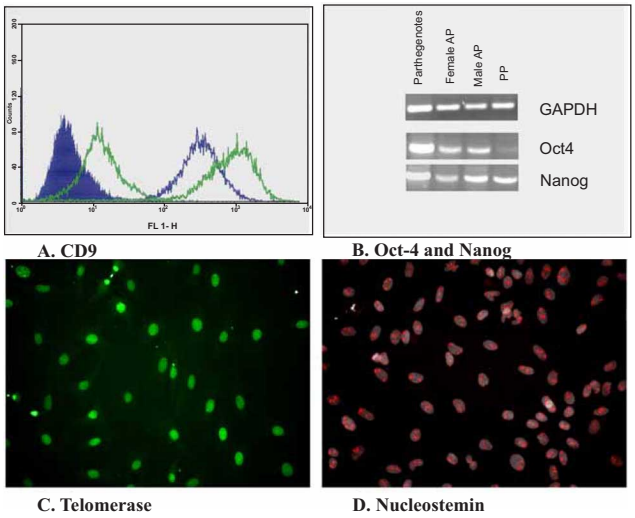

Fig. (7). Expression analysis of cell markers and genes associated with embryonic self-renewal and pluripotency in antler stem cell populations. A. Cell surface expression of the embryonic marker CD9 by AP (blue trace) and PP cell populations (green trace) (for color, refer to the e-version), determined by flow cytometry following standard indirect immunocytochemical (ICC) labelling. The first peak of each pair represents the 'no-primary' control (secondary antibody binding only), while the second peak of each pair represents the binding of anti-CD9 to both populations following fixation. Note that the peaks for each cell type have been offset for clarity, given the remarkable similarity in expression levels between AP and PP cells. B. Detection of the 'pluripotency' genes Oct3/4 and Nanog in antler stem cell populations, measured by standard RT-PCR and using probes designed against known bovine sequences. Expression levels are compared to that detected in bovine embryo parthegenotes (positive control) at blastocyst stage (embryonic day 7 in culture) and measured with reference to the house-keeping gene GAPDH. C. Expression of telomerase, the enzyme which regulates telomere length, in AP cells as uniformly detected by ICC. D. Expression of nucleostemin in AP cells as uniformly detected by ICC.

for both pedicles and antlers, chondroblastic and osteoblastic lineages would be expected outcomes from the differentiation of AP and PP cells [16, 35]. Interestingly, when AP and PP are exposed to linoleic acid (Fig. **8B** unpub. observation), or AP to rabbit serum [31] in culture medium, they also differentiate into adipocytes. Recently, we have successfully induced transdifferentiation of AP cells to multinucleated muscle precursor cells (unpub. observation) by *in vitro* co-culture with the established C2C12 muscle progenitor cell line (Fig. **8C**), or in medium supplemented with galectin 1. AP cells have also been shown to form neuronal-like cells with neurite-like structures projecting from each cell body (Fig. **8D**, unpub. observation) when cultured in N2 medium, a formula that promotes neural differentiation. This result may not be totally unexpected as AP cells have been demonstrated as being possible derivatives of the neural crest lineage [23]. Overall, these observations offer a tantalizing hint at a far broader potency for deer antler stem cells, particularly the AP population.

3. Stem Cell Niche

Antler stem cells have the ability to replenish the pool of fast proliferating cells for sustaining each round of antler regeneration over the life span of deer life; they must be located in their niche. In general, the maintenance of a quiescent stem cell population through the lifetime of the organism requires that they be localized at specific anatomical sites defined as the 'niche'. As a point of anchorage within the tissue, direct cell-cell contact between stem cells and with closely associated differentiated populations, is a principal element in the organization of the stem cell niche. A range of extrinsic factors, including specific extracellular matrix components and associated growth factors contribute further elements to this specialized microenvironment.

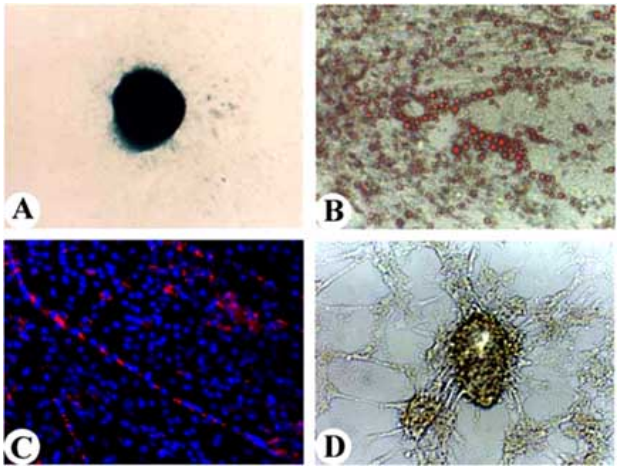

Fig. (8). Multipotency of antler stem cells. **A**. Cartilage nodule (blue) formed by PP cells in a micromass culture and stained with alcian blue. **B**. Adipocytes differentiated from PP cells in the culture medium containing linoleic acid and stained with Oil Red. **C**. Myotube (arrows) formed from AP cells (red color, labelled with fluorescent dye DiI) (for color, refer to the e-version) when co-cultured with C2C12 cells (no labelling), and the nuclei of both cell types stained with DAPI (blue color). **D**. Neuronal-like cells differentiated from PP cells when cultured in N2 medium. Note the extended neurites (arrows) from each cell body (8B and 8D: reproduced with permission from [31]).

Finally, particular physiological conditions contribute additional physiological elements to support the distinct milieu characteristic of the niche environment. The primary roles of the niche in adult tissues are to limit the proliferation of stem cells and to protect them from pro-differentiation stimuli. However, intermittently in tissues which undergo regularly renewal, or episodically in response to appropriate cues such as tissue damage, the stem cell population of the niche is activated to provide the necessary transit populations required for tissue rebuilding. Specific interactions within the domain of the niche are thought essential for triggering this expansion.

Our detailed anatomical and histological knowledge of antlerogenic processes suggests that more than one such niche is required for the generation of the 'first' antler and its subsequent annual renewal. The AP cell population represents the likely embryological source from which all future antler tissue is generated and is identifiable as a transient tissue mass on the frontal bone of the skull. It is no longer distinguishable once it has given rise to the pedicle. The PP cell population positioned between the pedicle skin and pedicle bone should perhaps be considered as the stem cell population proper, from which both the first structure and subsequent antlers are generated with each annual renewal. The microenvironment adjacent to the inner surface of pedicle skin may therefore constitute the true antler stem cell niche. Critically, it is the PP population which is repeatedly recruited for the initiation of antler regrowth, at least through the reproductive life-span of the animal. To qualify as a functional niche population, the PP cells must undergo seasonal asymmetric divisions, such that upon division one daughter cell is retained in the niche to support the self renewal process, while the other daughter cell becomes a transient amplifying cell and ultimately contributes to the regenerating structure. Asymmetric division in the PP cells remains to be demonstrated formally however. With the initiation of regeneration the transient amplifying daughters of the PP population relocate to the growing apical tip of the growing antler, positioned immediately underneath the apical skin, and known as antler mesenchyme. It is these mesenchymal cells which provide the necessary tissue bulk for the regenerating antler [36].

Establishment of mesenchymal stem cell niche begins when the mesenchyme becomes in close juxtaposition with the overlying skin (Fig. **9A-9D**). There is evidence to believe that the niche is located in the immediate vicinity of the inner surface of the overlying skin. It is only when the mesenchyme becomes closely adhered to the skin that the growth centres for antler generation (Fig. **9B**) or regeneration (Fig. **9D**) can start to form [7, 37]. These centres consist of histologically discernable layers (Fig. **9E-9G**) distant from the skin: a mitotic-quiescent cell layer (stem cells), an intensively cell dividing layer (transiently amplifying cells), and a pre-chondroblast layer (differentiating toward the chondrogenic pathway) [36]. Physical interruption of the interactions between mesenchyme and the skin inhibits antler generation or regeneration (see the following section of "Endocrine factors and local interactions"). Specifically, it is only competent regions of deer skin that can provide antler stem cells this unique microenvironment, a property not shared by three regions of deer skin, snout, tail ventral surface and back [38], or nude mouse skin [39, 40]. Significantly, the differentia-

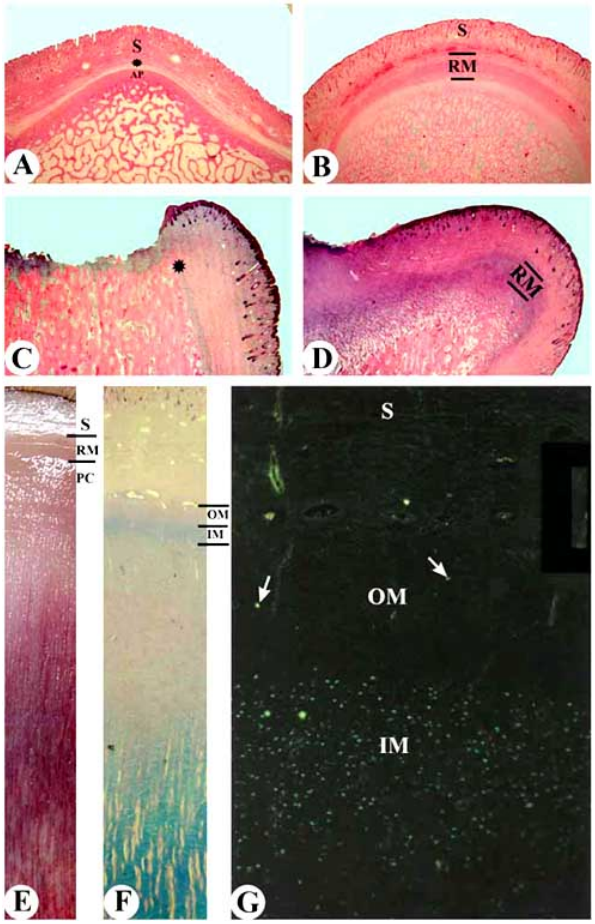

Fig. (9). Antler stem cell niche (vertical histological sections). **A**. A frontal crest. Note that AP and the overlying skin (S) were well separated by a thick layer of loose connective tissue (asterisk). **B**. A developing pedicle at transitional (from endochondral to intramembranous) ossification stage. Note that the AP-derived reserve mesenchyme (RM) and the overlying skin (S) had become closely associated at this stage and AP now has transformed into broad zone (RM) that consists of distinctive layers (refer to Fig. **9E -9G**). **C**. A posterior corner of a pedicle stump immediately after the hard antler casting. Note that PP (asterisk) at this stage is still thin, although the distal skin has become closely contacted with PP. **D**. A posterior corner of a pedicle stump/early antler bud at the late wound healing stage. Note that the thin layer of PP has differentiated into a broad zone of mesenchyme (RM) containing distinctive layers (refer to Fig. **9E -9G**). **E**. Central portion of an antler tip. Layers of skin (S), mesenchyme (RM) and precartilage (PC) can be distinguished morphologically. **F**. An antler tip with haematoxylin and Eosin/alcian blue counterstaining. Note that the layer of mesenchyme can be readily divided into two sublayers: outer (OM, no blue staining) and inner (IM, light blue staining) (for color, refer to the e-version) mesenchyme. **G**. BrdU incorporation in OM and IM. Note that majority of the cells in the IM sub-layer (IM) were stained with BrdU antibody, whereas only few cells (arrows) in the OM sub-layer (OM) stained.

tion fate of AP cells is dependent upon their position relative to the inner surface of the overlying skin. These immediately adjacent, become mitotic-quiescent stem cells (outer sublayer of AP); whereas those more distant away develop into transient amplifying cells (inner sublayer of AP); and yet further away, they differentiate to give rise to pre-cartilaginous layer. This regulative property of deer skin was discovered in our recent AP transplantation experiments. In the experiment when AP was transplanted subcutaneously in an inverted orientation (with the AP cellular layer facing the skin), the inner AP sublayer cells that would naturally differentiate into transient amplifying cells became quiescent stem cells, whereas the outer AP sublayer cells that are destined for stem cells developed into transient amplifying cells (unpublished observation).

The foregoing established attributes of AP, PP and antler tip mesenchymal cells (astonishing self-renewal capacity, expression of critical stem cell markers, multipotency and clearly defined niche system) make these cells well qualified to be regarded as "antler stem cells".

ENDOCRINE FACTORS AND LOCAL INTERACTIONS

1. Endocrine Factors

Deer antlers and their antecedents, the pedicles, are male secondary sexual characters, and as such their development is under the control of androgen hormones (Fig. **10A** and **10B**). The relationship between the antler growth cycle and the seasonal change in male gonads has been known for over 2000 years since the era of Aristotle (cited by [41]). Further, since antlers are arguably the fastest growing mammalian organ, nutrition and growth factors would inevitably play an indispensable role in the process (Fig. **10A** and **10B**).

1. Androgen Hormones (Testosterone)

Development of pedicles and antlers are closely associated with fluctuations of testosterone levels: positively for pedicles and negatively for antlers. Pedicle initiation takes place during a rapid increase in circulating testosterone levels when deer are approaching puberty, and pedicle growth occurs while testosterone levels remain high. Generation of the first antlers from the fully formed pedicles [42-44] and regeneration of the subsequent antlers from the pedicle stumps [45, 46] both coincide with a decline in testosterone level. Indeed, antler growth occurs in a period when circulating testosterone is barely detectable. Antler calcification and subsequent velvet shedding occur when testosterone levels increase sharply, just before the onset of the rutting season; a subsequent decrease in testosterone levels in spring is linked with the casting of previous hard antlers and the initiation of new antler regeneration [45, 46].

The close association of pedicle and antler development with changes in androgen hormones has been functionally confirmed by manipulation of the *in vivo* availability of these hormones. Castration of prepubertal male deer abolishes future pedicle and antler formation, while castration of adult males prevents full antler calcification and velvet shedding. The abnormalities of castration can be overcome by administration of exogenous testosterone [42, 47, 48]. This striking regulation by testosterone explains why female deer do not grow pedicles and antlers despite having AP (see above).

Despite the full dependency of pedicle and antler development on androgen hormones *in vivo*, our *in vitro* studies [49, 50] have failed to demonstrate direct mitogenic effects

of testosterone on AP cells, even though the isolated AP cells maintained in culture expressed specific binding sites for testosterone, as demonstrated by both autoradiography (Fig. **10C**; [51]) and *in vitro* binding assays (Fig. **10D**; [50]). Further studies are required to unveil the underlying mechanism as to why androgen is an absolute prerequisite for pedicle and antler formation *in vivo*, but does not play a role in AP cell proliferation *in vitro* in serum free medium.

2. Growth Factors (IGF1)

Both pedicle and antler growth are also influenced by additional growth and nutritional factors. Rapid antler growth always takes place when circulating testosterone level is low, but the level of insulin-like growth factor 1 (IGF1) is increasing or high [52, 53]. Furthermore, castration of a stag during the antler growth phase (while IGF1 level is high) does not seem to affect the process of antler growth [45, 47]. In addition, pedicle initiation tends to occur when deer reach a species-specific body weight (approximately 56 kg in red deer), irrespective of their age or the season [54, 55]. From an evolutionary point of view, it is conceivable that deer precedentally utilize the available nutrients for survival, i.e. body building, rather than channelling them to the precocious development of secondary sexual characters, i.e. pedicle formation. However, this phenomenon clearly indicates that besides androgen hormones, pedicle initiation also relies on nutrition, although the pathway through which nutrition regulates pedicle initiation and antler growth is, thus far, unknown.

Based on his observations that 1) antlers could continue to grow after castration, 2) antlers began their annual renewal at a time when the testes and seminal vesicles were least active, and 3) antlers became hard and velvet was shed when deer testes and male accessories were rapidly enlarging, Wislocki [48] advanced a hypothesis that there must be a non-gonadal origin "antler growth stimulus (AGS)" for the seasonal antler growth. In 1985, Suttie *et al.* reported [52] that the circulating IGF1 levels correlate significantly and positively with growth rates of a pedicle and a first antler (Fig. **10A**), suggesting that this growth factor may function as the putative AGS. Subsequently, the same team [56] found that plasma concentrations of IGF1 were significantly elevated in stags that had had their growing antlers removed, indicating that antlers are the target, rather than the source of circulating IGF1. Supporting this notion, both Type I and Type II IGF receptors have been localised in the antler growth centre [57, 58], and a direct dose-dependent mitogenic effects of IGF1 on antler tip mesenchymal cells [59, 60], AP cells [49, 50], and PP cells (unpublished results) have subsequently been demonstrated in serum-free *in vitro* culture assays.

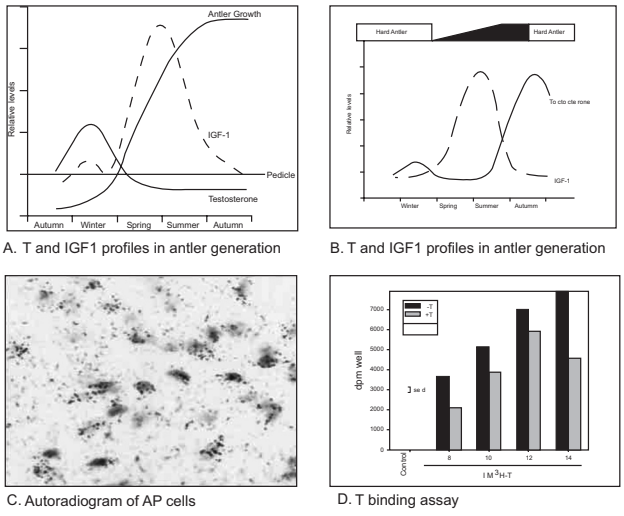

Fig. (10). Endocrine control of antler development. **A**. Relationship between profiles of testosterone and IGF1, and pedicle/antler generation (refer to the text). **B**. Relationship between profiles of testosterone and IGF1, and antler regeneration (refer to the text). **C**. Autoradiogram of AP cellular layer cells. Note that specific distributions of silver grains on each cell nucleus. **D**. Testosterone (T)-binding assay. +T, with 200-fold cold testosterone; -T, without cold testosterone; sed, standard error of difference. Note that with the presence of 200-fold cold testosterone, the counts (dpm/well) significantly reduced compared to those without it [50].

In view of the facts that nutrition promotes body growth mainly through the IGF1 signalling [61, 62] and IGF1 concentration is correlated significantly and positively with pedicle and first antler formation [52, 63], Li and Suttie [64] suggested that nutrition may promote pedicle and antler growth through the IGF1 signalling pathway. However, increasing the level of nutrition does not rescue the failure pedicle initiation and antler growth caused by pre-pubertal castration (unpublished observations); and to date there is no report of a pre-pubertally castrated but well-nourished stag growing pedicles or antlers, given that it should have at least normal IGF1 profiles. Consequently, IGF1 (the potent mitogen *in vitro*) does not seem able to activate quiescent AP cells and thus to initiate pedicle formation *in vivo*, in the absence of appropriate levels of androgen hormones. Thus far, the mechanism underlying the action of nutrition/growth factors in concert with sex hormones on pedicle initiation is unknown. In our view, to properly address the question how androgen hormones and nutrition/growth factors work synergistically to activate AP cells for the initiation of pedicle formation, efforts should combine the study of endocrine control with the investigation of antler stem cells.

2. Local Interactions

Since the late last century, the focus of antler research has shifted from the study of endocrine control to that of local signaling factors and tissue interactions. As comprehensive reviews have addressed the subject of local factors signaling on antler development [13, 33, 65], we have chosen here to highlight the potential significance of local interactions between antler stem cells and adjacent cell populations and associated extracellular matrices [66, 67].

1. Local Interactions in Antler Generation

As an organ, antler generation must rely on heterotypic cell/tissue interactions [68]. While carrying out AP transplantation experiments, Goss [38] noticed that ectopic antlers cannot be induced unless the antlerogenic tissue derived from the grafted AP became closely associated with the overlying skin, which led him [69] to think that this close apposition is indispensable for first antler generation. Detailed histological analysis of pedicle and first antler forma-

tion [37] support Goss's notion. In the study, the first antler generation only takes place when the interposing wide subcutaneous loose connective tissue layer is essentially compressed into a narrow strip, thus allowing the putative interactions to take place.

To experimentally test the foregoing hypothesis and to learn the nature of these interactions, a membrane insertion experiment was carried out. In study, different types of membrane have been interposed between the two interactive tissue types, AP and the overlying skin by Li *et al.* [67], prior to initiation of the pedicle. Insertion of an impermeable membrane prevented skin transformation from scalp to velvet type and subsequent antler formation for up to two years (Fig. **11A** and **11B**). In contrast, insertion of a permeable membrane (0.45 μm pore size) did not block, but delayed for a year the process of skin transformation to antler velvet (Fig. **11C** and **11D**). These experiments clearly demonstrate that diffusible factors mediate the interactions occurring between AP and skin prior to first antler formation. Interestingly, these interactions appear to be only transient in nature, as interposing a piece of impermeable membrane between the two interactive tissue types after initiation of the first antler formation did not arrest further antler elongation (Fig. **11E** and **11F**).

The membrane insertion experiments, while demonstrating the nature of the interactions that prevail in antler generation, do not allow identification of the precise origin of the initial inductive signal (AP fibrous layer, cellular layer or both), or give indication whether all the tissue types (subcutaneous loose connective tissue and entire dermis) intercalated between AP (inducer) and epidermis (responder) of the overlying skin are required for the establishment of the interactions. To address these questions, we have recently taken a xenograft approach to co-transplant AP and deer skin onto the forehead region of a nude mouse [40]. AP-derived tissue convincingly transformed the deer scalp epidermis into antler velvet epidermis (Fig. **12A** and **12B**) in the absence of subcutaneous loose connective tissue and its attached partial dermis (up to the level of hair roots). Thus, AP, epidermis and its adhered partial dermis on their own would appear to be necessary for the establishment of these interactions. In addition, when AP was transplanted in the inverted way (with cellular layer facing the skin), epidermal transformation from scalp to velvet phenotype took place faster (Fig. **12C**) than that in the non-inverted (its normal orientation) way (fibrous layer facing the skin) (Fig. **12D**), suggesting that the initial inductive signals may be derived from the more distant AP cellular layer rather than the fibrous layer that naturally lies adjacent to the skin.

2. Local Interactions in Antler Regeneration

The importance of heterotypic tissue interaction during annual antler regeneration was first proposed by Li and Suttie [70]. While developing a technique for sampling PP tissue, they noticed that there were differences in the degree of association between the enveloping skin and the PP along a pedicle shaft in young deer. The skin of the proximal two thirds of the total pedicle length is loosely attached to PP; whereas on the distal third of the pedicle, the skin is tightly bound to PP [70]. Further, they found that the association between the pedicle skin and PP was dynamic. When the cast/regeneration plane of a pedicle stump has gradually

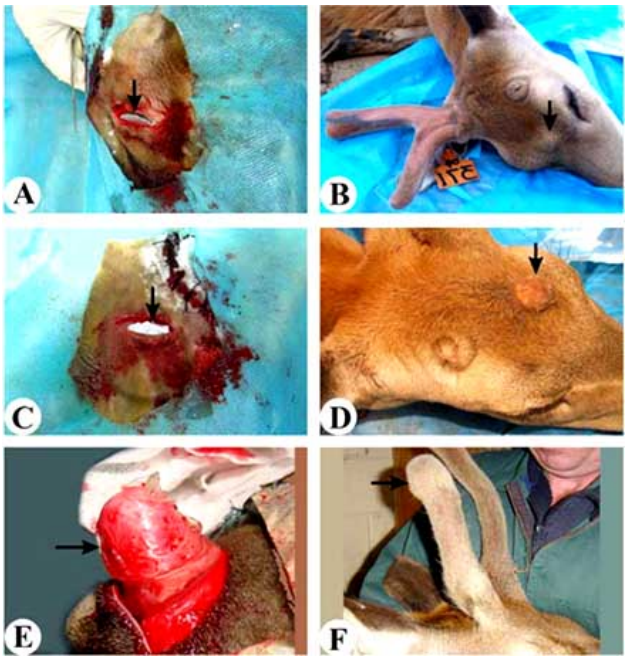

Fig. (11). Tissue interactions in antler generation (reproduced with permission from [67]). **A**. Impermeable membrane (arrow) was subcutaneously placed over the grafted AP on a sika deer forehead. **B**. Dome-shaped bump (arrow) was formed 2 years after AP and impermeable membrane transplantation. Note that the skin overlying the bump remained in scalp skin feature. **C**. permeable (0.45 μm pore size) membrane (arrow) was subcutaneously placed over the grafted AP on a sika deer forehead. **D**. Dome-shaped bump (arrow) was formed 2 years after AP and permeable membrane transplantation. Note that the skin overlying the bump had transformed into typical antler velvet type. **E**. Rubber sheath was surgically capped onto an exposed full-grown-pedicle tip (arrow). **F**. the antler generated from the sheath capped pedicle was somewhat shorter than the sham-control, but significantly elongated (arrow) compared to the time when it was sheath-capped.

shortened and approaches to the proximal two third region in an aging deer (as discussed in the "Pedicle periosteum" section), the two tissue types also become intimately apposed (unpub. observation). These observations indicate that antler regeneration also requires the close association between PP and the enveloping skin. Since close contact between AP and the overlying skin is a prerequisite for the establishment of the tissue interactions, which in turn triggers initial antler generation [37, 67, 69], Li and Suttie [70] hypothesized that the distal closely associated region of a pedicle stump is in a more advanced or primed state for antler regeneration compared to the proximal loosely attached region; they further suggested that the distal part be termed the "potentiated region", and the proximal part the "dormant region".

This hypothesis has been again tested using membrane insertion experiments [66]. Firstly, two types of pedicle stumps were created by removing off the distal parts of yearling stags at two different levels: "Type 1" stump was cut at the junction between a pedicle and an antler (Fig. **13A**); and "Type 2" stump at the junction between the potentiated and the dormant regions (Fig. **13D**). An impermeable membrane was then inserted between the enveloping skin and PP in each type of resulting pedicle stumps. In the first instance,

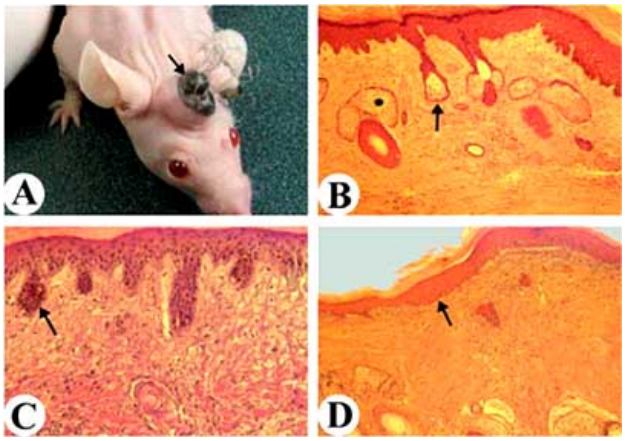

Fig. (12). Tissue interactions in xenografts (reproduced with permission from [40]). **A**. Antler-like protuberance on the forehead of a nude mouse is capped with velvet-like skin (arrow), which is transformed from grafted deer scalp skin. **B**. Histological section of the velvet-like skin in 12 A to show the typical features of velvet skin: thickened epidermis, multi-lobed sebaceous glands (asterisk), *de novo* formation of hair follicles (arrow) and absence of sweat glands. **C**. Histological section of the apical skin of a protuberance formed from the inverted (with AP cellular layer facing the deer skin) co-transplantation to show that the deer skin has acquired some features of velvet-like skin: the thickened epidermis and *de novo* hair follicle formation (arrow). **D**. Histological section of a protuberance formed from the non-inverted (with AP fibrous layer facing the deer skin) co-transplantation. The protuberance was sampled at the same time with the one in 12C, but the transformation from deer skin epidermis (arrow) to antler velvet has not yet started.

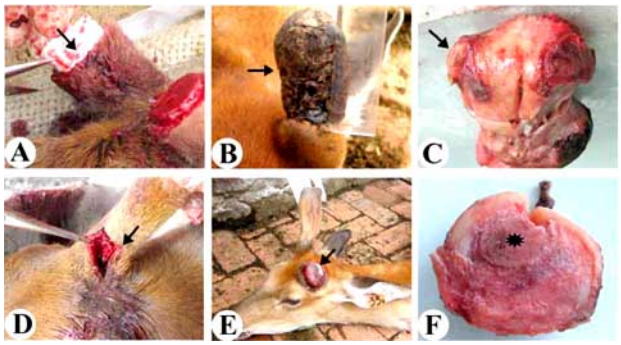

Fig. (13). Tissue interactions in antler regeneration (reproduced with permission from [66]). **A**. Impermeable membrane (arrow) was surgically interposed between PP and the enveloping skin in the potentiated region (refer to the text) of a pedicle stump. **B**. Skin-less-antler (arrow) was formed from the membrane-interposed potentiated pedicle stump, and covered with scab (arrow). **C**. Rudimentary branch (arrow) on the skin-less-antler was found after removing the scab layer. **D**. Creation of a dormant (refer to the text) pedicle stump by making a vertical skin incision to identify the very point (arrow). **E**. Membrane-inserted dormant pedicle stump (arrow) did not give rise to antler tissue at late antler regeneration stage. Note that the pedicle increased in thickness. **F**. The thickened pedicle was found to be caused by excessive soft tissue growth around the existing pedicle bone (asterisk).

regeneration gave rise to a skin-less and scab-covered antler (Fig. **13B**), with one such antler even developing a rudimentary branch (Fig. **13C**). In sharp contrast, insertion of the impermeable membrane completely blocked Type 2 pedicle stumps giving rise to antler tissue (Fig. **13E**), although one pedicle stump from the group became thickened due to an excessive growth of soft connective tissue (Fig. **13F**). Therefore, these experiments clearly demonstrate that interactions between PP and the enveloping skin are also indispensable for antler regeneration.

3. Schematic Summarization of Local Interactions

Based on the currently available information, we have schematically summarized the local interactions (Fig. **14**). Prior to the initiation of antler generation or regeneration, a wide subcutaneous loose connective tissue (SLCT) layer separates the two interactive tissue types: AP or PP and the competent skin [38] (Fig. **14A1**). When SLCT layer is fully compressed due to the proliferation of AP or PP cells, the cellular layer of AP (in the case of antler generation), or PP (in antler regeneration) releases instructive diffusible factors, which traverse the periosteal fibrous layer, compressed SLCT layer and the associated partial dermal tissue. These molecules firstly act in a long-range paracrine manner upon dermal cells resident in the dermal tissue at the level of hair follicle roots. These induced dermal cells then exert their influence *via* paracrine and juxtacrine [71, 72] mechanisms on the overlying epidermis, to induce the transformation to antler velvet (Fig. **14A2**). In turn, the transformed epidermal cells provide instructive reciprocal feedback to the AP/PP cellular layer cells in a reversed order (Fig. **14B1**) to initiate antler generation or regeneration (Fig. **14B2**).

BLASTEMA-BASED *VS* STEM CELL-BASED EPIMORPHIC REGENERATION

The apparent resemblance between antler and newt limb regeneration has prompted some biologists to suggest that antler regeneration is realised through the same mechanism used by the lower vertebrates. Goss stated [73] that "Very much the same mechanism is utilized in the epimorphic regeneration of all appendages. In each case, be it the fin of a fish, the limb of an amphibian, the tail of a lizard, or the antler of a deer, regeneration is made possible by the development of a blastema". Because blastema formation is the hallmark of epimorphic regeneration, this mode of regeneration is also referred to as a "blastema-based" process. However, there is significant controversy over the definition of "blastema" [5, 13, 74]. Goss [15] considered that "The aggregation of these dedifferentiated cells at the end of the stump leads to the production of the blastema, a rounded mass of cells endowed with the capacity to develop into a structure replacing that which was lost". However, recent studies show that the annual antler renewal is not achieved through cell dedifferentiation but rather *via* the *de novo* proliferation and differentiation of pedicle periosteal cells [5, 7, 14, 30], and is therefore a "stem cell-based", rather "blastema-based", epimorphic regeneration [5]. To fully appreciate the differences between classic "blastema-based" epimorphic regeneration (such as the newt limb, the current gold standard) and the "stem cell-based" epimorphic regeneration (such as antler regeneration), it is necessary to compare these two processes at the organ, tissue and cellular levels.

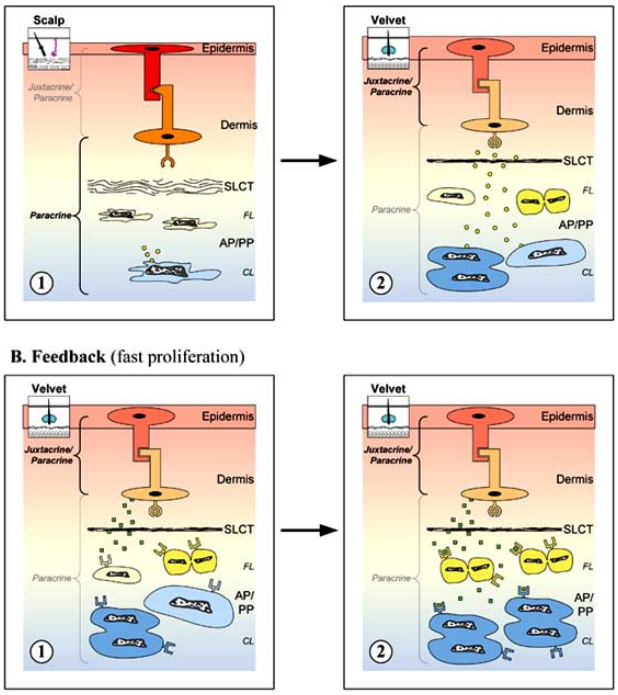

Fig. (14). Schematic summarization (refer to the text) of the interactions operating in antler generation and regeneration (modified from [40]).

A. Induction process. 1) Prior to the initiation of antler generation or regeneration, the two interactive tissue types (AP or PP and the covering skin) are interposed by a thick layer of subcutaneous loose connective tissue (SLCT). 2) Once the two interactive tissue types become closely contacted, the cellular layer (CL) cells of AP in the case of antler generation or PP in antler regeneration release the instructive diffusible molecules, which traverse the AP/PP fibrous layer (FL), compressed SLCT and its associated partial dermal layer to act on the dermal cells resident in the level of hair roots. Subsequently, the altered dermal cells exert their influence *via* paracrine and juxtacrine mechanisms on the overlying epidermis, which then is transformed into antler velvet.

B. Feedback process. 1) In turn, the transformed epidermal cells give the instructive feedback signals, which eventually act on the AP/PP cellular (CL) cells. 2) The feedback signals drive the AP/PP cells into the mode of rapid proliferation to initiate antler generation or regeneration.

AT ORGAN LEVEL

Regeneration of the newt limb proceeds through four distinct morphological stages, referred to as initial wound healing (Fig. **15A**), cone (blastema, Fig. **15B**), palette (the flattened cone; Fig. **15C**) and notch (2-3 digits; Fig. **15D**) [75] stages. However, the contour over the distal end of a pedicle stump during the course of wound healing and early antler regeneration undergoes characteristic changes from flat to deeply concave, due to the rapid augmentation of tissue mass from the anterior and posterior growth centres (Fig. **3D** and **3F**). No cone-shaped structure is formed during the initial period of antler regeneration in contrast to the regeneration of a newt limb.

Wound healing has been considered as a prerequisite for newt limb regeneration, the continuous interactions between healing epithelium and the mesenchyme on the amputation surface initiates the epimorphic regeneration [2, 9]. Indeed, in the absence of the healing wound epithelium newt limb regeneration does not occur [76]. In contrast, we have found that antler regeneration occurs even if the pedicle skin is physically prevented (by insertion of an impermeable membrane) from participating (after interaction with the pedicle periosteum) in the healing process (Fig. **13A** and **13B**). Hence, wound healing is not an obligate requirement for antler regeneration.

The initial formation of the blastema on a newt limb stump is a nerve-dependent process, and denervation at this stage completely inhibits epimorphic regeneration [77]. However, denervation of the future antler growth regions [78, 79] does not affect the formation of pedicles and first antlers, indicating that innervation is dispensable for antler development. In addition, antler regeneration from the denervated pedicle (Fig. **16A**) takes place in a similar manner to that from the non-denervated one (Fig. **16B**). Therefore, both antler generation and regeneration seem to be independent of nerve supply, and hence distinct from blastema-based newt limb regeneration.

It is known that blastema formation requires participation of all cell types located on the immediate amputation plane of the newt limb stump [77]. This does not seem, however, to be the case for antler bud regeneration. In typical deer farm practise, velvet antlers are sometimes removed at their mid-late growth stage 2 cm above the pedicle and antler junction in order to maximize economic returns. The remaining antler stumps may still retain the potential for partial antler regeneration (Fig. **16C**). In such cases, the antlers al-

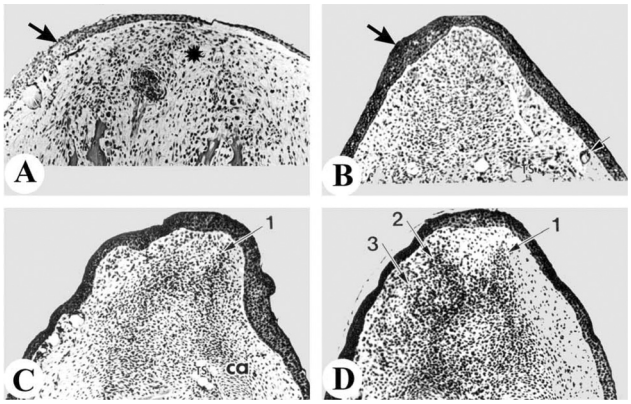

Fig. (15). Blastema formation and early limb regeneration over a newt limb stump (reproduced with permission from Neufeld, 1982; Devel Biol. 93:36-42). **A**. Early stage of a blastema formation. Note that limb stump wound was sealed solely with epithelium (arrow) beneath it are the embryonic-like cells (asterisk) that were dedifferentiated from all the cell types on the immediate amputation plane. **B**. Cone-shaped- structure that was directly covered with the further thickened epithelial layer (arrow), a typical initial regenerate known as blastema. **C**. Palette stage of regeneration. At this stage, dedifferentiated cells began to redifferentiate and form the lost structure of the first toe (1). **D**. Notch stage of regeneration, a stage further advanced from palette stage. Note at this stage, three toes had already taken shape (1, 2, 3).

ways develop from the distal pedicle periosteum and the enveloping skin without contribution from the central bony tissue of the antler stumps (Fig. **16D**). This distinction is further supported by an unusual phenomenon called "double-head" antler formation [80]. This phenomenon is caused if a previous hard antler fails to drop off when new antler regeneration starts, and the nascent antler arises from the dead antler/living pedicle junction (Fig. **16E**). In this case, there is no casting surface onto which the different types of cells in the distal end of the pedicle can migrate, proliferate and de-differentiate. Therefore, the whole bony portion on the distal plane of a pedicle cannot participate in new antler regeneration, the only tissue types giving rise to regenerating antler tissue being the distal pedicle periosteum and the skin. The distal antler/pedicle periosteum and the skin are thus sufficient to give rise to regenerating antlers, which is hence different to newt limb regeneration that requires all tissue types from the stump to participate.

Newt limb regeneration through initial blastema formation is a scar-less process [75]. During antler regeneration, however, an obvious scar located somewhere on the regenerating antler is sometimes the final outcome of the wound healing (Fig. **16F**).

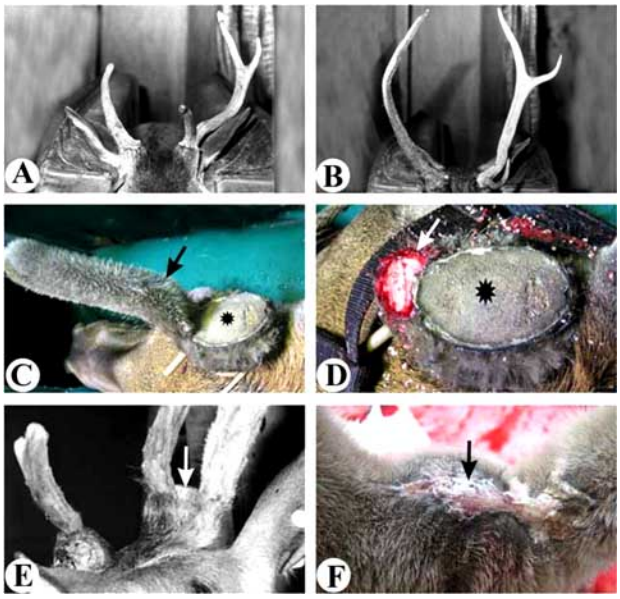

Fig. (16). Characterization of antler regeneration at organ level. **A**. The left antler regenerated following amputation of the previous antler from the base at early growing stage (control deer). **B**. The left antler regenerated following the similar treatment in 16A, but the antler formed from the denervated antler growth region (treated deer). Note the similarity. **C**. Partial antler regeneration (arrow) from an antler remnant (asterisk), which was created by removal of the 55-day-growth antler at the level 2 cm above antler/pedicle junction. In this case, only a small spike antler regenerated from the anterior margin of the antler remnant. **D**. Remnants of the proper antler (asterisk) and the small regenerated antler (arrow) that was created by removing the antler at the same level of the proper antler remnant. Note that the small antler only regenerated from the peripheral tissues including the skin and the periosteum. **E**. Double-head formation (refer to the text). Note that the new antlers could only regenerate from the pedicle periosteum and skin as previous hard antler (arrow) failed to cast. **F**. scar (arrow) was formed on a 55-day-growth antler at the place where main beam and brow tine were bifurcated. (16A and 16B: reproduced with permission from [78]; 16C-16E: reproduced with permission from [5]).

AT TISSUE AND CELLULAR LEVELS

The blastema formed on a newt limb stump is a type of avascular tissue; capillaries do not invade the blastema until it is fully formed [77]. However, histological and immunohistochemical data (Fig. **17A** and **17B**) demonstrate that early regenerating antler buds are richly vascularised.

In the newt limb stump, proliferating cells are evident throughout the blastema [75]. In marked contrast, in the early regenerating antler buds, dividing cells are found predominantly in the mesenchymal layer (Fig. **17C**) and in the vascular walls of the precartilage zone (Fig. **17D**), suggesting a regenerating antler bud contains localised growth centres, which is hence different to the typical regenerating blastema.

The ability to delay the basal lamina formation until after a newt limb blastema has fully formed is the feature that distinguishes regenerative from non-regenerative appendages [77]. The basal lamina is a thin layer located between the epidermis and the dermis. Therefore, if antler regeneration were to take place through initial blastema formation, it might be expected that the basal lamina be absent in the healing skin over a pedicle stump. However, this is not the case [13, 34], a well-developed basal lamina being evident throughout the healing skin over the pedicle stump (Fig. **17E** and **17F**).

It is well established that metalloproteinase enzyme MMP9 plays a major role in histolysis [77], and is crucial for cells at the amputated surface of a newt limb stump to migrate, dedifferentiate and accumulate under the wound epithelium, to form the blastema. The wound healing phase in newts is therefore always associated with high levels of MMP9 expression [77]. MMP9 signal, however, is not detectable in the regions of wound healing over the cast surface of a pedicle stump by *in situ* hybridisation [34], although it is clearly evident in the newly formed cartilage region underneath each newly established growth centre (Fig. **17G** and **17H**). This finding suggests a possible role of MMP9 in cartilage degradation and in facilitating tissue remodelling. These observations further support the notion that migration and dedifferentiation of pedicle periosteal cells are not significantly, if at all, involved in the process of antler regeneration.

Collectively, these comparisons demonstrate that stem cell-based antler regeneration is fundamentally different from the blastema-based newt limb regeneration process.

ANTLER RENEWAL-A MODEL FOR MAMMALIAN EPIMORPHIC REGENERATION?

The ultimate goal of studying antler regeneration is not to satisfy one's curiosity about this unique phenomenon, but to learn whether it can be used as a useful model for the investigation of epimorphic regeneration in mammals including human beings. Through the course of evolution vertebrates have gradually lost the ability to replace their missing appendages [75], such that mammals now retain only a negligible potential to regenerate digit tips after damage [81]. The consequences of mammalian limb loss has been studied his-

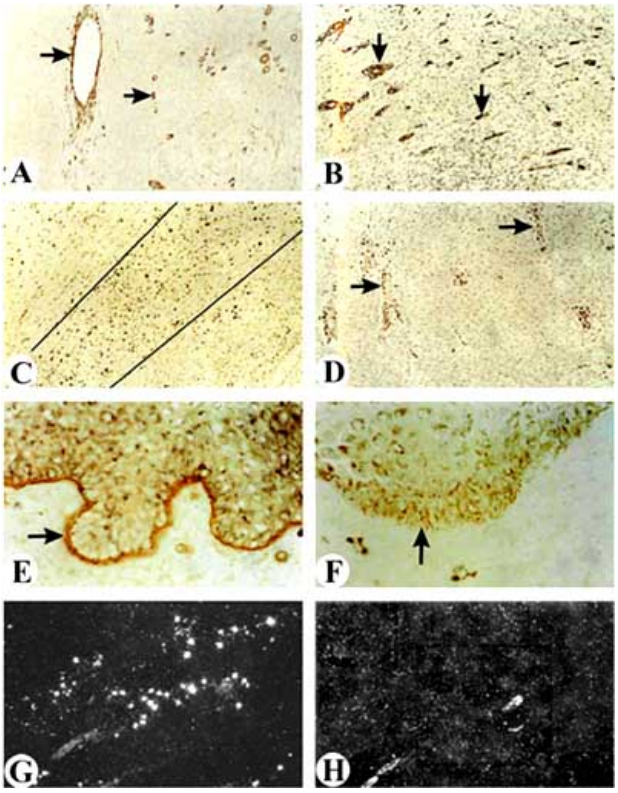

Fig. (17). Characterization of antler regeneration at tissue/cell level (A-F, Immunohistochemistry (IHC); G-H, in situ hybridization). **A** and **B**. Stained with smooth muscle actin antibody to show blood vessels (arrows). Note the growth centre are rich in vascular system. **A**. lateral to the newly established growth centre; **B**. In and above a newly established growth centre. **C** and **D**. Stained with Ki67 antibody to detect dividing cells. **C**. Mesenchymal layer (within the solid line); **D**. Precartilage layer. Note that most of the cells in the mesenchymal layer and the cells of the vascular channels (arrows) in the precartilage layer are positively stained. **E** and **F**. Stained with laminin antibody to detect the basal lamina layer. **E**. Stained with laminin antibody, a well-formed basal lamina layer is evident (arrow). **F**. Stained with IgG (negative control), the basal lamina layer is absent (arrow). **G** and **H**. *In situ* hybridisation using a matrix metalloproteinase 9 (MMP9) probe to detect MMP9 expressing cells. **G**. Hybridised with MMP9 anti-sense probe and MMP9 expressing cells (arrows) located in the vascular channels within the newly formed cartilage tissue. **H**. Hybridised with MMP9 sense probe and no MMP9 expressing cells were detected (Reproduced with permission from [34]).

tologically in some detail using a mouse model [82]. Externally, a full thickness of skin heals the stump wound, with scar formation being the final outcome. Internally, the distal periosteal cells of the stump are activated by the mechanical trauma and enter a rapid phase of proliferation and differentiation (Fig. **18A**). Subsequently, a significant amount of cartilage (Fig. **18B** and **18C**) is formed around the distal end of the stump by these activated periosteal cells. At the same time, a limited amount of cartilaginous cells migrate centripetally over the amputation plane to seal the open end of the long bone (Fig. **18B**). Shortly thereafter, the newly formed cartilage is quickly remodelled into bone (Fig. **18D**). Interestingly, the processes occurring in the stage of early antler regeneration are surprisingly similar to those observed in the healing processes over the wound of the mouse limb stump. In both cases, 1) the wounds over the stumps are healed with the full thickness of skin with scar formation, although in most cases scars formed over pedicle stumps are much less obvious [34]; 2) cells from both distal stump periostea are activated to enter the mode of rapid proliferation and differentiation to form cartilage; and 3) significant amounts of cartilaginous tissue is formed surround the distal ends of the stumps, and a very limited amount of cartilage is found to cover the amputation/cast planes. The most notable difference to set these two processes apart is the potential of the periosteal cell populations to proliferate: in the mouse limb, proliferation ceases as soon as the newly formed cartilage seals the open end of the amputated long bone; whereas, in the deer PP cell populations continue to expand until the entire antler is fully regenerated.

Recently, Gargioli and Slack [83] reported that the regeneration of the Xenopus (anuran amphibian) tadpole tail adopts a mechanism that is completely different from that previously found in the appendage regeneration of newts (urodele amphibian). Notably, regeneration of the Xenopus tail does not involve the processes of cell dedifferentiation or metaplasia (conversion of a type of differentiated cells to another), and each compartment (spinal cord, notochord, and muscle) regenerates through the proliferation and subsequent differentiation of tissue-specific reserves of undifferentiated cells (stem cells). This has led to the predication by these authors that any epimorphic regeneration that might be stimulated in mammals will be closer to the anuran amphibians (stem cell-based), rather than to those occur in the urodeles (blastema-based). The growing body of data on deer antler renewal, the only acknowledged instance of naturally occurring epimorphic mammalian regeneration, would seem to support this contention. In any case, the understanding of antler replacement mechanisms should provide valuable insights into the future possibilities for the rapidly developing fields of human regenerative medicine.

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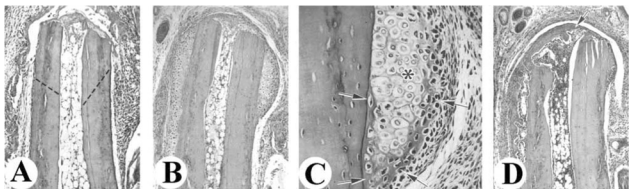

Fig. (18). Bone healing over a mouse limb stump (vertical histological sections; reproduced with permission from [82]). **A**. Distal end of a limb stump only minutes after amputation. Note that death and disappearance of bone cells have already started to the dashed lines. **B**. A week later after amputation, periosteum continued to thicken and substantial amount of hyaline cartilage was formed surrounding the distal end of the stump bone. **C**. Higher magnification of the newly formed cartilage region in 18B to clearly show the impressive quantity of cartilage tissue mass (arrows and asterisk). **D**. Over 18 days, healing process was stabilized and all the cartilage tissue has converted into bone (arrow).

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