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REVIEW ARTICLE

New physiological insights into the phenomena of deer antler: A unique model for skeletal tissue regeneration

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SUMMARY

Generally, mammals are unable to regenerate complex tissues and organs however the deer antler provides a rare anomaly to this rule. This osseous cranial appendage which is located on the frontal bone of male deer is capable of stem cell-based organogenesis, annual casting, and cyclic de novo regeneration. A series of recent studies have classified this form of regeneration as epimorphic stem cell based. Antler renewal is initiated by the activation of neural crest derived pedicle periosteal cells (PPCs) found residing within the pedicle periosteum (PP), these PPCs have the potential to differentiate into multiple lineages. Other antler stem cells (ASCs) are the reserve mesenchymal cells (RMCs) located in the antlers tip, which develop into cartilage tissue. Antlerogenic periosteal cells (APCs) found within the antlerogenic periosteum (AP) form the tissues of both the pedicle and first set of antlers. Antler stem cells (ASCs) further appear to progress through various stages of activation, this coordinated transition is considered imperative for stem cell-based mammalian regeneration. The latest developments have shown that the rapid elongation of the main beam and antler branches are a controlled form of tumour growth, regulated by the tumour suppressing genes TP73 and ADAMTS18. Both osteoclastogenesis, as well as osteogenic and chondrogenic differentiation are also involved. While there remains much to uncover this review both summarises and comprehensively evaluates our existing knowledge of tissue regeneration in the deer antler. This will assist in achieving the goal of in vitro organ regeneration in humans by furthering the field of modern regenerative medicine.

The Translational potential of this article: As a unique stem cell-based organ regeneration process in mammals, the deer antler represents a prime model system for investigating mechanisms of regeneration in mammalian tissues. Novel ASCs could provide cell-based therapies for regenerative medicine and bone remodelling for clinical application. A greater understanding of this process and a more in-depth defining of ASCs will potentiate improved clinical outcomes.

Introduction

Tissue regeneration is the physiological process by which tissues repair or replace themselves, enabling the regrowth of organs and body structures associated with aging or injury [45]. Physiological regeneration, such as the seasonal replacement of deer antlers, occurs for the maintenance of homeostasis during the life of an organism; whilst reparative regeneration occurs in response to injury [45]. The basis of tissue renewal in most species is dependent on the proliferation and differentiation of stem cells [28,79,104]. Advances in stem cell biology have shown that regional populations of stem cells reside within specialized and instructive anatomical microenvironments, called 'niches' [93,98], and are regulated by external signals from the extracellular matrix (ECM), and internal signals controlled by intracellular gene expression [20]. Regenerative tissue capability varies across organs, organisms, and species [89]. For example, the axolotl, an urodele amphibian, has astounded researchers by its extraordinary regenerative ability, and is capable of re-growing a spinal cord, heart and entire limbs

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into adulthood [96]. In comparison, mammals possess very limited organ and tissue regenerative capacity compared to lower vertebrates. Further, the capacity of mammalian tissues to regenerate generally declines into adulthood, together with the decreased regenerative capacity of stem cells [117]. Deer antlers are a unique cranial appendage belonging to the phylogeny Cervidae, and have remarkable properties enabling their periodic regeneration throughout the life of an individual. Rapid growth rates approximating 1.7 cm/day, and generating up to 30 kg of bone tissue within a few months, are reported for deer antlers, which exceeds the growth of certain cancerous tissues [92,112]. Thus the phenomenal interaction of cell growth factors and physiological systems regulating this process have led to the establishment of deer antlers as a model for the advancement of regenerative medicine. Recent advances have identified this cyclical regrowth as an epimorphic-stem cell based process as opposed to regeneration through the formation of a blastema [59,68]. In contrary, blastema-mediated epimorphic regeneration is impingent on the proliferation and accumulation of localised progenitor cells that are confined to a specific lineage [45]. Antler studies in vitro have successfully isolated and cultured novel antler stem cells (ASCs) found within the antlerogenic periosteum (AP), pedicle periosteum (PP) and reserve mesenchyme (RM) that are crucial for tissue regeneration. Histological and morphological examinations have also showed the presence of multiple tissue types within the growth region of the antler [61]. The aim of this review is to elucidate the current understanding of the biological processes underpinning tissue regeneration in deer antlers and to assess the future trajectory of this research. Uncovering the molecular basis and evolutionary origins of tissue regeneration in deer will not only allow us to better understand the specialised process of tissue regeneration in humans, but will also aid in therapeutic interventions for cancer as well as bone and cartilage repair.

Histogenesis of pedicle and first antler development

Deer antlers are paired outgrowths of true bone found on the foreheads of male deer, which are covered externally in a layer of hair-like skin known as velvet. In contrast to normal deer scalp skin, velvet contains a thickened epidermis, larger sebaceous glands and an extraordinary potential for expansion [36]. Antlers grow directly from pedicles, a permanent protuberance of the frontal bone, which provides the base for antler growth [10,60]. Deletion and insertion experiments showed that without the pedicle, no subsequent antler growth takes place [38], suggesting that it is the pedicle bone that gives rise to the tissues of the antler and therefore highlighting its significance [64]. Both pedicle and antlers are enveloped in periosteum, a thick membrane located on the surface of bone [37]. This layer of tissue functions to support bone growth and repair [25]. The pedicle and antler are structurally comprised of fibrous tissue, bone, cartilage, nerves and blood vessels [8,59,60,92]. The development and histogenesis of pedicles occurs at puberty during the first year of life, and the timing of this process varies across the different deer species; [52]. The mechanisms of pedicle formation are largely unknown, however it is known that the pedicle originates from the AP [67]. This is a special type of periosteum located in a distinct region of the frontal bone [67]. It is approximately 2.5 cm in diameter, 0.25–0.30 cm in thickness and has been proposed to be a postnatally retained embryonic tissue [67];. The formation of the antler (the first set) occurs after birth at age one, it is also derived from the AP [50].

Male deer antlers are used for fighting and display, and their cyclical growth is thought to be linked to the reproductive cycle. During antler regeneration, an increase in systemic testosterone level is thought to initiate histogenesis of the pedicle, and to provide the activation signal for the antlerogenic periosteal cells (APCs) in the AP (Fig. 1). However, experimental findings indicate that the role of the sex hormones in regulating the cycling and differentiation of APCs is complex, and possibly modulated by the effect of additional signals from hormones, such as oestrogen. Additional non-sex hormones also appear to affect cyclical antler growth, such as vitamin D, thyroid hormones, and cortisol [13,101,103,105,108]. The AP exhibits profound regenerative and genetic properties by continuing to grow antler tissue upon ectopic transplantation, and by the generation of entire cloned deer calves from the process of somatic cell nuclear transfer (SCNT) [8,37]. The regenerative properties and genetic potential of APCs (and ASCs) are further demonstrated by their capacity for deer antler organ formation, their expression of mesenchymal stem cell (MSC) markers (CD73, CD90, CD105 and Stro-1), osteogenic lineage differentiation, and partial attributes of embryonic stem cells (ESCs). APCs have the ability to give rise to mammalian organs and tissues, including the skin, blood, nerves, eyes, cartilage, connective tissue and bone [8]. The potential for exogenous antler generation by the pedicle and APCs was investigated by a in vivo xenograft approach in mice, which demonstrated that antler formation requires reciprocal interaction of the pedicle and overlying deer skin [97]. Whilst the transplantation of the AP into a nude mouse head results in the formation of a proportionally sized ectopic pedicle, mouse skin was incapable of inducing antler formation [63]. The nude mouse transplantation model by Li et al. [97], is considered to be of lower quality and

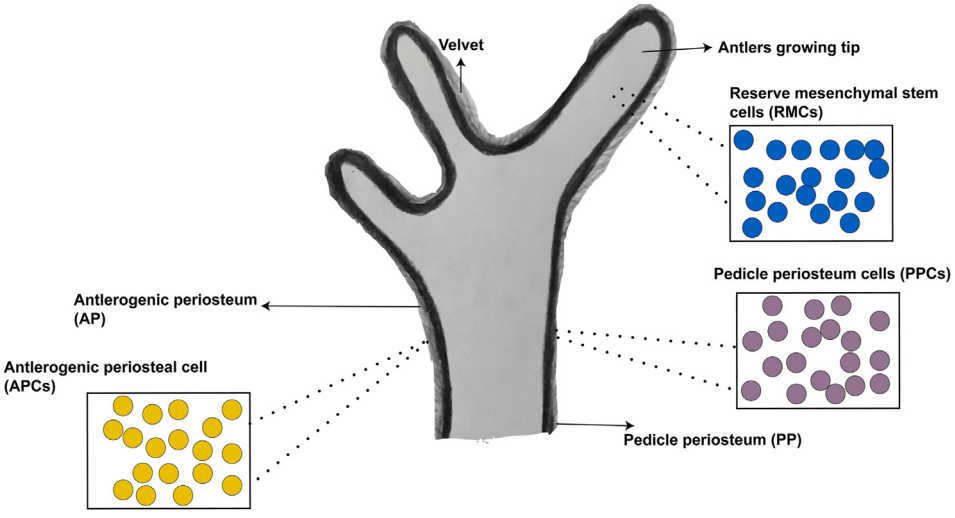

Figure 1. Types of potential antler stem cells. Antlerogenic periosteal cells (APCs) are found within the antlerogenic periosteum (AP), these cells function in pedicle and first antler formation. Reserve mesenchymal stem cells (RMCs) are located in the antlers growing tip and form the cartilage tissue of the regenerating antler. Pedicle periosteal cells (PPCs) are localised within the pedicle periosteum (PP) and form the antler growth centre (AGC).

is highly susceptible to error, despite its convincingly strong conclusions on the heterotypic tissue interactions involved in antlerogenesis and pedicle formation. However, it is unclear if the interplay between the two tissues types (the AP and deer skin) are required for the initiation of pedicle formation or for sustaining its growth.

Pedicle development is thought to occur in four stages, the first stage is intramembranous ossification whereby recruited APCs proliferate and differentiate into osteoblast-lineage cells [66,92]. These osteoblast cells subsequently form the trabecular bone of the periosteum under the regulation of genes, including osteocalcin (OCN) or secreted protein acidic and rich in cysteine (SPARC), transforming growth factor beta-1 (TGFβ-1), and S100A4 [62,92]. The second stage occurs once the pedicle has reached 5–10 mm in length and is termed transitional ossification [92]. In this stage APCs differentiate into chondrocyte forming the interior osseocartilaginous tissue of the pedicle [92]. The third stage is endochondral ossification where chondrogenesis takes place solely in the pedicle [92]. In the final stage of pedicle formation APCs sustain their chondrogenic differentiation pathway until the completion of first antler formation, termed antler endochondral ossification [92].

Although APCs have been anatomically and histologically reasonably well characterised, the specific growth factors, signalling pathways and genes regulating their self-renewal, proliferation, and differentiation during antler and pedicle development have not been fully determined [8]. In vitro studies have demonstrated that insulin-like growth factor-I (IGF-I), a hormone produced in the liver, might have a role in pedicle and first antler formation and growth, and appears to stimulate APC proliferation and differentiation during each ossification stage [92,97,106]. Retinoid acid (RA), a derivative of vitamin A, appears to play an important role in regulating deer antler pedicle formation, growth and development, and could potentially increase the rate of proliferation of APCs in vivo [48]; [2,3]; [90]. Analysis of proteome and molecular signalling pathways of ASCs found that several pathways, including PI3K/Akt, ERK/MAPK, and p38 MAPK are activated during proliferation, as are ESC transcription factors, such as POU5F1, SOX2, NANOG and MYC [62]. Characterization of the growing deer antler proteome identified approximately 130 distinct proteins, and indicates the need for further research to determine and discover vital regulatory pathways of antler development [86].

Morphological characteristics of deer antlers vary between species. For example, pedicle length in Sika deer (*Cervus nippon*) will grow to be between 40 and 50 mm, whereas in Red deer (*Cervus elaphus*) its length ranges between 50 and 60 mm [33,63]. Growth at the primary antler tip appears to occur once the pedicle reaches a certain species-specific threshold, and may broadly coincide with a decrease in individual testosterone level [51]. At this stage, across species, presumably in response to functional demands, a change in the skin's composition from pedicle scalp skin to velvet occurs [60]. These primary antlers are much smaller and unbranched in comparison to the seasonally regenerated antlers, whose lateral branches and complexity tend to increase with age across species [10,52,60]. Collectively, findings indicate that the growth of first antlers and subsequent seasonal or secondary antlers is regulated by a combination of mechanical interaction between the antlerogenic tissue and overlying skin, and intercellular communication and molecular signalling mechanisms potentiated by ASCs [60,63,71]. Further research is needed to elucidate the species-specific histogenetic characteristics of deer antler formation, growth and development.

The anatomy and physiology of antler tissue regeneration

Antler regrowth has been anatomically well described by several publications [92];. Following considerable debate and extensive research, this form of tissue regeneration is considered to be stem-cell based [4,59,68]. Cyclical regrowth of antler appears to be coordinated with the reproductive cycle, and to be regulated by both the endocrine system and various environmental factors (Fig. 2) [57]. The molecular mechanisms regulating antler stem-cell based regeneration, such as the interactions between localized tissue, endocrine and environmental factors, are largely unknown. Regeneration begins annually in late spring following the casting of the previous 'hard' antlers, which are essentially dead bone, coinciding with the testosterone cycle [37,88,105];. Antler casting involves the shedding of the overlying velvet and periosteum, mineralisation of the existing antler bone, and osteoclastic bone resorption in the distal pedicle at the abscission line between pedicle and antler [39,54];. Observational studies indicate that immediately following antler casting, the pedicle bleeds substantially, and a scab covering approximately half of the casted surface is produced within 1–2 days, which is eventually smoothed by osteoclastic activity within the distal pedicle [39]. A swollen ring of skin containing a shiny outer surface known as 'wound epithelium', and consisting of epidermal and mesodermal-derived cells, subsequently migrates to cover the pedicle stump, which serves as the first indicator of antler regrowth [39]. The antler bud that branches and elongates is thought to be derived from the tissues of the pedicle stump/PP [39,59]. The precise cellular mechanisms affecting the early stages of the antler regeneration (~14 days) are yet to be elucidated, and highlight a vital area of future research. During early summer rapid longitudinal, bone-tissue growth of the main beam and lateral branches, referred to as 'tines', occurs at an astounding growth rate approximating 2.75 cm/day [54];, which coincides with low levels of circulating testosterone, reproductive inactivity of the deer, and an abundant food supply [36]. Throughout this growth period, the antlers become enveloped by an integument which supplies the growing antler with blood from the arteries of its vascular layer, and is critical for the metabolic demands of the regenerating tissue [30]. The integument is highly innervated by sensory fibres of the trigeminal nerve, whose exons grow at a pace accompanying that of the antler (2 cm/day) [83];. Rapid antler bone growth occurs through a combination of modified endochondral ossification within each antler distal tip following cartilage formation, and intramembranous ossification within the shaft which increases antler diameter [6,54]. Modified endochondral ossification involves extensive resorption of mineralised cartilage by osteoclast or chondroblast cells [30], followed by the secretion of bone matrix by osteoblasts, and subsequent mineralisation. In vitro findings suggest that antler osteoclast-like multinucleated cells (MNCs) are phenotypically comparable to osteoclasts of mammalian bone, as exemplified by their

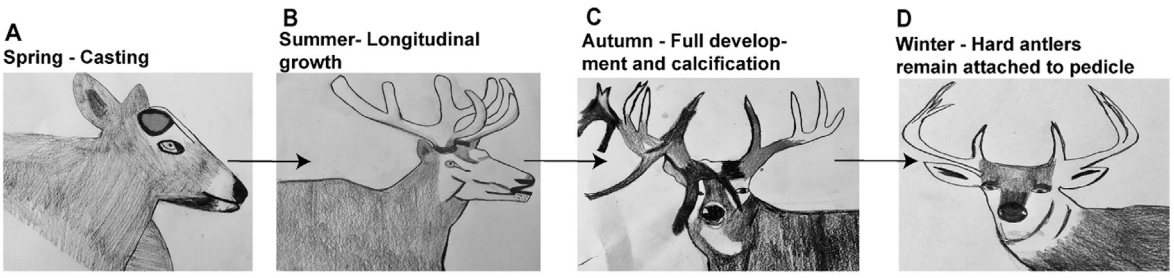

Figure 2. Timeline of the different stages of deer antler regeneration (A) Spring – Casting **(B)** Summer -Longitudinal growth **(C)** Autumn - Full development and calcification **(D)** Winter - Hard antlers remain attached to pedicle. AP.

expression of tartrate-resistant acid phosphatase (TRAP) with mineralized tissue resorptive activity [30]. However, the culture conditions provided by antler non-mineralized cartilage cells were sufficient to induce osteoclastic differentiation of MNCs, and the expression of factors normally required for osteoclastogenesis, receptor activator of nuclear factor-kappa B (RANKL) and macrophage colony stimulating factor (M-CSF) [30]. Further, osteoclast differentiation in the regenerating tissue of deer antlers seems to be regulated by parathyroid hormone (PTH)-related protein (PTHrP)/PTH(rP) receptor (PPR) signalling [29], Indian Hedgehog (IHH) and transforming growth factor-beta 1 (TGF-β1) [31]. When the rutting season approaches in late summer/autumn the developing antlers remain attached to the pedicle, their blood and nerve supply recedes, and the antlers undergo full mineralisation and maturation [72]. Further research is needed to investigate the unique bone and cartilage mineralisation and remodelling processes, including cellular differentiation and signalling pathways, affecting deer antler regeneration.

The antler growth centre (AGC), located at the tip of the antler, is a primary growth centre [97]; [90]. Morphological and histological studies indicate that the AGC consists of four distinct zones [97]; [90], which may be considered to recapitulate the distal zones of the differentiating embryonic endochondral long bone. In distoproximal order, these zones are the proliferation, maturation, hypertrophy and calcification zones [61]. Within the proliferation zone (where a majority of antler growth takes place), three main tissue types may be discerned: the reserve mesenchyme (RM), pre-cartilage (PC) and cartilage (CA) [5,61], although the partitioning between these tissues may not be clearly defined histologically, due to their immense vascularisation [53]. Li et al. by utilising standardised sampling techniques rapidly and accurately showed that these tissue layers appear to be structurally and functionally distinct, and vary in gene expression [61]. The identification of these highly organised tissue types within the antlers tip might provide new regenerative insights by enabling future molecular and functional analyses [61]. The figures included in the Li.et al. study, however, only allow for visualisation at the microscopic level [65]. Further, the established tissue dissection methods/protocols are specific for the red deer species, limiting their reproducibility [61].

Rapid growth at the tip of deer antlers is sustained by distinct populations of ASCs. Reserve mesenchymal stem cells (RMCs) within the RM undergo intense and sequential proliferation [83]; [112,114,115], mediated by signals from growth factors, including melatonin and IGF-1 [118,119]. Chondro/osteogenic differentiation of RMCs is regulated by the increased expression of genes which are characteristic of robust mammalian cartilage and bone development, including fibroblast growth factor (FGF), TGF-β, Wnt, and bone morphogenetic proteins (BMPs) [41, 92]. Perichondrium-derived cells (PER) are multipotential progenitors found within the perichondrium, a fibrous tissue layer continuous with the periosteum that is the site of intramembranous bone formation; [92]. In contrast to RMCs, PER cells in vitro were found to express high levels of type I collagen mRNA and protein, a phenotypic marker for cells of the osteoblast lineage; [90], however, their low levels of the enzyme alkaline phosphatase (ALP) are indicative of a pre-osteoblastic state [92].

Morphological and histological studies of antler regeneration have documented the importance of stem cells within the PP, called pedicle periosteal cells (PPCs). During annual antler regeneration, PPCs are recruited for proliferation and differentiation for the formation of antler tissue [59];. PPCs are a multipotent type of ASC, which are relatively few number and reside exclusively within the PP, a membrane derivative of the AP; [59]. The PP membrane is found along the rim of the pedicle stump and underneath the newly grown velvet, developing from the differentiation of approximately 5 million APCs. During the early wound healing stage following antler casting, the PP situated at the distal pedicle becomes markedly thickened. This thickening is indicative of the high proliferative activity of the PPCs. Wang et al., conducted the first comprehensive molecular characterisations of the ASC lineage (PPCs, RMCs, and APCs), and the findings from this study are considered to be of excellent quality. The study successfully confirmed the ability of ASCs to form large colonies in vitro, identified their potent immunosuppressive capabilities, and verified the expression of several MSC receptors. Similarly, Seo et al. [102], previously isolated, cultured, and partially characterised and defined ASCs, by utilising fluorescence-activated cell sorting (FACS), immunophenotyping, and immunostaining. The quality of this study is considered fair, however, it is important to carefully interpret many of their results due to certain limitations and unexpected findings. For example, ASCs surprisingly did not express the markers Abcg2, CD90, AP, Nanog, and SSEA1 [102]. Furthermore, due to the current limited availability of stem cell markers, various animal-specific and human host antibodies were used in this experiment, instead of deer-specific antibodies [102]. It is suggested that for future ASC characterisations studies and to verify ASC identification, deer-host species antibodies should be developed and utilised [102]. PPCs express classic mesenchymal markers, CD73, CD90, CD105 and Stro-1, and appear to have certain ESC characteristics, including the expression of Tert, Nestin, S100A4, nucleostemin and C-Myc, indicating their potential for mammalian tissue regeneration. Additional in vitro findings showed that PPCs express pluripotency genes, Oct4, Sox2 and Nanog, and could undergo differentiation towards mesenchymal (osteo-, chondro-, and adipocyte) and neural cell lineages [70]. PPCs appear to initiate the formation of growth centres, and to influence the development of both the external (nerves, blood vessels and skin) and internal (cartilage and bone) tissue components of the regenerating antler [59];. The growth centres are crescent-shaped and consist of bundles of cartilage covered in a layer of perichondrium, forming the main beam and first tine of the antler [59];. Deletion and insertion experiments have demonstrated the importance of the PP (and PPCs) for annual antler regeneration; [59]. For instance, surgical removal of the PP membrane prior to regeneration inhibited antler regrowth, indicating that PPCs are essential for the initiation of antler regeneration and subsequent bone and cartilage tissue formation [59]. The full and partial PP deletion experiments conducted by Li et al., indicated that the low potential risk of error is partly due to significant technical challenges associated with tissue deletion and transplantation experiments. The study selected a moderate sample size of eight deer (four yearling and four 2-year old stags), however further studies with larger deer numbers are indicated due to the inconsistency and variability of the results. For instance, full deletion of the PP membrane in some deer delayed regeneration, whilst in others it entirely inhibited antler regeneration. Further research is necessary to develop the immense potential of PPCs for regenerative medical applications.

Several in vitro studies have demonstrated that ASCs transition through various states of activation (dormant, active and post active), during distinct phases of deer antler tissue regeneration. Critical investigation and accumulating evidence on the activation and regulation of ASCs indicates that stem cell activation might be essential during antler regrowth. ASC activation and differentiation are thought to be induced and maintained by several biological systems and signalling pathways. Thus, antler regeneration provides an exceptional opportunity to study the proteomic control of neural derived stem cell niches during organ regeneration. A recent in vitro study utilised label-free quantification and ingenuity pathway analysis (IPA) to investigate the regulation of ASC activation by examining specific protein expression in situ obtained dormant PP, activated ASCs within the AGC, facial periosteum cells and post activated mid beam AP [24]. The immunohistochemical findings of this study indicate that many unique mesenchymal markers such as CD73, CD90 and CD105 are discerned in the RM, PC and C tissues within the antlers tip, and upregulated during specific periods of regeneration, subsequently controlling ASC activation [24]. The Hippo and canonical Wnt signalling are also considered to be prime candidates for mediating ASC activation. The overall quality and external validity of the results obtained from the Dong et al. study [24], are considered sufficient but do not entirely reflect true values. This is due to changes in the antler stem cell niche during the isolation and culture of ASCs in vitro, which affect protein expression [24]. To verify these findings, future in vitro studies

could be designed with culture conditions designed to recreate the in vivo microenvironment.

Clinical application and therapeutic potential of ASCs

In stem cell biology and regenerative medicine, different stem cells isolated from a variety of large animals such as, horses and pigs, are highly sought after [102]. Human embryonic stem cells (hESCs) are considered to be the "gold-standard" in cell-based regenerative therapies, however, there are many significant challenges for clinical translation [55]. The recent identification and partial molecular characterisations of ASCs in deer antlers, and their speculated wide-ranging potential for therapeutic application in regenerative medicine and bone/osteoporosis research has prompted avid interest amongst clinicians and scientists [102];. Isolated ASCs, such as PPCs are unique and could provide a novel source of stem cells [102]. In contrast to other stem cell types, ASCs are considered advantageous owing to their easy isolation, high proliferative and differentiation ability, and ex-vivo expansion [95,102]. Recent preclinical in vivo studies show promising results by indicating that ASCs are not animal species-specific, and can stimulate cutaneous wound healing and reduce liver fibrosis in rat models [94]. Both studies by Rong et al. [94,95], tested on suitable animal models, presented a fair level of evidence, an elementary exploration of interventions, and further provided a sound basis for potential clinical translation. However, ASCs are heterologous to humans and are not able to be directly administered by injection [22]. Furthermore, the efficacy of ASC cell-based therapies in vivo rat models does not ensure its successful administration in human patients. These preclinical animal experiments still remain controversial as they cannot be used as a means to predict the efficacy or safety of ASCs in clinical trials. Therefore, ASCs cannot move towards future human trials without a systematic analysis of all preclinical data from animal testing. To date, there is no existing data from human clinical trials on the efficacy and/or safety of potential antler derived stem cell-based regenerative therapies or combination therapies, specifically for bone remodelling in patients [102]. Prior to the potential widespread use of deer antlers in a clinical setting, a concerted effort is required to expand our knowledge of ASCs, and the potent regulatory systems which control the antler stem cell regenerative process. This is due to the inadequate quantity and quality of studies on ASCs, and thus additional preclinical animal model studies are essential [102]. Further to this, comparative studies contrasting ASCs to various human cells, such as human mesenchymal stem cells (hMSCs) are required [42]. Additionally, identifying and overcoming the barriers in the translation of ASCs to the clinic at an early stage is considered to be crucial and of great duty to society.

Despite the deer antler being recognised as a suitable experimental model for skeletal tissue regeneration, and a promising therapeutic strategy for human disease, there are many constraints towards its wider application and clinical use [92]. Firstly, xenogeneic stem cell transplantation treatments are contentious, raising major ethical and legal concerns [95]. These preliminary concerns include animal rights, man intervening with nature, the potential introduction of pathogenic agents into humans, obtaining patient informed consent, and the combining of human and animal cells/genes. Secondly, there is profound variability between human skeletal tissue and antler tissues, and major differences in their lifespan, which could potentially lead to incorrect physiological functioning [113]. Thirdly, the clinical application of ASCs presents a safety hazard, and is limited by the risk of immune rejection, sensitivity under toxic conditions, senescence, and low availability [44]. The long-term effects of transplanted stem cells are also unclear and require extensive research. Additional shortcomings include the potential rigorous use of a large, wild, non-model organism, which is of great ecological importance and complexity [92]. Antler regeneration appears to be initiated seasonally, by systemic levels of reproductive hormones, as opposed to being induced by injury or trauma, which limits their use in research and regenerative medicine [92].

Growth factors and signalling pathways regulating antler tissue regeneration

The growth factors and cellular signalling pathways controlling antler regrowth are largely unknown. Both local and systemic factors have been linked to antler regeneration, and the molecular mechanisms affecting antler growth are thought to be similar to those involved with the regulation of embryonic development [31]. Antler regrowth is linked to the reproductive cycle and appears to be regulated systemically by the sex hormones, including testosterone and oestrogen (Table 1). Additional systemic factors, such as vitamin D, calcium, and melatonin seem to affect antler growth [108];. Locally, numerous growth factors have been found to regulate the cellular processes of antler regeneration, such as PTHrP, RA, and IGF-1 [106];. Therefore, there appears to be an array of growth hormones affecting antler regeneration, and here we explore the possible genetic elements, molecular mechanisms and signalling pathways mediating their effects.

The annual regrowth of male deer antlers is timed to coincide with the reproductive cycle, and is thought to be regulated by systemic levels of reproductive hormones [32,105];. Testosterone appears to be the principal regulator of the timing of antler growth, however, the intense growth of antlers is likely to be affected by the interaction of numerous hormones, such as oestrogen, gonadotrophin-releasing hormone (GnRH), luteinizing hormone (LH), cortisol, and prolactin [7,32]. Of the reproductive hormones, oestrogen appears to limit progenitor cell proliferation and to promote their differentiation in the antler tip. Further, the interaction of growth factors, such as IGF-1, with testosterone, is consistently found to affect the intense growth of deer antlers in vivo [7,106]. Autoradiographical histological findings support the notion that IGF-1 is an endocrine factor involved with regulating the growth of deer antlers by interaction with testosterone [26,27]. Further research is needed to improve our understanding of the precise roles, timing, and interactions of reproductive hormones and growth factors in regulating cyclical deer antler growth.

Parathyroid hormone-related peptide (PTHrP) appears to have local effects on deer antler growth. In humans, PTHrP signalling via its receptor, PTH type-1 receptor (PTHR1 or PPR), is considered to regulate skeletal development, bone metabolism, and mineral ion homeostasis [18]. Classical animal and in vitro studies indicate that PTHrP coordinates the process of endochondral ossification during skeletal development by regulating chondrocyte differentiation [21,58,109].

Table 1
Hormones, growth factors, and signalling pathways mediating antler tissue regeneration.

Hormones, growth factors, and signalling pathways	Role in antler tissue regeneration	Reference
Testosterone	• The predominant regulator of the timing of antler renewal.	[32]
Oestrogen	• Limits progenitor cell proliferation and promotes its differentiation within the antlers tip.	[90]
IGF-1	• Interacts with testosterone to affect intense antler growth. • Proliferation of ASCs in antlers tip	[106]
Canonical Wnt signalling	• RMC apoptosis and survival • Regulates osteoblastic bone formation and chondrogenesis	[80]
Melatonin (MLT)	• Increases IGF-1/IGF-1R signalling • Promotes proliferation of antler mesenchymal cells	[119]
Parathyroid hormone-related peptide (PTHrP)	• Regulator of osteoblast, chondrocyte, and osteoclast differentiation. • Controls the self-renewal and timing of chondrocyte.	[21]
Retinoid acid (RA)	• Controls the differentiation of chondrocyte, osteoblasts, and osteoclasts.	[90]

PTHrP mediates the effects of IHH on chondrocyte by an autocrine/-paracrine negative feedback loop [109], which affects the rate and timing of chondrocyte differentiation, and osteoblast differentiation of the growth plate [21]. Mutations leading to the dysregulation of PTHrP/PTHR1 signalling are known to cause skeletal disorders, such as chondrodysplasias [100]. Subsequent research indicates that PTHrP regulates chondrocyte proliferation, differentiation, and maturation both by receptor-mediated signalling, and directly by nucleolar translocation [3]. In deer antlers, in vitro findings indicate that PTHrP might regulate the differentiation of osteoblasts, chondrocyte, and osteoclasts. In situ hybridization and immunohistochemical findings indicate that PTHrP may promote the proliferation and affect the timing of chondrocyte differentiation, leading to the regulation of antler growth [40,100]. Subsequent in situ hybridization studies indicate that PTHrP appears to attenuate chondrocyte differentiation, maturation, and cartilage matrix degradation by inhibiting the expression of matrix metalloproteinases, MMP9 and MMP13 [113]. During the antler rapid growth period PTHrP/PPR signalling is thought to regulate the differentiation of both osteoblasts and osteoclast cells within the perivascular tissues of antler cartilage. PTHrP/PPR signalling appears to be unique by its direct effect on osteoclast differentiation during deer antler regeneration, whereby PPR appears be localized to the nucleus, and PTHrP may affect osteoclastogenesis independently of RANKL [29]. Interestingly, the PTHrP/IHH/TGF-β signalling axis of endochondral ossification appears to be conserved during deer antler regrowth [31], whereby the developmental signalling pathways affected by IHH and TGF-β may form a PTHrP/PPR negative feedback loop for the regulation of bone and cartilage regeneration. Therefore, further research is needed to investigate the unique effects and signalling mechanisms of PTHrP involved with the regulation of cellular processes controlling deer antler regeneration.

Retinoid acid, RA, is an active hormone metabolite of retinol (vitamin A, or all-*trans*-retinol) which plays a critical role in vertebrate embryonic development and organ formation, such as the eye. A number of studies suggest the importance of RA for limb regeneration in amphibians, for example, high performance liquid chromatography based analysis of axolotl limb regeneration elucidated the effect of an anteroposterior RA gradient, which is involved with patterning of the limb [99]. Histological, immunolocalization, and in situ hybridization evidence indicates that RA is synthesized, and its receptors are expressed in the blastema and MSCs of developing deer antlers. Further in vitro findings support the effects of RA on regulating the differentiation of chondrocyte, osteoblasts, and osteoclasts during deer antler regrowth, however, further research is needed to determine the precise cellular signalling mechanisms and molecular pathways mediating these effects. Additional high-performance liquid chromatography, immunohistochemical, and in situ hybridization evidence suggest that RA is involved with the regulation of deer antler regeneration [3]. RA was found to be significantly expressed by deer antler tissues, including the skin, perichondrium, bone, cartilage, and periosteum [3]. Receptors for RA of the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families are characteristically expressed within the area of the antler tip that undergoes growth by endochondral ossification, including the velvet and perichondrium, and chondrocyte and osteoblast-lineage cells [3]. Further, immunolocalization of retinaldehyde dehydrogenase-type 2 (RALDH-2), a prime enzyme of RA synthesis, detected its expression within skin, perichondrium, and perivascular cells, including chondrocyte and osteoblast-lineage cells [3]. Subsequent in vitro findings indicate that RA may inhibit chondrocyte differentiation, and promote osteoblast differentiation, suggesting the need for further investigation [3]. RA treatment was also found to increase the growth of the pedicle and size of the first antlers in deer, and further research of the perichondrium cellular response is required to determine the mechanisms of this effect [48]. Together these findings suggest that RA regulates cyclical deer antler regeneration, and further research will help to determine the potential of RA for related regenerative medical applications.

Wnt ('wingless-type') are a large family of genes which are conserved by evolution, and encode numerous proteins across species (approximately 19 in humans), for the regulation of vital biological processes of embryonic developmental [56,85,116]. Wnt signalling affects diverse processes of embryogenesis, including patterning, organ formation and systems development, and cell fate [56]. Wnt signalling is mediated by complex intracellular pathways leading to the regulation of gene transcription within the nucleus, and today, our knowledge of the intricacies of Wnt pathways continues to evolve [116]. Wnt signalling is considered to be activated by the binding of Wnt proteins to surface transmembrane receptors, called Frizzled or Fz (a family of approximately 10 in humans), with co-activation of receptors, such as low-density-lipoprotein-related protein 5/6 (LRP5/6) [43,56]. Following Wnt binding, activation of the receptor complex appears to effect transduction of the signal to the phosphoprotein, Dishevelled (Dsh/Dvl), within the cytoplasm, where it may branch along one of three accepted pathways: canonical Wnt, non-canonical planar cell polarity, and non-canonical Wnt/Ca^{2+} [56,110]. Canonical Wnt signalling is classically mediated by the translocation of β-catenin to the nucleus for the regulation of gene transcription, and appears to be fundamentally important for bone homeostasis, by regulating osteoblastic bone formation and osteoclastogenesis for bone remodelling [11,116,120]. Wnt signalling (Wnts 10a, 7a, 5a, and 5b) also appears to regulate the regeneration of lost structures in lower vertebrates, such as the tail of urodele amphibians [16]; and Wnt signalling was investigated for its potential role in the regulation of deer antler regeneration. Immunocytochemistry and in vitro findings indicate that canonical Wnt signalling via β-catenin appears to be crucial for the survival, and possibly apoptosis, of ASCs within the mesenchymal tissue of growing antlers, and might regulate endochondral and intramembranous bone formation by osteoblasts and chondrocyte in a tissue-specific manner. The Mount et al., study is considered novel, well-designed and conducted, providing a strong evidence base with sound conclusions. However, it is important to recognise that the localisation of β-catenin for investigation of canonical Wnt signalling may potentially impact the validity of the results, since the expression of β-catenin is thought to be impacted by many Wnt-independent mechanisms. Recently, next-generation sequencing (NGS) technology was used to detect the expression of microRNAs (miRNAs) within deer ASCs from PP [4]. Wnt, together with mitogen-activated protein kinase (MAPK) and TGF-β, was one of 167 conserved and 20 deer-specific miRNA families that appear to be upregulated for the initiation of ASC potentiation within the PP during antler regeneration [4]. Therefore, further research is necessary to elucidate the complex regulatory network and effects of Wnt signalling involved with the regulation of cellular processes, including osteoblastic bone formation and chondrogenesis, specific for deer antler regeneration. The study by Ba et al. [4], appears to be of fair quality, as the paper accurately and quantitatively estimated miRNA expression profiles of PPCs during the potentiated (PPP) and dormant (DPP) stages of antler regeneration. It coheres to and extends upon a series of previous studies, and further used an appropriate sample size of six tissues obtained from three male sika deer [4]. This selected sample size enabled biological replication, which improved the reliability of the RNA-seq results.

Melatonin (MLT) is a hormone secreted by the brain's pineal gland in humans and is considered to affect the timing of circadian rhythms in response to darkness. In deer, melatonin appears to influence the reproductive cycle and onset of the breeding season, also depending on darkness, and might regulate antler growth and regeneration [1,14,74,115]. The mechanism of MLT/melatonin receptor 1 (MT1) signalling for the regulation of deer antler mesenchymal cells was studied in vitro. MT1 is thought to be expressed by the cambium layer of deer antler, and was detected in antler mesenchymal cells. Further, exogenous MLT lead to the proliferation of mesenchymal cells via MT1 signalling, and MLT also appeared to increase IGF-1/IGF-1R signalling, indicating a possible mechanism of co-activation of IGF-1/IGF-1R signalling by MLT for the proliferation of antler mesenchymal cells. However, the in vitro findings

reported by Yang et al., appear to be inconclusive, which detracts from the reliability and quality of the study. The localisation of the MLT receptors was investigated and determined, however, it is not accurate nor indicative of the full potential function of MLT in regeneration. Additional research detected single-nucleotide polymorphisms of the melatonin receptor 1A (MT1A), which may increase antler yield [119]. Thus, MLT appears to affect the regulation of deer antler regeneration, and further investigation is required to determine comprehensively its potential mechanisms and therapeutic applicability.

Genetic basis of antler regeneration and cancer resistance

Antler regeneration is considered to be a neural crest stem cell-based epimorphic regenerative process;. Currently little is known about the genes regulating the rapid tissue growth observed in deer antlers, and recent studies have utilized next generation sequencing technology to analyse the genomic basis of deer antler regeneration [5,113];. Further, the rapid growth rates observed in antlers (in excess of certain cancers), and the low cancer rate of cervids, indicate that distinct oncogenic inhibitory genetic processes of deer antler regeneration may provide clues for gene-based attenuation and treatment of human cancers. Following transcriptomic analysis of 221 cervid and bovid genomes, the presence of headgear (antlers and horns) is characterized by defined genetic elements, termed lineage-specific positively selected genes and conserved elements (HCEs), including 761 antler-specific and 201 shared genes. Genes distinctly expressed in antlers and horns (headgear genes) are often co-expressed within the tissues of the nerve, testis, bone and skin. Antlers are considered to derive from neural crest stem cells, and from an evolutionary perspective, findings from this study suggest that headgear-specific HCE-associated genes, TWIST1, SOX9, SNAI2, and the HOXD cluster may be involved in the reprogramming of neural crest cells for the development of headgear (Table 2) [9];. Additionally, headgear-specific genes included genes related to the migration of the neural crest cells and genes involved with neural processes of antler regeneration (SOX10, SNAI1, SNAI2, TFAP2A, NGFR, and COL11A2). Nine genes highly expressed in headgear as detected by this study (ALX1, VCAN, COL1A1, SATB2, RUNX2, POSTN, SP7 or OSX, TNC, and COL4A2) are classified as bone development genes by gene ontology (GO) [19];. The dataset of this large-scale in vitro genomic and transcriptomic study is thought to be of excellent quality and great statistical reliability. This is indicated by the large sample size of 16 tissues, that were obtained from 20 roe deer and 20 sika deer, including comparative analyses of the genetic profiles of both bovids and cervids within the ruminant mammalian group. In future studies, the combination of functional, physiological, and transcriptomic analyses with an extensive sample range across the various periods of antler renewal is necessary to verify the function of the identified antler-specific neural genes. SP7 (also called OSX) is a transcription factor known for its role in regulating osteoblast differentiation and bone formation [82]. RUNX2 gene expression is an established promoter of osteogenesis, and regulator of OSX transcription [34,84]. Additional in vitro RNA-seq model based findings identified the unique involvement of previously unknown genes in deer antler proliferation (40) and mineralization (91), including UHRF1 and S100A10 [113]. This simplistic and smaller-scale study was the first to identify unique genes that are expressed in the regenerating antler, and it appears to be of fair quality. Furthermore, the RNA-seq datasets comprise a large percentage of unannotated genes, which could be refined by utilising orthology-driven blast mapping [113]. Additional improvements to the study are suggested which might potentiate its impact, and refine future replication of this research [113]. Together these findings provide an extensive characterization, suggesting that deer antler regeneration is regulated by a network of hundreds and thousands of genes and HCEs, which is incompletely defined. Therefore, further investigation of the complex genetic regulatory networks, molecular signalling pathways, and their biological outcomes affecting deer antler growth, development, and regeneration is required.

Despite the rapid growth rates observed in deer antlers, the rate of cancer in cervids has been reported to comparatively lower than other mammals, leading researchers to seek explanations for the biological basis of this paradox [35,75]. Rapid antler growth is essentially driven by the processes of cell proliferation, bone, and cartilage formation, and the gene expression profiles of antlers and osteosarcoma are more highly correlated than those of antlers and normal bone. Further, the cell proliferation programs of antlers have similarities with those of cancer cell growth, however, in contrast to cancer tumours whose rapid growth is uncontrolled and pathological, antler tissue regeneration is a highly coordinated physiological process. Recent findings detected the positive selection of proto-oncogenes (FOS, FAM83A, and REL) in cervids during evolution, and the antler-specific expression of five growth factor and receptor genes (FGF19, FGF21, FGFBP3, PDGFD, and PDGFRL), which are implicated in cancer development [107];. Recent findings also indicate that cancer resistance of antlers appears to be regulated by tumour suppressor genes. Tumour suppressor genes that are positively selected in cervids and highly expressed in antlers include PML, TP53 pathway-related, and ADAMTS family members, such as ADAMTS18 [46,47,87];. Additionally, three p53 cofactor genes (PML, NMT2, and CD2AP) and five p53 regulator genes (ELOVL6, S100A8, ISG15, CNOT3, and CCDC69) were found to be positively selected in cervids and expressed in antlers. Interestingly, the ADAMTS family antler-specific genes have higher expression levels in deer antler than in osteosarcoma. Additionally, genes TP73 and TP53I13 are expressed distinctly in antlers, and may inhibit tumour growth through the p53-mediated DNA damage response pathway [77];. Answer to the paradox of deer antler regeneration therefore appears to be explained by the tumour suppressor gene regulatory network. Further research is required to demonstrate the molecular mechanisms and signalling pathways regulating the profound cell proliferation and differentiation unique to deer antlers, whilst providing cancer resistance.

Table 2
The genetic control of antler tissue regeneration.

Gene	Gene function and/or role in antler regeneration	Reference
SNAI2, HOXD cluster, TWISTI1, SOX9	Neural crest stem cell migration	[111]
OLIG1, OTOP3, COL11A2	Neural crest stem cell differentiation	[111]
FOXD3, SOX10, SNAI1, TFAP2A, NGFR	Neural crest stem cell genes	[111]
ALX1, VCAN, COL1A1, SATB2, RUNX2, POSTN, SP7, COL4A2	Bone development	[111]
NGFR	Neural growth	[111]
RXFP2, SOX10	Neural function	[111]
UHRF1	Bone cell proliferation	[112]
S100A10	Bone mineralisation	[112]
TP73, TP53I13	Inhibition of tumor growth	[111]
ELOVI6, S100A8, ISG15, CNOT3, CCDC69	Regulation of cellular division and inhibition of tumor growth	[111]
ADAMTS18	Inhibition of tumor growth by regulation of tumor microenvironment	[111]
RXFP2	Headgear specific gene	[111]
FGF19, FGF21, FGFBP3, PDGFD, PDGFRL	Cancer cell proliferation and survival	[111]
S100A4	Expressed in ASCs Cell proliferation and tumor growth	[62]
NOVA1	Tumor growth and telomerase activation	[111]
FOS, REL, FAM83A	Proto-oncogene FOS = cell proliferation and differentiation FAM83A = EGFR signalling pathway	[111]

Evolutionary origin of regeneration in deer antlers

Antlers are a fascinating and elaborate adaptation thought to have

evolved by natural selection for use by male deer as weapons, for display and competition, and for defence against predators [12,36,76]. In deer the size and symmetry of antlers may serve as an indicator of strength and hierarchy, ultimately determining fitness and reproductive success [17]. Further, some species, such as the Irish Elk, are thought to have faced extinction from disproportions between antler size and body mass [78]. Recent findings show that deer antler morphology may be predicted by phylogeny and body mass [15]. Phylogenetic based studies of deer antler evolution have provided valuable understanding of this topic, which remains an area of scientific contention.

Osseous cranial appendages, such as deer antlers (in cervids), are a form of ruminant headgear, also including horns (bovids), pronghorns (antilocaprids), and ossicones (giraffids), that appear to have evolved in Pecora families during the Neocene, approximately 23.3 to 20.8 million years ago [19,23]. Common robust morphological features of ruminant headgear include their prescience in males, pairing and positioning on the frontal bone, a bony core, covered by skin and related connective tissues [23]. Antlers are found only in cervids and are unique mammalian appendages which are regenerated annually [23,36]. Further, the evolutionary origin of ruminant headgears remains a question of current vigorous debate, seeking to explain, for example, the morphological variation of headgear across Pecora families: can a single common ancestral origin of ruminant headgear be defined, or have ruminant headgear independently evolved from multiple ancestors? The challenges in determining the origin of headgear are considerable, and stem from discrepancies amongst phylogenetic trees, and the confounding results from studies in headgear development [23]. Phylogenetic trees, fossil records, and transcriptome profiling have indicated that ruminant headgear may have originated from a single Pecora ancestor and later diversified, however, the complexity of patterns and inconsistencies of phylogeny contradict a unified theory to explain the evolution of headgear [19], therefore indicating the need for further research.

Summary

Although biologists have made considerable progress in the past decade there is still a paucity of knowledge in the cellular mechanisms and molecular pathways that govern antler renewal. This is surprising given the tremendous value deer antlers hold as the only model of mammalian organ regeneration. A major breakthrough in antler research has stemmed from the discovery and molecular characterisations of novel ASCs, cells that appear to closely resemble ESCs. This finding has dramatically shifted our previous understanding of antler regeneration, which was once thought to be comparable to that of limb regeneration in urodele amphibians. Scientific advances in genome sequencing technologies have allowed for the recent identification of several antler-specific genes that are involved in this rapid longitudinal growth. There further exists heterogeneity in the quality of the literature, with many of the published evidence considered unreliable and possibly misleading. Hence, it is important that studies are interpreted with caution. To formulate precise and valid results, future research must strive to provide higher quality evidence from well-designed and executed studies, for the development of the safe and effective potential application of ASCS for therapeutic use. The future challenge in the field is in deciphering how these environmental and endocrine factors, for example testosterone interact with local factors to orchestrate antler regeneration. Additionally, an understanding of the interactions between the various signalling pathways, and of the mechanisms that regulate first antler growth may provide further fundamental insights into antler renewal. Conclusively, in depth research in antler wound healing has also been suggested to be beneficial for application in diminishing scarring during the human wound healing process.

Author contributions

M.F. contributed by devising and writing the original draft. S.B. critically revised and edited the manuscript. J. C., X. H., and D.W. provided discussion, advice and revision points during the process of review paper formation. D.W. and J.X. discussed and formulated the idea. J.X. coordinated this review and revised manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare no conflict of interest.

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