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Stem cells, stem cell niche and antler development

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Abstract. Annual full regeneration of deer antlers has been proved to be a stem cell-based process, and antler stem cells (ASC) reside in both antlerogenic periosteum (AP) and pedicle periosteum (PP). In this review, we first put forward a hypothesis that the closely associated skin is the primary component of ASC niche and then provide results testing this hypothesis. Membrane insertion experiments confirmed that interactions between ASC and the associated skin are indispensible for both antler generation and regeneration, and these are achieved through exchanging diffusible molecules. Intradermal AP transplantation study demonstrated that both epidermal and dermal papilla cells are involved in these interactions. Further, the AP inversion experiment indicated that the initial inductive signal originates from the ASC resident in the AP cellular layer, although the AP fibrous layer is naturally adjacent to skin. Experimental manipulation to the niche has profound effects on antler development. We believe that eventual identification of these interactive molecules will not only greatly enhance our knowledge of antler development, but also have significant impacts on regenerative medicine in general.

Additional keyword: pedicle.

Introduction

Organ regeneration is the 'Holy Grail' of modern regenerative medicine. To realise this dream, regenerative medicine must be underpinned by regenerative biology, which seeks to understand the mechanism of regeneration through investigation of different model systems.[1] Among these systems, deer antler (Fig. 1) stands out as the only mammalian appendage capable of complete renewal. Therefore, it offers a unique opportunity to explore how nature has solved the problem of regeneration of a complex mammalian organ including tissues of bone, cartilage, blood vessels, nerves and full thickness of skin.[2]

Antlers are deer cranial appendages that are cast and fully regenerate each year. Antlers do not renew directly from a deer's head; but instead from the apices of permanent protuberances, known as pedicles (Fig. 1). Deer are not born with pedicles, which begin to develop from frontal crests (behind the eye sockets) as male deer approach puberty (around 56 kg in red deer). When a pedicle reaches a species-specific height (5–6 cm for red deer), shiny skin which is sparsely populated with hair starts to emerge from its apex, indicating the commencement of antler transformation. Growing antlers are then enveloped by this newly differentiated skin that has a velvet-like appearance, and is hence called velvet skin. When the breeding season approaches, these first antlers become fully calcified and the blood supply is occluded, which causes the demise of velvet skin. The dead velvet skin is subsequently shed to expose the bare bone of hard antlers. Hard antlers are cast in the following spring, and regeneration of the second-set antlers on their living pedicle stumps follows immediately. From then on annual renewal of subsequent antlers enters a well defined cycle: previous hard antler casting and new soft antler regeneration take place in spring, rapid antler growth and maturation in summer, full antler calcification and velvet skin shedding in autumn, and the bare bony antler phase in winter.[3] Both antler generation and regeneration are unique zoological phenomena; understanding the mechanism underlying these processes may help to unlock the secrets of organogenesis as well as mammalian organ regeneration.

Antler stem cells

We consider that the era of antler stem cell (ASC) research started when Hartwig and Schrudde[4] discovered the uniqueness of the antlerogenic periosteum (AP), a bone membrane that overlies the frontal crests of pre-pubertal deer. Removal of AP abrogates future pedicle and antler development from the original place; whereas, transplantation of AP elsewhere on deer body induces ectopic antler formation. Subsequently, Goss[5,6] greatly extended this discovery by defining the threshold mass and orientation of AP, and regional skin competency for ectopic antler induction. Li *et al*.[7] successfully isolated and cultured AP cells, which laid the foundation for subsequent characterisation of AP cells.

Although the tissue-type AP that gives rise to first antlers was successfully identified through tissue deletion and transplantation, a similar approach failed to pinpoint the tissue type for antler regeneration.[8] Both Wislocki[9] and Goss[2] believed

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Fig. 1. Deer antlers at growth phase. Arrow points to the antler pedicle.

that pedicle skin provided the cell source for antler regeneration. Based on the 'double-head' formation (an unusual antler growth phenomenon) and inspired by the discovery that it is the periosteum that gives rise to first antlers, Kierdorf *et al.*[10] suggested that it is the pedicle periosteum (PP) that is likely to induce subsequent regenerating antlers. Successful identification of the cell source for antler regeneration was not forthcoming until the time when a breakthrough was made via serial morphological and histological examinations.[11–13] In these examinations, establishment of the initial growth centres for antler regeneration were found to be solely derived from the distal periosteal cells of a pedicle stump. To functionally confirm this finding, Li *et al.*[14] deleted the PP before antler regeneration either totally or partially. The results showed that properly timed total PP removal can effectively inhibit pedicle stumps from initiating antler regeneration (Fig. 2*a*, *b*), and antler regeneration can only take place on the pedicle shaft of the partial-PP-deleted pedicles at the area that the distal end of the PP remains, which was markedly distant from the pedicle apices where normal antler regeneration occurs (Fig. 2*c*, *d*). Therefore, PP is shown convincingly to be the precise tissue that gives rise to regenerating antlers.

The unique attributes of AP and PP prompted us to think that the cells resident in them must be ASC.[15] In order to provide evidence for this claim, we subsequently characterised these cells, and found that both of them express key embryonic stem cell markers Oct4, Sox2, Nanog, CD9, telomerase and nucleostemin (Fig. 3); and are multipotent as they could be readily induced to

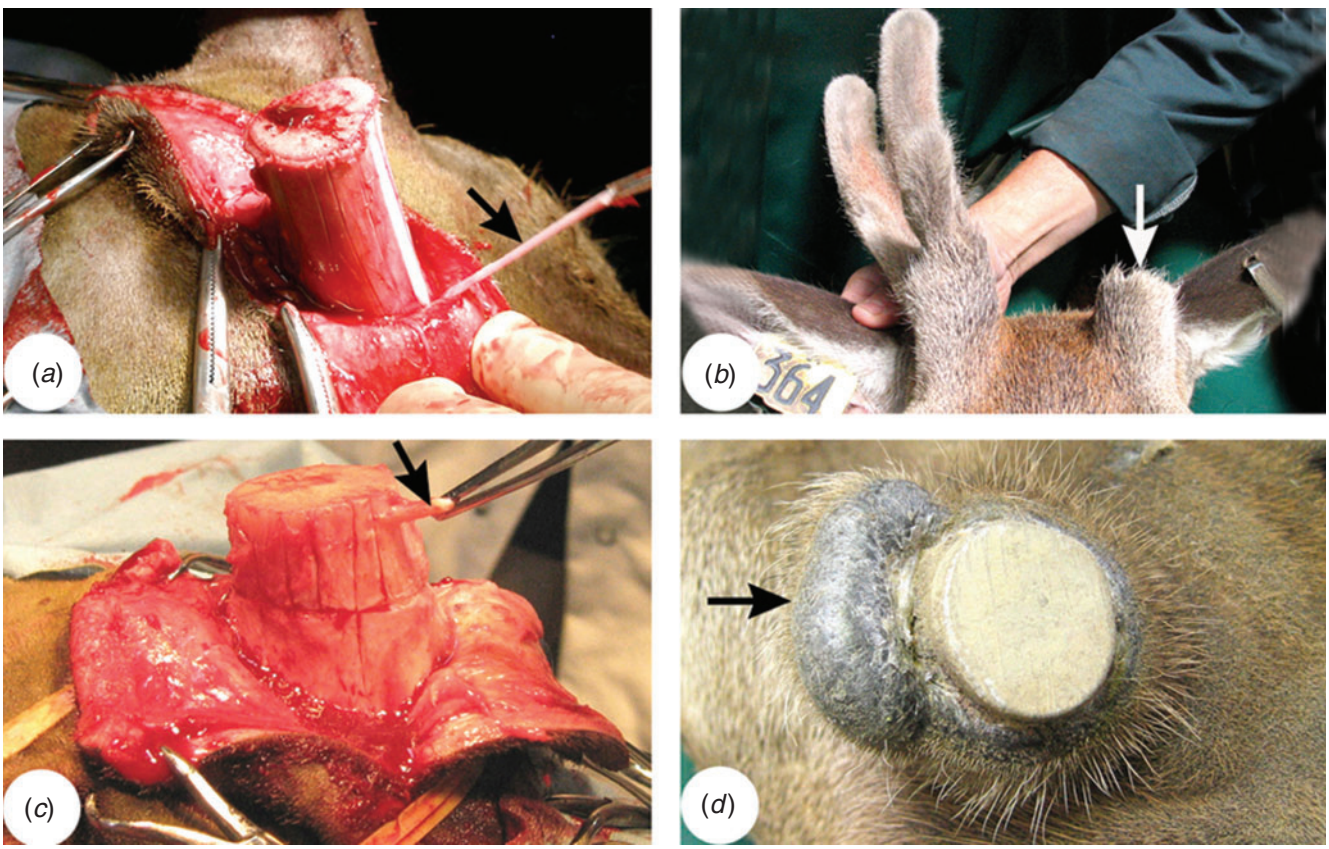

Fig. 2. Pedicle periosteum (PP) deletion in yearling red deer stags (after Li *et al.*[14]). Following the exposure, PP (arrows) was peeled off the pedicle bone either totally (*a*) or partially (*c*). A total PP-deleted pedicle failed to regenerate antler (arrow), although the sham-operated pedicle formed a branched antler (*b*). Interestingly, a partial PP-deleted pedicle regenerated an antler bud (arrow) on the pedicle shaft from the distal end of remaining PP (*d*), which was markedly distant from the normal regeneration surface.

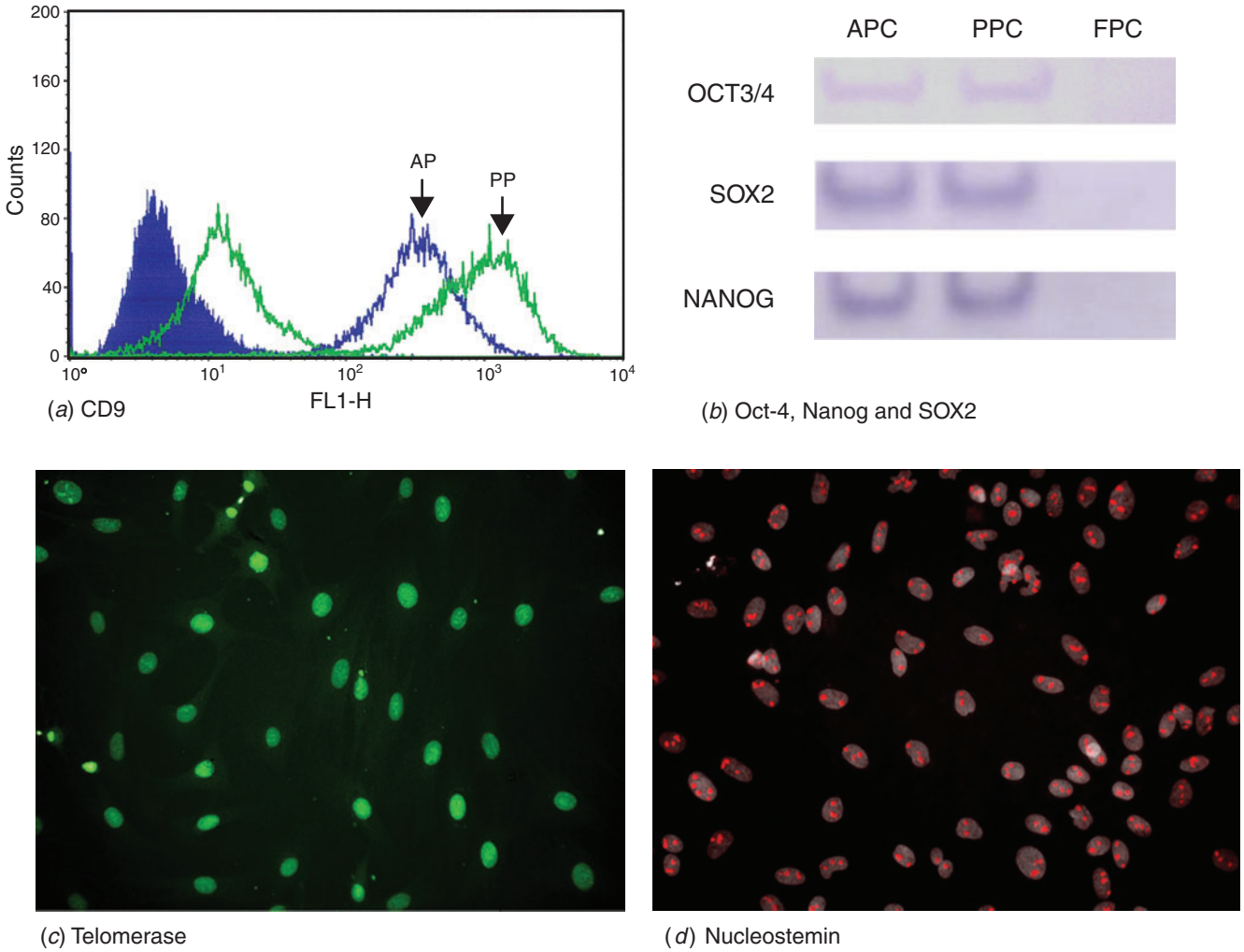

Fig. 3. Expression of key embryonic stem cell markers (after Li *et al.*[17]). (*a*) CD9 by AP (blue trace) and PP (green trace) cell populations determined by flow cytometry. (*b*) Detection of the 'pluripotency' genes Oct3/4, SOX2 and Nanog by Western blot analysis. APC, antlerogenic periosteal cells; PPC, pedicle periosteal cells; FPC, facial periosteal cells. (*c*) Expression of telomerase by AP cells detected by immunohistochemistry. (*d*) Expression of nucleostemin by AP cells detected by immunohistochemistry.

differentiate into chondroblasts, adipocytes, myoblasts and neuronal-like cells (Fig. 4).[16–18] Consequently, we concluded that AP and PP cells are ASC.

Advancement of the ASC niche hypothesis

Pre-existing experimental results implicate skin as the niche candidate

As stem cells, ASC must reside in and interact with their niche. While carrying out tissue transplantation experiments, Goss[6] noticed that AP could induce ectopic antler formation only when AP-derived tissue came in close contact with the overlying skin, which led him to think that interactions between AP-derived tissue and skin were indispensible for the initiation of antler formation, and this close association seemed to facilitate the establishment of these interactions. Detailed histological examination of skin transformation from scalp to antler velvet during initial antler generation strongly supported Goss' claim.[19] This was because velvet skin transformation does not take place until the AP-derived perichondrium and the overlying skin become intimately bound together through total compression of the interposing subcutaneous loose connective tissue layer. Likewise, antler regeneration may also rely on the interactions between PP and the enveloping skin. While carrying out PP sampling, Li *et al.*[12] noticed that the degree of association between PP and the enveloping skin varies considerably from the distal to the proximal end: intimately bound at the distal end where antler regeneration occurs and loosely attached at the proximal end. Interestingly, antler regeneration also takes place when pedicles shorten into the proximal end as deer age; however, by then PP and the skin have become closely associated at the proximal end (C. Li, pers. obs.). Overall, all these observations indicate that close association between PP and the enveloping skin are indispensible for antler regeneration. Therefore, the closely associated skin is likely to be the primary element of ASC niche for both antler generation and regeneration.

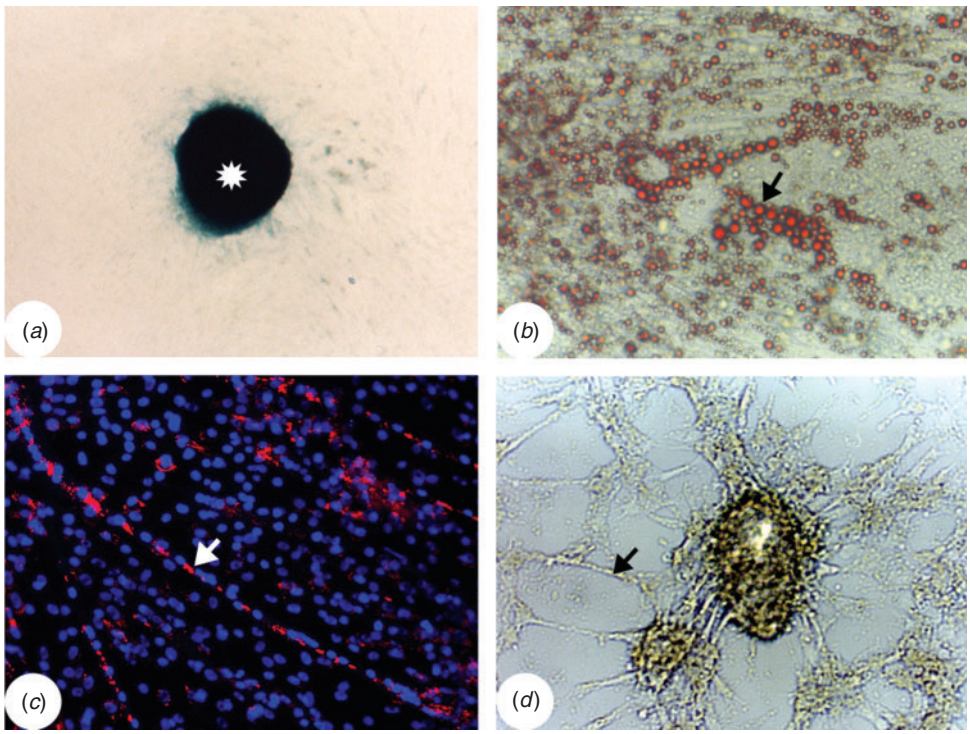

Fig. 4. Multipotency of antler stem cells (after Li *et al.*[17]). (*a*) Cartilage nodule (asterisk) formed by antlerogenic periosteum (AP) cells in a micromass culture. (*b*) Adipocytes (arrow) differentiated from AP cells in the culture medium containing linoleic acid. (*c*) Myotube (arrow) formed from AP cells when co-cultured with C2C12 cells. (*d*) Neuronal-like cells differentiated from AP cells when cultured in N2 medium. Note the extended neurites (arrow) from each cell body.

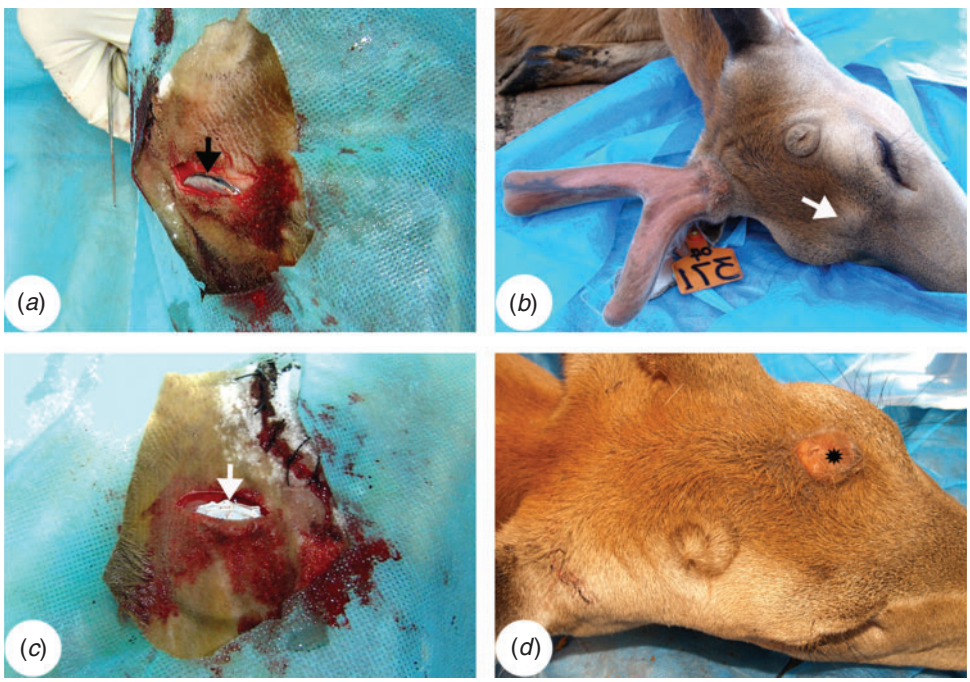

Fig. 5. Tissue interactions in antler generation (after Li *et al.*[20]). (*a*) Impermeable membrane (arrow) was subcutaneously placed over the grafted antlerogenic periosteum (AP) on a sika deer forehead. (*b*) Dome-shaped bulge (arrow) was formed 2 years after AP and membrane transplantation. Note that the skin overlying the bulge remained in scalp skin feature. (*c*) Permeable membrane (arrow) was subcutaneously placed over the grafted AP on a sika deer forehead. (*d*) Dome-shaped bulge (asterisk) was formed 2 years after AP and membrane transplantation. Note that the skin overlying the bulge had transformed into typical antler velvet type.

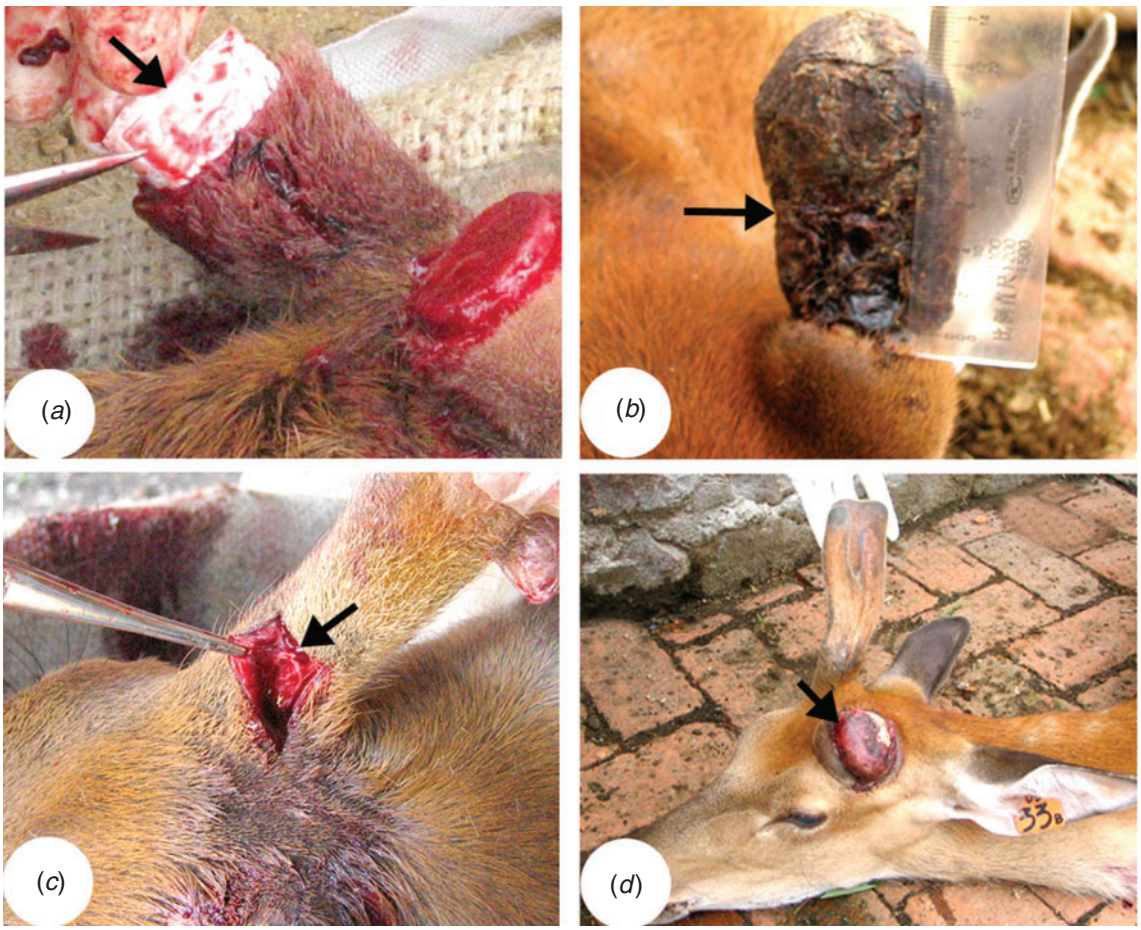

Fig. 6. Tissue interactions in antler regeneration (after Li and Suttie[21]). (*a*) Impermeable membrane (arrow) was surgically interposed between pedicle periosteum (PP) and the enveloping skin in a full-length pedicle stump. (*b*) Skin-less antler (arrow) was formed from the membrane interposed full-length pedicle stump, and covered with scab. (*c*) Creation of a 2/3-length pedicle stump (refer to the text) by making a vertical skin incision to identify the very point (arrow) where transition occurs from tight to loose association between PP and the enveloping skin. (*d*) Membrane-inserted 2/3-length pedicle stump (arrow) did not give rise to antler tissue at late antler regeneration stage.

ASC niche hypothesis

Based on the aforementioned observations, we put forward an ASC niche hypothesis: antler development including generation and regeneration is triggered by the interactions between ASC and the niche, i.e. the closely associated skin cell populations. ASC transform the skin from scalp type into antler velvet; instructive feedback from the transformed skin triggers the proliferation and differentiation of ASC to initiate antler formation.

Testing of the ASC niche hypothesis

Dependence on interactions between ASC and the niche for antler development

To functionally test whether the interactions between ASC and niche cell populations are indispensible for antler development, we carried out two experiments: one was for antler generation (i.e. the first antler)[20] and the other for antler regeneration (subsequent antlers).[21] In the experiment for antler generation, either impermeable or semi-permeable (0.45-μm pore size) membranes were inserted between ectopically grafted AP and the overlying skin. Interposition of the impermeable membrane completely inhibited antler formation from the grafted AP (Fig. 5*a*, *b*), whereas the semi-permeable membrane only significantly delayed (for a year), but did not stop eventual velvet skin transformation (Fig. 5*c*, *d*). In the experiment for antler regeneration, two types of pedicle stumps were first created: full-length and 2/3-length pedicle stumps. The first type was generated by removing antlers through the junction between pedicles and the antlers. PP and the enveloping skin were in tight contact at the distal end of a full-length stump. The second type was generated by cutting through the transition point where the tight and loose association between PP and the skin are met. PP and the enveloping skin were in loose association in 2/3-length stumps. Insertion of the impermeable membrane into PP and the enveloping skin of full-length stumps did not prevent antler regeneration, although skin failed to participate in the process (Fig. 6*a*, *b*). However, for 2/3-length stumps, it completely stopped antler regeneration (Fig. 6*c*, *d*). These results indicate that when PP is separated from skin before the interactions (still loosely associated) occur, antler regeneration cannot take

place; whereas, after the establishment of interactions (already closely contacted), skin is no longer required for antler regeneration. Taken together, interaction with the closely associated skin is indispensible for ASC to initiate antler generation or regeneration, and these interactions are achieved through exchanging diffusible molecules as semi-permeable membrane cannot completely stop the process.

Identification of niche cell types

ASC niche (i.e. deer skin) is not homogenous but consists of epidermis and dermis, and dermis contains a few cell types. Epidermal cells must participate in interactions with ASC, as during the initiation of antler formation, scalp epidermis is transformed into antler velvet epidermis. It is not currently known whether the interactions between ASC and the epidermal cells are accomplished through directly exchanging diffusible molecules or via dermal cell mediation. In his serial AP transplantation experiments, Goss[22] found that all areas of deer skin, except those that cover the snout of the nose and the tail ventral surface, are competent to interact with the grafted AP for the initiation of antler formation. The common feature of snout and ventral tail skin is that it is devoid of hair follicles. Therefore, dermal papilla cells (DPC), the only dermal component of hair follicles, may participate in these interactions by relaying the signals between ASC and epidermal cells.

To confirm the DPC involvement, Li *et al.*[23] transplanted AP and partial deer skin, which had been sutured together, onto a nude

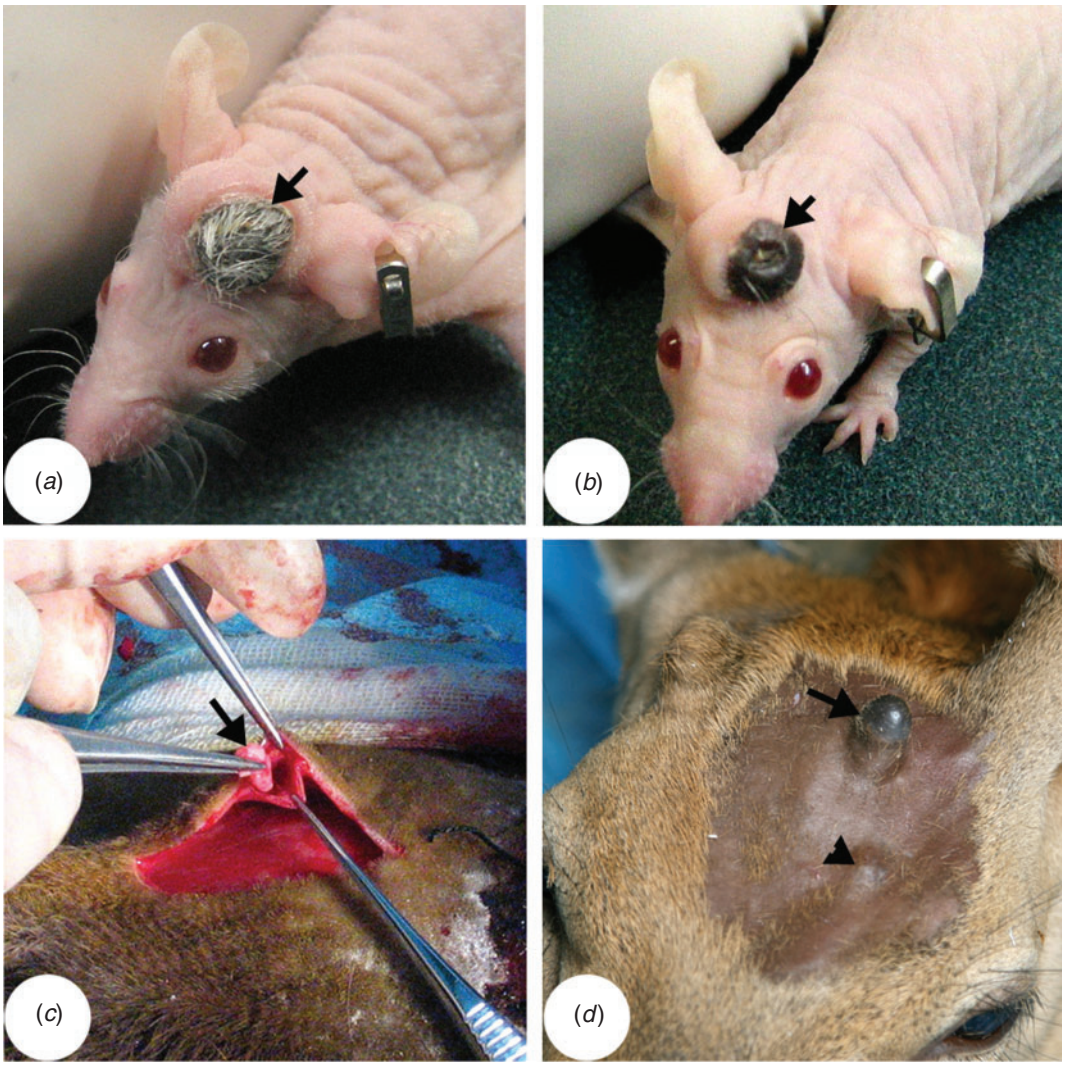

Fig. 7. Tissue interactions in xenograft transplantation (*a* and *b*, after Li *et al.*;[23] *c* and *d*, after Li *et al.*[24]). (*a*) A protuberance was formed on the forehead of a nude mouse 3 weeks after the operation from the co-transplanted antlerogenic periosteum (AP) and deer skin. Note the protuberance was capped with typical deer scalp skin. (*b*) The same protuberance in (*a*) but 10 weeks after the operation. Note that the scalp deer skin was transformed into typical antler velvet skin (arrow). (*c*) A pellet of finely minced AP (arrow) was carefully loaded into an intradermal pocket. (*d*) Antler formation (arrow) took place 60 days after AP transplantation at the intradermal transplantation site and reached 14.5 mm high at the time of tissue sampling. Only a small bulge (arrowhead) was found at the subcutaneous transplantation site by the same quantity of AP tissue.

mouse head. The partial skin was only composed of epidermis and the dermal portion which contained hair follicles. Interestingly, the epidermis of the partial deer skin was fully transformed into antler velvet epidermis by the attached AP in a xenograft model (Fig. 7*a*, *b*). However, this experiment did not provide evidence for the direct participation of DPC in these interactions, although it demonstrated that the interposing subcutaneous loose connective tissue and non-hair-follicle-containing dermal tissue are not required for the establishment of interactions. Therefore, we conducted another experiment, within which AP tissue was directly delivered underneath the hair follicles (DPC) through an intradermal transplantation approach, and at the same time used subcutaneous transplantation as a Control.[24] The intradermal approach showed that for 1/8 of AP tissue mass or more there was a 100% success rate of antler induction at the grafted sites; whereas for the subcutaneous approach, 1/4 of AP tissue mass or less did not induce ectopic antler formation (Fig. 7*c*, *d*). Because removal of the interposing tissue barrier between AP and hair follicles greatly stimulated antler formation, DPC of hair follicles must have been involved in the interactions between ASC and epidermal cells.

Origin of initial inductive molecules

Just like deer skin, AP tissue is also heterogeneous and consists of two layers: a fibrous layer (closest to the skin) and a cellular layer (abuts the bone). It is not known whether the initial inductive molecules are derived from the fibrous, the cellular or both layers. To clarify this, we conducted the following experiment.[25] In the study, AP from both sides of the future antler growth regions on a deer head was detached (Fig. 8*a*) and then put back in a different orientation. On one side, AP was directly put back without changing orientation as a Control (Fig. 8*b*), and on the other AP was inverted before being replaced, i.e. allowing the cellular

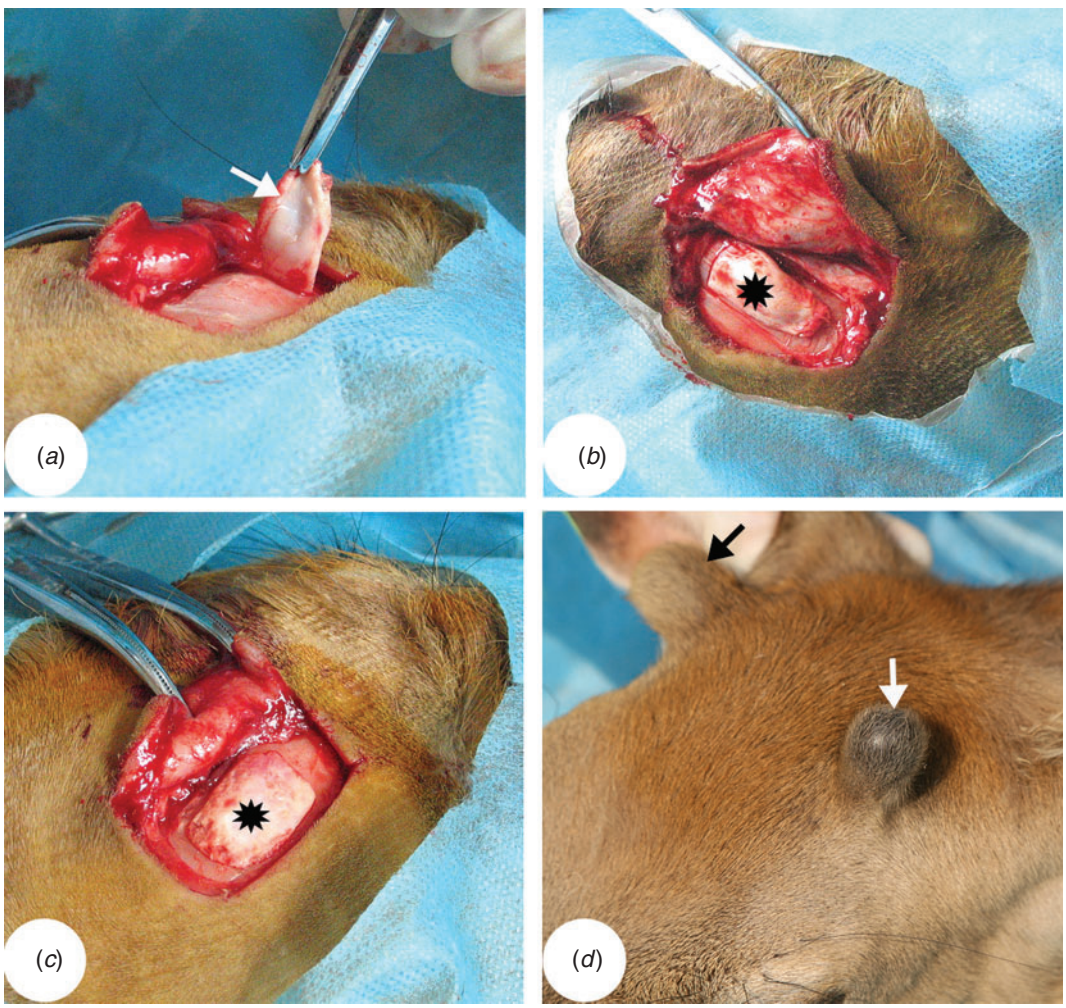

Fig. 8. Antlerogenic periosteum (AP) inversion and antler formation (after Gao *et al.*[25]). (*a*) AP overlying a frontal crest was peeled off (arrow) from the underlying bone using a pair of rat-toothed forceps. (*b*) The detached AP was directly replaced onto the area (asterisk) from which it was removed. (*c*) The detached AP was replaced upside down onto the area (asterisk) from which it was removed. The inversion was carried out by reversing the medial and lateral sides and keeping anterior and posterior axis in register. (*d*) Seventy-four days after surgery, antler growth (white arrow) took place from the inverted-AP side; whereas no velvet skin could be observed on the 28-mm-high pedicle from the non-inverted AP side (black arrow).

layer face the overlying skin (Fig. 8*c*). The results showed that in the AP inverted side, antler generation took place without passing through a distinguishable pedicle stage; whereas, in the AP non-inverted (natural) side antler formation occurred only when its pedicle grew up to the species-specific height (Fig. 8*d*). These results indicate that the AP cellular layer cells are the origin of inductive molecules that initiate the interactions between ASC and the niche cell populations and subsequent antler development.

Explanation of some unusual phenomena using the ACS niche theory

In the antler biology field, some unusual phenomena have been reported but never been properly explained. Mechanical wounding to both skin and AP usually causes direct antler formation without passing through a visible pedicle stage (Fig. 9*a*, *b*;[26]), this would be because the trauma has effectively disrupted the interposing subcutaneous loose connective tissue, thus greatly facilitating the interactions between ASC and the niche, and triggering direct initiation of antler formation. Sometimes it takes a few seasons for a transplanted AP to initiate an ectopic antler formation. The most extreme case reported so far is 9 years (Fig. 9*c*;[27]). This would be caused by the weak inductive signal, low permeability of the interposing physical barrier or both. Antler initiation sometimes can take place in a very localised manner on an antler growth region (Fig. 9*d*;[27]). This would be because only the physical barrier in these localised areas allowed sufficient interactive molecules penetrating through to establish the interactions between ASC and the niche.

Effects of ASC niche manipulation

Very recently, we experimentally manipulated the ASC niche and found antler development was profoundly affected.[28] When permeability of the interposing tissue layers (subcutaneous loose connective tissue and partial dermal tissue) between ASC and essential skin cell populations were physically increased through liquid nitrogen spray, antler transformation was greatly advanced (Fig. 10*a*). Normally antler transformation takes place when its pedicle reaches 5–6 cm in height in red deer; after the increase in permeability of the interposing tissue barrier in this case, antler transformation took place from the precocious pedicles, (which were fully grown at ~2 cm in height; Fig. 10*b*). Destruction of AP cells in the central region, from which a pedicle develops, with or without damaging the interposing tissue layers resulted in completely opposite outcomes: antler formation from the marginal AP was inhibited when the interposing tissue layers were kept intact (Fig. 10*c*); whereas, antler formation from the

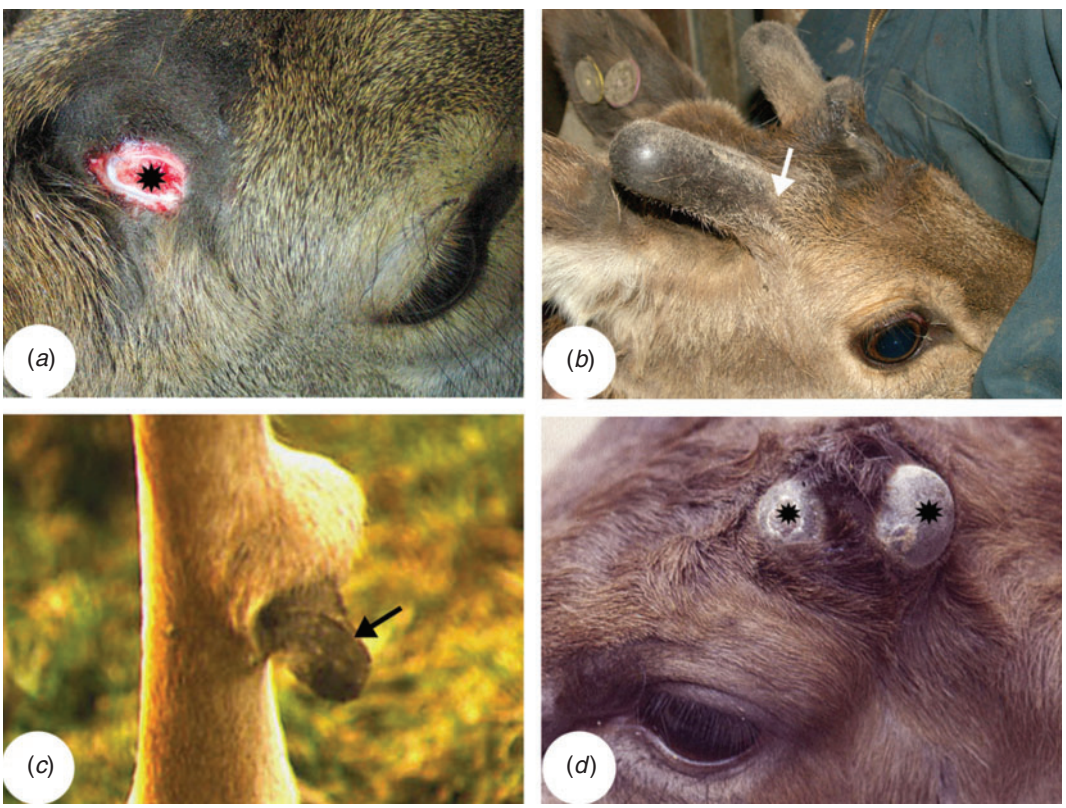

Fig. 9. Unusual phenomena of antler development (*c* and *d*, courtesy of Dr Uwe Kierdorf). (*a*) Mechanical removal of partial antlerogenic periosteum (AP) and the overlying skin (asterisk). (*b*) An antler was formed subsequently from the operated site without going through a distinguishable pedicle stage (arrow). (*c*) An ectopic antler (arrow) was formed on a deer hind leg from the transplanted AP 9 years after the operation. (*d*) Localised antler transformation (asterisks) from an antler growth region.

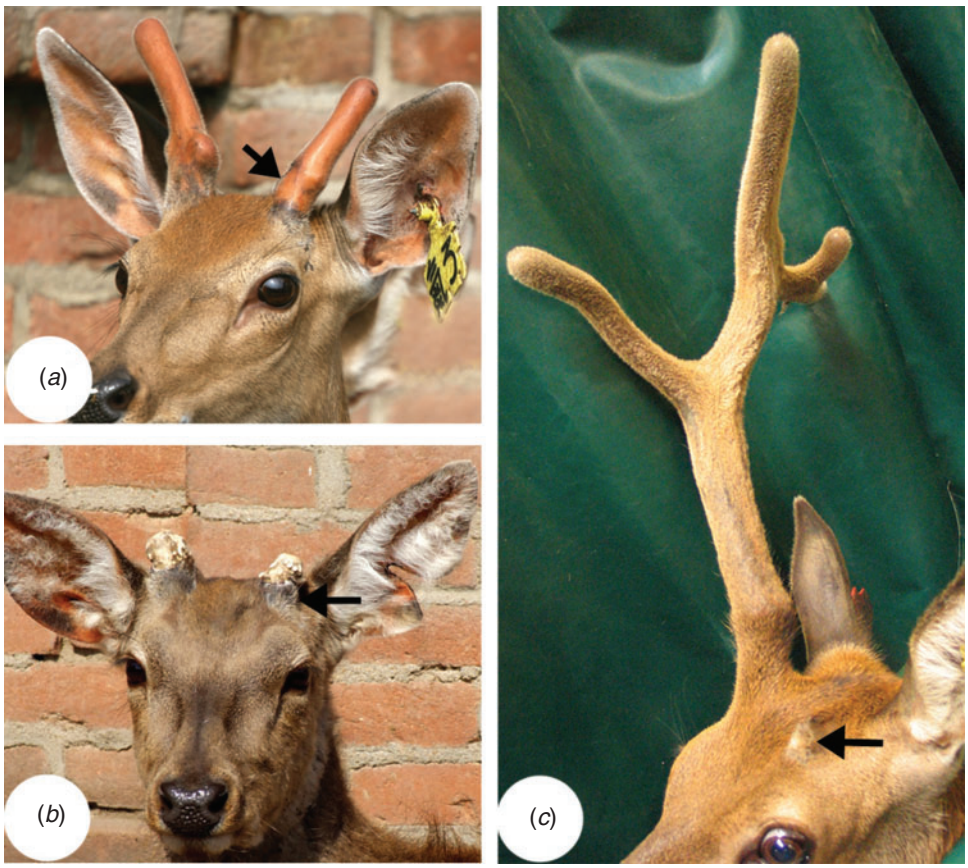

Fig. 10. Antler formation after the manipulation of antler stem cell niche through liquid nitrogen spray. (*a*) Antler formation was significantly advanced (arrow, evidenced by precocious skin transformation from pedicle type to antler velvet) after the increase in permeability of the physical barrier between antler stem cells and the niche cell populations. (*b*) Antler (remnant) calcification and velvet shedding confirmed the early transformation of antler formation, which is evidenced by the short pedicles (arrow). (*c*) No pedicle or antler was formed from marginal antlerogenic periosteum (AP) on the site where central region AP had been destroyed while leaving the interposing physical barrier intact, although the Control site formed a branched antler.

marginal AP was promoted when the layers were disrupted (Fig. 9*b*). Thus, we conclude that we have identified the primary components of the ASC niche.

Summary and future work

Antler generation and regeneration are triggered by the interactions between ASC and the niche cell populations (DPC and epidermal cells) through exchanging diffusible molecules. In other words, the putative diffusible molecules play a key role in antler generation and regeneration. To help identify these molecules, we recently established a co-culture system within which all the essential cell types were placed together *in vitro* in a way that can maximally mimic the *in vivo* situation. Eventual identification and isolation of these molecules will not only greatly enhance our knowledge of antler development, but will also have significant impacts on regenerative medicine in general.

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