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Review Article

Techniques for Research in Deer Antlers, the Sole Mammalian Organ which undergoes Epimorphic Regeneration

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Abstract

Annual renewal of deer antlers offers the only opportunity to learn how nature has solved the problem of mammalian organ regeneration. Promotion of the antler model to the field of regenerative biology and medicine, however, requires understanding the mechanism underlying the generation and regeneration of this unique organ. During the course of nearly four decades of antler research, we developed a number of techniques specifically for carrying out the investigation of deer antler biology. In this paper, I summarized six of them including **1)** Mechanical disintegration of antler stem cell (AnSC) tissue; **2)** In vivo investigation of the interactions between antlerogenic tissue and overlying skin; **3)** In vivo identification of skin tissue components required for establishing interactions with AP; **4)** Alternative transplantation technique to reduce AP quantity required for antler induction; **5)** In vivo evaluation of the role of interposing tissue layers in antler generation; **6)** In vitro identification of the interactive molecules between AnSCs and niche cell populations. I believe if these techniques are adopted in the antler research field, it would greatly facilitate the progress for revealing the mechanisms of antler development, and ultimately benefit regenerative medicine in general.

Keywords: Epimorphic Regeneration; Deer antlers; Pedicle; Tissue interactions, Periosteum

Introduction

Full restoration of lost organs/appendages including both structure and function (known as epimorphic regeneration) is the "Holy grail" of regenerative medicine. The latter is underpinned by regenerative biology. Regenerative biology seeks to understand the mechanisms underlying organ regeneration through use of model animals, such as planarians, zebra fish and newts [1]. Thus far, suitable mammalian models of organ regeneration are lacking but highly desirable.

Deer antlers are the only mammalian organ that can fully regenerate once lost, and thus offer the sole opportunity to explore how nature has

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solved the problem of mammalian organ regeneration. Although antlers hold the potential to be developed into a unique model for mammalian organ regeneration, to unleash this potential, a comprehensive understanding of basic antler biology is required. During almost four decades of antler research, we have developed a series of approaches and a method including tissue sampling [2] and custom-built tools [3].This paper provides a summary of enabling techniques. Our aim is to promote the unique antler model to the field of regenerative biology and medicine thus benefiting human health.

1. Mechanical disintegration of AnSC tissues for molecular study

- **Freezer Mill**

1.1 Purpose

As previously reported by our group and others, the potential for antler generation resides in the antlerogenic periosteum (AP) [4], and for antler regeneration in the pedicle periosteum (PP) [5]. Deletion of the AP abrogates pedicle and antler formation, whereas transplantation of the AP induces ectopic antler growth (Figure 1A and 1B). Likewise, with removal of the PP, PP-less pedicle fails to give rise to regenerating antler (Figure 1C and 1D).

Cells for both generation in the AP and regeneration in the PP have been demonstrated to have stem cell attributes [6,7], so they are termed AnSCs. Thus, understanding of the underlying mechanisms necessitates study of molecules associated with AnSCs. However, this is problematic given the fact that the tissue within which AnSCs reside, the periosteum, is very tough fibrous in nature and relatively resistant to disintegration. The dilemma is a need to properly prepare (grind) this tissue to fine particles while, at the same time, not impairing the biological activity of molecules resident in the periosteum. Historically, standard techniques using a mortar and pestle [8,9] or an Ultra-Turix homogenizer (unpublished) have been used, but these did not provide the degree of disintegration required for applications using molecular biology techniques (e.g. protein-based: Western blot, 2-dimensional electrophoresis, LC-MS; RNA-based: RT-PCR, qPCR, in situ hybridization). Consequently, a more robust approach was required, and via trial and error, we have found that the SPEX SamplePrep freezer/mill (Figure 1C) is arguably the best tool to disintegrate periosteal tissue without compromising biological activity.

1.2 Principle

The SPEX SamplePrep freezer/mill (Figure 1E and 1F) is a cryogenic laboratory mill that cools samples to cryogenic temperatures and pulverizes them by mechanically shuttling a steel impactor back and forth against stationary end plugs. Since the vial is closed, the integrity of its contents is maintained, critical samples are easily controlled, clean-up is simplified, and cross-sample contamination is eliminated. Because the vial is immersed in liquid nitrogen

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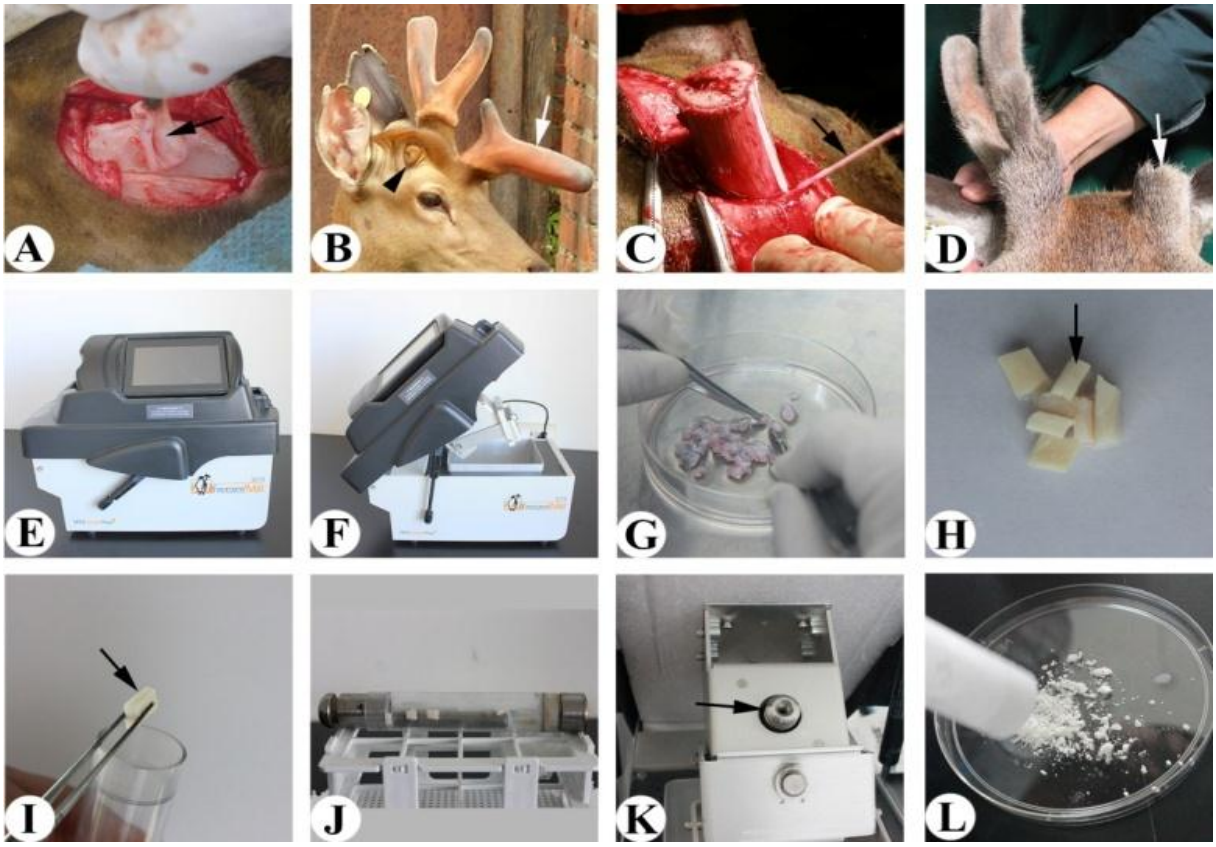
throughout the grinding cycles, the sample is kept at cryogenic temperatures and key aspects of molecules in the tissue preserved. It is claimed that SPEX SamplePrep freezer/mills are the "mills of last resort" for many problematic samples, or samples whose composition or structure is altered by the heat generated by conventional grinding. For detailed specification and general operation of the apparatus, refer to the manual (www.spexcsp.com/sampleprep).

1.3 Procedure

The technique that we apply to the preparation of periosteum is: immediately after sampling, cut the periosteal tissue into small pieces

Figure 1: Disintegration of antler stem cell tissues (AP). **A:** AP tissue deletion (arrow). **B:** Antler formation status; note that the original site failed to grow pedicle and antler after the AP was lost (arrow), but an ectopic pedicle and antler formed from the transplanted AP (arrowhead). **C:** PP deletion (arrow). **D:** Antler regeneration; note that the pedicle failed to give rise to regenerating antler once the PP tissue was lost prior to antler regeneration (arrow). **E and F:** SPEX Sample Prep freezer/mill. **G:** Minced periosteal tissue in a culture dish using two handheld scalpels. H: Frozen AP pieces (arrow). **I:** Loading a frozen piece of AP (arrow) into a cryogenic vial from the open end. **J:** A cryogenic vial containing both tissue and metal impactor. **K:** Insertion of a cryogenic vial (arrow) into the holder inside of the mill. **L:** Fine periosteum powder ground using the freezer / mill.



(around 2 mm^2/piece) using two hand-held scalpels in a 100 mm Petri dish (Figure 1G); then freeze them (Figure 1H) in liquid nitrogen (LN_2) before loading into a freezer mill cryogenic vial from the open end (Figure 1I); then

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forcefully insert a plug into the open mouth to seal the cryogenic vial (Figure 1J), and place the assembled vial into the vial holder inside of the mill (Figure 1K). The size of vial chosen depends on the quantity of tissue sample to be ground (using small- or micro-vial for our purpose, refer to the manual). For example, the procedure for grinding whole piece of AP from one side of the presumptive antler growth region into a fine powder (Figure 1L) the following parameters are sufficient: 2 min precooling time; 3 grinding cycles with 1 min/cycle; impactor rate: 8CPS.

1.4 Comments

The key factors are the need for safety with handling of LN2, and the need to select the smallest practical size of cryo-vials (to avoid wasting sample and LN2). It is necessary to cut periosteum into small pieces before transferring them into the vials. One must handle each item with extreme care to avoid burning hands or feet by LN2 (ware protecting shoes and gloves), and work in an open area, not get suffocated as LN2 disperse oxygen.

2. In vivo investigation of the interactions between antlerogenic tissue and overlying skin

- **Membrane insertion**

2.1 Purpose

Although transplantation experiments demonstrated that the antlerogenic potential is exclusively held in the AP, elsewhere autologously transplanted AP can only form an possible if it is not directly placed underneath the competent skin, for example inserted in the muscle or underneath the hair-less skin (ventral surface of the tail) [4]. These results strongly suggest that there are indispensable reciprocal interactions being taken place between the AP and competent skin during initial antler generation. Notably, this interaction can only be achieved when the AP-derived tissue mass and the overlying skin become intimately bound together [4].

Likewise, antler regeneration may also require the interactions between the PP and the enveloping skin. In tissue sampling, we have found that the association degree of skin to the PP along the pedicle shaft varies: thus, in the proximal pedicle portion (about two-thirds of the total pedicle length) the skin is loosely attached to the PP, whereas on the distal third of the pedicle, the skin is tightly bound to the PP. Importantly, antler regeneration can only occur from the distal end where skin is tightly bound to the PP [10,11]. Interestingly, antler regeneration can also take place when pedicles shorten into the proximal portion (loosely attached region) as deer age; however, when that to happened PP and the skin have already become closely associated [12].

Obviously, to understand the nature of this type of interaction, including the pathway through which the interaction is realized (cell-cell, cell-extracellular matrix, or diffusible substances) and duration (transient or permanent) is paramount important for the study of

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mechanism underlying antler generation and regeneration; and fields of organogenesis and regenerative medicine in general. To achieve these purposes, we recent years developed a membrane insertion technique and successfully applied this technique to investigate the nature of the interactions in both antler generation and regeneration.

2.2 Principle

Theoretically, it is straight forward to experimentally test this interaction in vivo just by inserting a piece of thin impermeable or semi-permeable membrane between the two interactive tissues at an appropriate timing depending on the purpose. Practically, it is a very challenging task, mainly is to find a way to effectively retain the inserted membrane in place during formation of a pedicle and antler. This problem is caused by the angled topology of the presumptive region of pedicle growth, and the widespread and uneven distribution of antlerogenic potential. This problem has been finally resolved by us [13]. Briefly, to solve the problem of uneven surface, we transferred the AP from the future presumptive pedicle growth region (rugged surface) to the forehead region (flat plane); and problem of localized antlerogenic potential, each inserted membrane is tied onto a stainless steel "O" ring before implantation. In so doing, we successfully investigated the nature of the interactions between antlerogenic tissue and the overlying skin, thus laid the foundation for identification and isolation of the putative interacting molecules.

2.3 Procedure

Detailed surgical procedures for membrane insertion in antler generation [13] or regeneration [14] are reported elsewhere. Briefly, for antler generation, AP is firstly sampled from the original site before pedicle initiation, and then autologously transplanted subcutaneously on the deer's forehead through a skin incision (Figure 2A). A subcutaneous tunnel is bluntly made for creating the space for membrane insertion (Figure 2B). A stainless steel "O" ring with (as a control) or without tied membrane (permeable or impermeable) is directly placed on top of the transplanted AP and underneath the skin (Figure 2C). The dimension of an "O" ring is as follows: 30 mm inner diameter, 1 mm wall thickness, and 3 mm height. A 0.5 mm deep groove is cut on the outer surface of each ring for firmly attaching the membrane using a thread.

For antler regeneration, a vertical pedicle skin incision is firstly made along the pedicle shaft, which facilitates the identification of the very point where transition occurs from tight to lose adherence of skin to the underlying bone (Figure 2D). This point marks the boundary between the potentiated and the dormant pedicle regions. To test whether the interaction between the PP and the enveloping skin is necessary for antler regeneration, an impermeable membrane should be inserted in the loosely adherent region. The enveloping skin is forcefully detached

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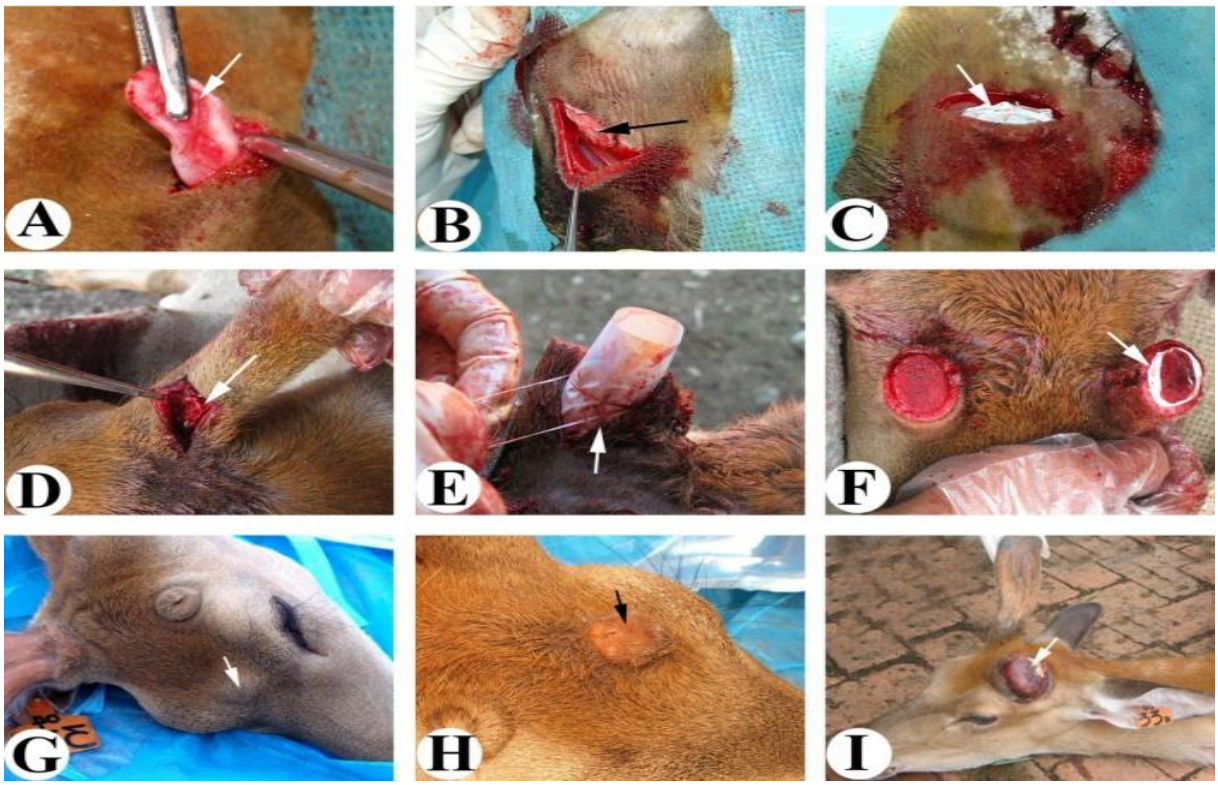
from the pedicle bone through the vertical skin incision, and membrane is used to wrap the exposed pedicle bone and the wrapped membrane is then tied in place by a thread (Figure 2E). After suturing of the skin incision, excessive membrane is trimmed away (Figure 2F).

The consequence of the membrane insertion, for antler generation, depends on the type of the membrane inserted: impermeable, completely stops antler formation (Figure 2G); and semi-permeable, delays but not stops antler formation (Figure 2H). For antler regeneration, it depends on inserted region: at loosely adherent dormant region, inserted membrane stops antler regeneration (Figure 2I); at the tightly adherent potentiated region, inserted membrane failed to stop antler regeneration, but form a skin-less antler.

Figure 2: Membrane insertion. **A:** AP tissue insertion through the skin incision (arrow) between deer two eyes. **B:** Implanted AP tissue (arrow) under the skin tunnel. **C:** Membrane insertion (arrow) between the implanted AP and the overlying skin; note that the membrane is tied to a stainless steel "O" ring. **D:** Identification of the transition point (arrow) from tight to lose adherent of skin to the underlying bone through a skin incision along the longitudinal shaft of the pedicle. **E:** Wrapped membrane around the exposed pedicle bone, which is tied in place by a thread (arrow). **F:** Inserted membrane (arrow), which is trimmed of excess tissue. **G:** Transplantation site of AP+ impermeable membrane 2 years after surgery; note that no skin transformation from scalp type to velvet type (arrow) has occurred. **H:** Transplantation site of AP+ permeable membrane 2 years after surgery; note that skin transformation from scalp type to velvet type (arrow) has taken place. **I.** A pedicle stump (arrow), which has failed to regenerate antler after separation of the PP from the enveloping skin using an impermeable membrane in the pedicle dormant region.



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2.4 Comments

When to tie a membrane onto an "O" ring, it should tie it to such a degree that, on the one hand, membrane is firmly held onto, and on the other hand, can separate from the attached "O" ring without breaking it by the expanding tissue mass pushing upwards from the underneath.

For antler regeneration, one should note that dormant pedicle stumps (skin loosely attached portion) are very short (2–2.5 cm), thus it is very hard to firmly hold the inserted membranes during the period of antler regeneration, given that surface of PTFE membrane is quite slippery; therefore, the membrane wrapped around a pedicle stump must be tied properly using a thread to prevent the membrane from being lost.

3. In vivo identification of skin tissue components required for establishing interactions with AP

- **Nude mouse transplantation**

3.1 Purpose

Previous experiments convincingly demonstrated that antler formation is triggered by interactions between AP-derived-tissue and the overlying skin, and possibly via exchanging (putative) diffusible molecules (Refer to Technique 2). However, it is still not known whether all components of the skin tissue or only some are involved in these interactions. It is known that the hallmark of antler formation is the change in the type of epidermis from a scalp-type (with a thick hair shaft, mono-lobed sebaceous glands and sweat glands) to a velvet-type (thin hair shaft, large multi-lobed sebaceous glands, absence of sweat glands) and presence/absence of a thickened epidermis [4,15]. Since only skin with hair follicles is competent for antler formation [15], then the two skin tissue components, epidermis and hair follicles, must be involved in the process.

Currently, the roles of the inner dermal portion (below the level of hair follicles) and the subcutaneous loose connective tissue (SLCT), which resides between the AP and outer dermal portion (containing hair-follicle), in the interactions are not known. That is, it is not clear whether these two interposing tissue layers are required for successful initiation of the interactions during initial antler formation. This is important if the ultimate goal is to identify and isolate the interactive molecules, which requires precise pinpointing of the key interactive tissue/cell components. Therefore, we developed and successfully applied a nude mouse transplantation model to address this question.

3.2 Principle

The key question with respect to initiation of antler formation is whether or not initiation results from direct interactions between the AP- derived tissue and the outer dermal portion of the skin (epidermis plus hair-follicle-containing dermal tissues). A technique

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has been exploited to address this question: transplantation of AP plus outer-dermal portion in a nude mouse model. In the transplantation, the interposing layers including SLCT and inner dermal portion (below the hair bulbs) are omitted; the AP and the outer dermal portion are then sutured together (tissue unit) before transplantation. In so doing, the AP and the outer dermal portion are brought together directly, hence addressing the question as to whether antler formation can be initiated without participation of the interposing layers.

3.3 Procedure

The procedure for the nude mouse transplantation of the tissue unit has been described [16]. Before developing this technique, we demonstrated in nude mice that subcutaneously transplanted AP (Figure 3A and 3B) can induce formation of pedicle-like, but not antler-like, bumps (Figure 3C) [17], indicating that nude mouse skin lacks the competency to interact with AP tissue. Consequently, to induce xenogeneic antler via AP transplantation, deer skin must be present either through co-transplantation with AP or have been transplanted prior. Initially we tried to suture outer dermal portion onto the head of a nude mouse (Figure D), but circulation between the deer skin and the host was failed to establish, and the deer skin eventually became desiccated (Figure 3E) and exfoliated.

To overcome the problem, we took an alternative approach directly suturing the AP and the outer dermal portion together; the combined tissue unit was then transplanted subcutaneously onto the heads of nude mice (Figure 3F). When the pedicle bumps from the transplanted deer tissue appeared and pushed up (Figure 3G), a-cross-skin-cut was made on the apex of each bump (Figure 3H); at the same time, and the excess mouse skin was trimmed to entirely expose the deer skin. After exposure, the hair on the deer skin started to grow (Figure 3I), and gradually the deer skin was transformed to antler velvet skin (Figure 3J). Histological examination (Figure 3K) and exogenous testosterone administration (Figure 3L) experiments confirmed the antler nature of the xenogeneic mouse antlers.

Figure 3: Nude mouse model through AP transplantation. **A:** AP insertion through a skin incision (arrow) between the two ears. **B:** AP subcutaneous transplantation onto the head of a nude mouse (arrow). **C:** A pedicle- shaped bump is formed at the AP transplantation site (arrow). **D:** Suture of outer dermal portion (dermis below the hair follicles trimmed off) onto nude mouse heads (arrow), where the equivalent area of mouse skin has been removed. **E:** Desiccated deer skin, which was previously sutured onto mouse head but did not survive. **F:** Subcutaneous transplantation of AP+ outer dermal portion (tissue unit) with epidermal upside onto the head of a nude mouse (arrow). **G:** A pedicle-shaped bump is formed at the tissue unit transplantation site; note the dark colour (deer hairs) at the apex of the bump. **H:** Crosscut made on the apex of the mouse bump to expose the implanted deer skin (arrow). **I:** Exposure of the deer skin on the entire apex of the bump by trimming off the excessive mouse skin; note the densely populated hairs. **J:** The deer scalp skin (hair densely populated) has been transformed to velvet skin (shinny and hair-sparsely populated) two to three weeks after exposure. **K:** Histological

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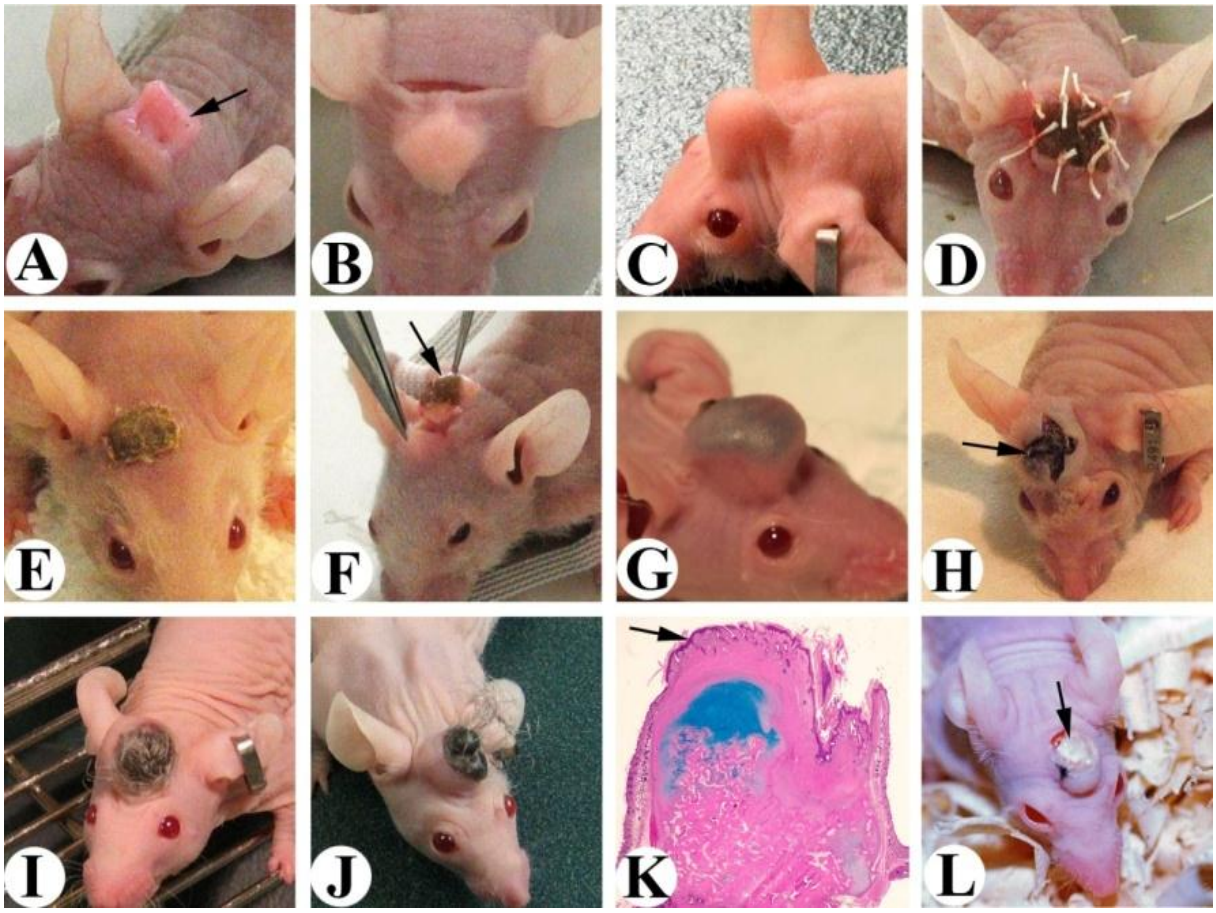
section of an antler-like bump formed on a nude mouse; note that the apical skin of the bump has acquired velvet skin features (thick epidermis and large sebaceous glands; arrow). **L:** A hard antler-like bump on a nude mouse (arrow), which was induced via administration of exogenous androgen.



3.4 Comments

Successful establishment of a nude mouse model to grow xenogeneic antlers has opened a totally new avenue for antler research. This has rendered some previously formidable tasks with live deer into manageable routine laboratory work. Examples include testing the role of tissue components in antler formation, and key tissue interactions during antler genesis through membrane insertion. It has also made some other tasks in the study of antler generation or regeneration possible; examples are the in vivo evaluation of the role of individual molecules identified through in vitro and/or in silico approaches (refer to Technique 6).

4. Alternative transplantation technique to reduce AP quantity required for antler induction

- **Intradermal approach**

4.1 Purpose

Now we know that initiation of antler formation relies on the interactions between the skin-components (hair follicle containing outer dermal portion) and the AP-derived tissue, and the tissue layers interposing these two

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interactive tissue types, including inner dermal portion (below the level of hair follicles) and SLCT, are not required (refer to Technique 3). Then it follows that if AP tissue can be delivered directly underneath the hair bulbs (i.e. carrying out intradermal implantation), substantially less quantity of AP tissue should be needed for successful antler induction compared to the conventionally subcutaneous transplantation, as in so doing it can bypass the step of close association between the interactive tissue types. Thus, a statistically viable experiment with multiple groups can be practically designed from the limited source of AP tissue (only two pieces can be sourced from a deer).

The ultimate proof for the involvement of candidate molecules identified from in vitro and/or in silico approaches (refer to Technique 6) is whether or not they are involved in triggering antler formation in vivo. To successfully establish an in vivo model for the purpose, the following criteria must be met: 1) number of groups that enough AP tissue can be allocated into must be statistically viable; 2) a means through which a target gene can be efficiently knocked-down or knocked-in in the AnSCs; and 3) an animal species that can sustain antler growth.

Gene silencing or overexpression studies are normally carried out on cells. Unfortunately, we have tried, but failed when using disaggregated singular AP cells to induce antler formation in deer. The reason for the failure, we believe, is that the extracellular matrix is also required for success. Therefore, we have focussed on tissue rather than cells to solve the problem and have achieved positive results. While the deer is the preferred species for study, there are practical limitations for the following reasons: 1) deer are large wild ungulates, which pose challenges in management in captivity and handling along with costs for each round of testing; 2) antler generation is once in a life-time event and regeneration takes place annually; hence there are considerable time costs in the evaluation of the effects of gene silencing/overexpression along with a narrow window of opportunity (seasonality) for such testing. Therefore, there is a strong case for consideration of conventional laboratory animals, although they do not naturally grow antlers. Interestingly, we have successfully used the nude mouse model to evaluate roles of tissue components in antler generation and regeneration. To test candidate genes, we established an in vivo model through combining intradermal AP implantation with nude mouse transplantation techniques. Intradermal implantation allows conservation of AP tissue opening up opportunities for experiments with statistically viable design. The nude mouse transplantation alleviates costs, experimental duration and complexity associated with large wild ungulates.

4.2 Principle

For intradermal implantation in vivo, AP tissue is delivered intradermally and directly underneath the bulbs of hair follicles; in so doing, the AP and hair follicles can physically form close

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associations bypassing the step of close association between the interactive tissue types. This enables an answer to the question as to whether the process of antler formation can be advanced (shorter pedicle) and less AP tissue used compared with the natural condition (i.e. with the interposing layers). As shown by Goss [4], minced (and mixed) AP tissue can successfully initiate ectopic antler formation when transplanted elsewhere in the body. Therefore, in our model, minced AP tissue is used for intradermal implantation, as antlerogenic potential is unevenly distributed within the AP.

Theoretically, the combination of intradermal implantation and nude mouse transplantation is straight forward, as the techniques of intradermal AP implantation [18] and creation of xenogeneic antlers on nude mice [16,17] have been established. However, practically there are still at least two hurdles to be overcome before one can successfully establish the model. The first is to make an intradermal pocket ex vivo in a piece of sampled deer skin for loading the AP tissue directly underneath the hair follicles. This means one must be able to horizontally cut into dermis of thin deer skin (<2 mm; soft but tough) sampled from a 1-year-old stag (adult deer skin is too thick for nude mouse transplantation) ex vivo. We have developed and successfully used a device for the task. The device is, however, only suitable for in vivo use, and does not suit for the ex vivo situation. The latter requires the skin to be held tightly during cutting in a sterile hood. Therefore, we specifically designed new gear for this purpose, namely the ex vivo intradermal pocket maker.

The second hurdle is that one must be able to efficiently infect the AnSCs resident in the periosteum using viral vectors (carrying one or more selected sequence (s)).This requires using AP tissue rather than disaggregated singular cells, as with induction of antler formation by AP, both cells and the extracellular matrix seem indispensable. Therefore, we have been working on tissue, based on the rationale that so long as AP tissue is cut sufficiently thin, viral vectors will be able to reach every cell, including those that are distant from both cut surfaces. We have succeeded in delivery of the FGP gene into majority of AnSCs, if not all, resident in the AP tissue, with the optimal thickness being about 200 μm. In order to properly cut AP into 200 μm thin sections, we developed and successfully used a periosteal tissue cutter (Patent No: ZL 201420335401.8).

4.3 Procedure

The detailed procedure for intradermal AP implantation in vivo has been described [18]. The major technical challenge for AP implantation is to create a suitable intradermal pocket. In this respect, it is virtually impossible to cut an intradermal pocket without damaging hair follicles or puncturing the pocket using a handheld scalpel, as the scalp skin of yearling sika stags (the only age which possesses an AP) is very thin (1.5-2 mm). Consequently, we developed and successfully applied [18] a custom-built intradermal pocket

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maker (Patent No: ZL 201402817l0.6) specifically for this task. A coronal skin incision is firstly made between the two pedicles using a scalpel, and then inserting the device subcutaneously into the skin anteriorly through the incision for making the intradermal pocket. Load the AP into the pocket (Figure 4A) and use subcutaneous AP transplantation as an appropriate control (Figure 4B). Subsequently, the mouth of the pocket is sutured before closing the first scalp skin incision (Figure 4C). Our results have shown that intradermal implantation approach has redefined the minimum quantity of AP tissue for antler induction. Only one-eighth of the total AP is required via intradermal transplantation for successful antler induction in the same year (Figure 4D) or one-twelfth for antler induction in the following year; whereas, about one-half of AP via subcutaneous transplantation is required. Therefore, intradermal transplantation is superior to conventionally subcutaneous transplantation as it conserves precious AP tissue.

To establish the method of intradermally implanting AP ex vivo, two longitudinal ends of sampled deer skin are tied to the two holders of the pocket maker and then the skin is tightened through adjusting the handle. Using a handheld scalpel to cut the dermis from the upper edge of the skin, an intradermal pocket is carefully made (Figure 4E). The minced AP is loaded into the pocket using two fine forceps (Figure 4F); two pockets can be made on one piece of deer skin, and open mouth of each pocket is sutured following the AP loading (Figure 4G). The skin tissue unit containing each pocket removed using sharp scissors (Figure 4H). The collected skin tissue units are placed in culture medium (Figure 4I) for subsequent transplantation. Each tissue unit with the epidermis upwards is inserted subcutaneously through the skin incision on a nude mouse head. When the tissue unit is survived and grows up, the mouse skin is opened by cutting, and the excessive mouse skin trimmed away to expose the deer skin (refer to Technique 3). Around 2-3 weeks later, the deer scalp skin will have been converted to velvet-like skin (Figure 4J). Transformation of scalp skin to velvet skin is the hallmark of antler formation. Histological structure of the mouse bumps further confirms the antler nature of transformed bumps (Figure 4K). If the antler-like bumps formed from the implanted thin AP slices (200 μm) that are infected with GFP-containing viral vectors, the tissue of the bump will express GFP (Figure 4L).

Figure 4: Intradermal implantation of AP in vivo (A-D) and ex vivo (E-L). **A:** Loading the minced AP tissue into an intradermal pocket (arrow). **B:** Loading the minced AP tissue subcutaneously (arrow). **C:** Suture of the open mouth of an intradermal pocket after loading minced AP (arrow). **D:** Ectopic antler formation from the intra- and sub-dermis transplanted AP; note that an antler is formed from the intradermal pocket site (arrow), whereas no antler was generated at the subcutaneous implantation site (arrowhead). **E:** Dermal pocket (arrow) made using a handheld scalpel, where the skin is tightly held in an ex vivo dermal pocket maker. **F:** Loading of the minced AP into the dermal pocket (arrow). **G:** Sutured mouths (arrows) after loading of the AP tissue. **H:** Trimming of the skin portion containing a

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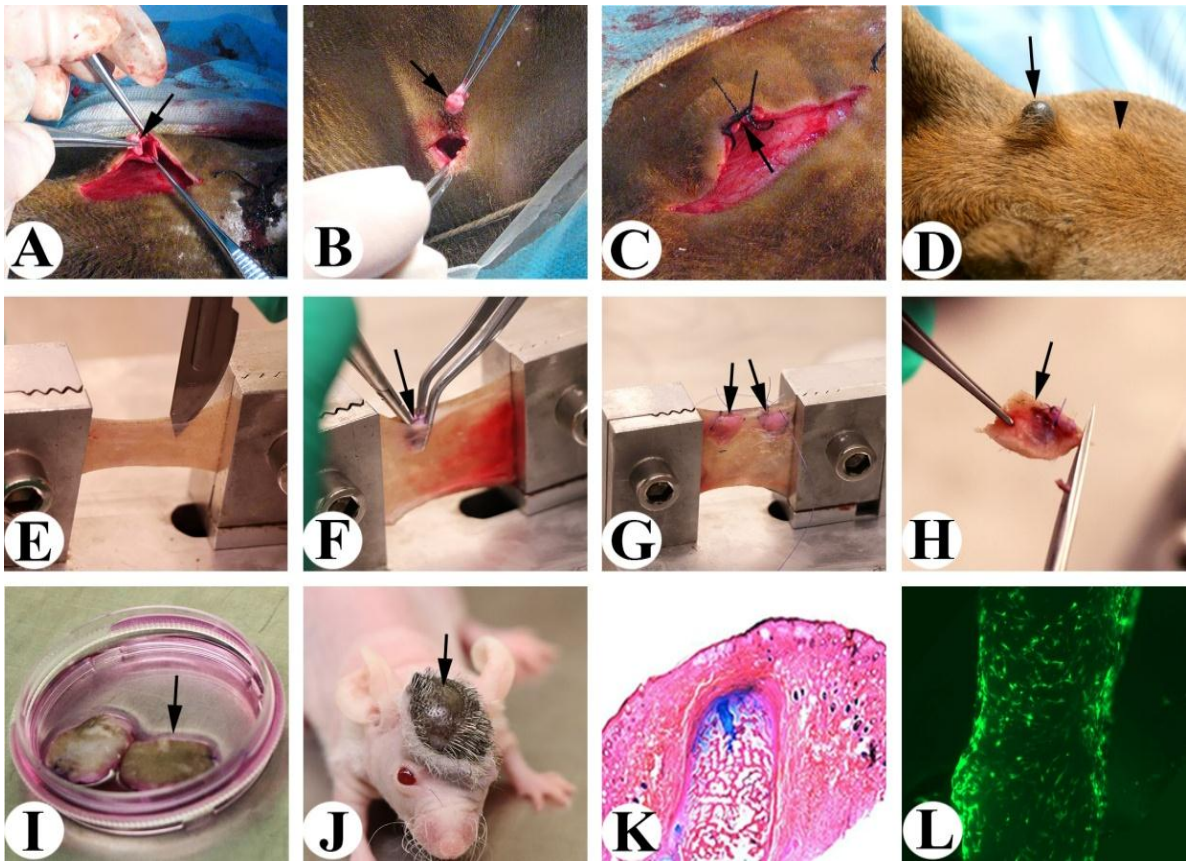
pocket using sharp scissors (arrow). **I:** Two tissue units containing AP intradermal (arrow). **J:** Apically exposed bump on a nude mouse head; note that velvet skin has transformed from typical scalp skin on top of the bump at the centre (arrow). **K:** Histological section of the nude mouse bumps; histologically the bump resembles the real antler. **L:** GFP-labelled AnSCs in the thin slices of AP; note that almost all the AnSCs are expressing GFP incorporated via Lentiviral vector infection.



4.4 Comments

Both the AP and PP are minced before transplantation. This is considered necessary as the antlerogenic potential of the AP in the future pedicle growth region is not evenly distributed [19,20]; this is also the case for PP from different aspects (anterior, posterior, lateral, and medial) of a pedicle [21,22].

Based on preliminary results of our work with the ex vivo model, AP from each side of the future antler growth region of a male deer can provide about 8 pieces, each of which can successfully induce a xenogeneic antler formation. Thus, each deer can provide at least 16 AP tissue mass which can sustain growth of 16 xenogeneic antlers. Therefore, our approach renders gene testing in vivo possible. Notably, we have established a method to successfully freeze down thin slices of AP tissue for subsequent implantation use, so that experiments can be carried out in year-round.

5 In vivo evaluation of the role of interposing tissue layers in antler generation

- **Liquid nitrogen spray**

5.1 Purpose

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Experimental studies show that antler formation relies on the interactions between the AP-derived-tissue and overlying skin. Successful establishment of the interactions requires the two interactive tissues to become closely associated, which is primarily achieved through expansion of the AP tissue mass due to rapid multiplication of the resident cells [23]. Naturally, only the central region AP (an area equivalent to the size of a pedicle base) forms a pedicle and first antler, although the marginal periosteum also has the similar potential to launch pedicle and antler formation (when mechanically wounded or in the case of the naturally disappearing pedicles in aging stags). This has been attributed to the reason that only the central AP has the ability to receive the seasonal stimulation of circulating androgen hormones [24]. Thus, only the central AP can build up the critical tissue mass to form a close association with the overlying skin, and so initiate antler formation. We know now that any putative molecules to be exchanged between the AP-derived-tissue and hair follicles in the overlying skin must traverse the interposing layers in order to reach the reciprocal targets [25]. Deletion of the central AP in a pre-pubertal deer (Figure 5A) or an entire pedicle in an adult deer by surgically cutting through the skin and SLCT can effectively stimulate the marginal periosteum to launch antler formation (Figure 5B). Our hypothesis is that the process of mechanical cutting cleared the interposing physical barriers, thus creating the opportunity for the two tissue types to interact directly, effectively bypassing the need for their close association. To test this hypothesis in vivo, we have exploited different approaches and finally found that cryosurgery can provide a successful means.

5.2 Principle

Cryosurgery is the most widely used method for destroying abnormal or diseased tissue, with liquid nitrogen (LN2) being the preferred cryogen. Interestingly, LN2 spray applied for less than 30 seconds does not result in scarring because the collagen layer of the dermis including SLCT is preserved, allowing for in-migration of the cellular components in the healing process to restore the normal integrity of the skin layers [26]. Because an appropriate LN2 treatment can effectively decellularize the treated tissue while, at the same time, keeping extracellular collagen components intact, cryosurgery offers an opportunity to directly test our hypothesis that mechanically breakage of the interposing physical barrier is the main reason why wounding can stimulate AP either from the central or marginal region to grow antlers. A CRY-AC 500 ml cryogun (Figure 5C) fitted with a size “A” nozzle (Brymill, Ellington, USA) is the preferred apparatus for delivering LN_2 to the central antlerogenic region prior to pedicle initiation. For detailed specification and general operation of the LN2 gun, refer to the

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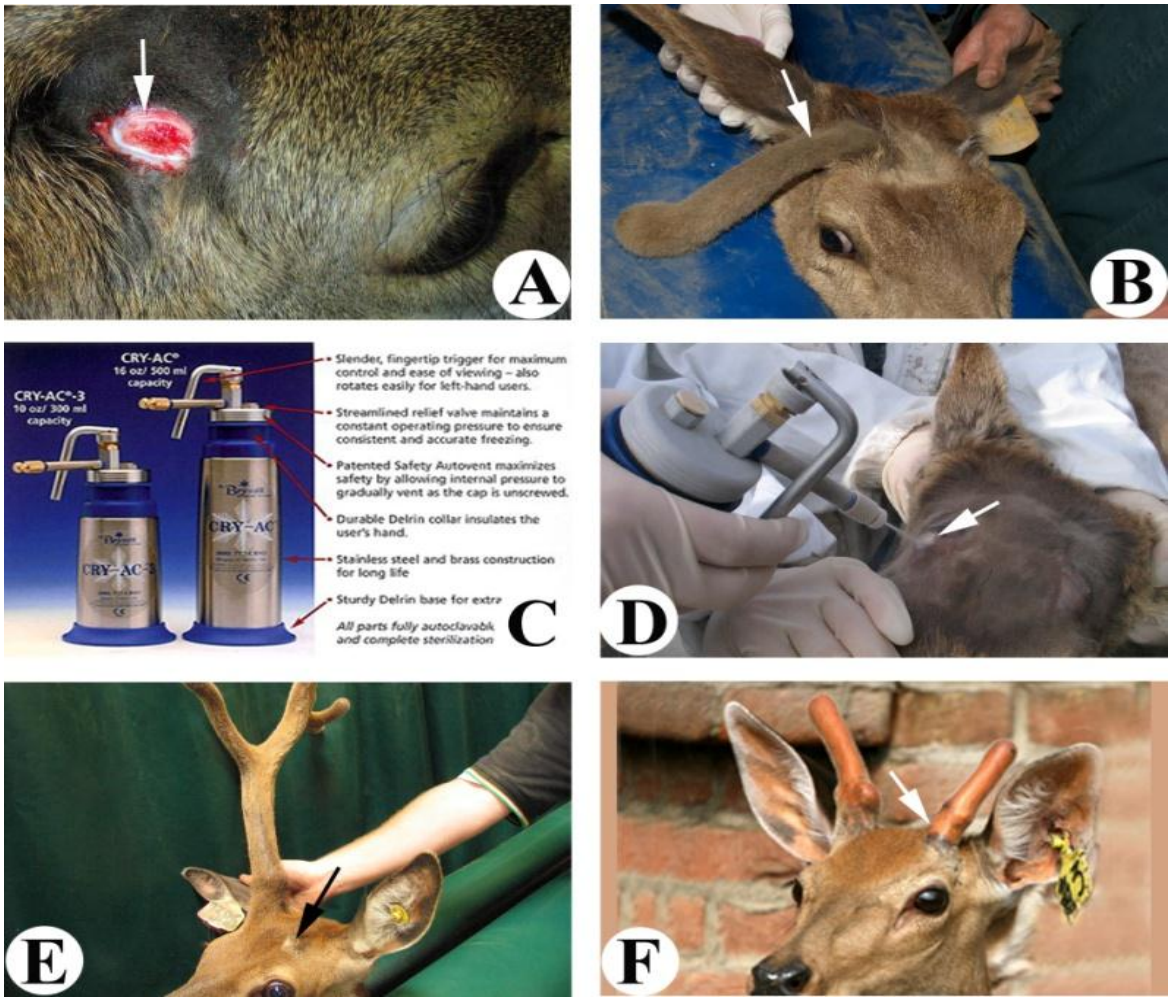
manufacturer's manual (www.boc.co.nz/shop/en/nz/brymill-cry-ac-cryogun-500ml-b-700).

5.3 Procedure

The detailed cryosurgery procedure for decellularizing central AP has been described [27]. Briefly, prior to each cryosurgery, the hair over the antlerogenic region is thoroughly shaved, and the nozzle of the cryogun is positioned 2-4 cm above the skin surface (Figure 5D). Duration is set at 15 sec/spray after the ice field has filled in the central region. For skin-only cryosurgery, skin over the AP is moved

Figure 5: Liquid nitrogen spray on deer heads. **A:** Deletion of the central AP in a pre-pubertal deer using a tissue cutter including the overlying skin and a sliver of underlying bone (arrow); the deleted area is equivalent to the average size of a pedicle base. **B:** Formation of a spike antler from the central AP deleted site (arrow). **C:** A CRY-AC 500 ml cryogen fitted with a size "A" nozzle. **D:** Liquid Nitrogen (LN_2) spray to the future pedicle growth region using a cryogen with the nozzle being positioned 2-4 cm above the deer skin surface (arrow). **E:** Antler has failed to form from the marginal AP (arrow) after the central AP was cryo-surgically destroyed using LN_2 spray, due to the preservation of the interposed collagen structure of the SLCT. **F:** Significant advancement of development of an antler caused by repeated LN_2 sprays while ensuring that the central region of the AP is kept intact; note that the pedicle formation step is bypassed (arrow).



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back and forth in the intervals (60 sec/interval) of LN_2 spraying to avoid cryo-damage to the underlying AP. For the skin plus AP cryosurgery, the spray duration is counted from the time when skin has firmly frozen to the underlying AP (skin can no longer be moved over AP by fingers). A single 3-FT (freeze and thaw)-cycle of spray (3 x 15 sec spray with 60 sec intervals) applied to a treated region can effectively kill the cells resident in the periosteum. Spray tests demonstrate that a marginal AP fails to initiate growth of a pedicle and an antler after the central AP is cryo surgically destroyed while with the preservation of the collagen structure of the SLCT (Figure 5E). In contrast, antler development is significantly advanced if the collagen structures of skin and SLCT layers are substantially attenuated by repeated LN_2 sprays while keeping the central region AP intact (Figure 5F). Consequently, the cryosurgery approach has supported convincingly our physical barrier theory, namely that the timing of antler development must be controlled primarily by the degree of permeability of the SLCT layer to the putative interactive diffusible molecules.

5.4 Comments

The 3-FT-cycle spray regime is recommended for testing this type of theory using cryosurgery, rather than 40 second continuous spray, because, although both regimes are found equally effective for decellularization of the AP tissue (evaluated using cell culture approach), the latter is highly likely to cause extracellular component damage (i.e. disruption of integrity of skin/SLCT). In contrast, the former is less likely to cause this problem as each spray is less than 30 seconds, the amount of time required to destroy the extracellular component [26]. Furthermore, repetition of the freeze-thaw cycles in 3-FT-cycle spray is known to be a more effective way of destroying cells than a continuous spray of the same duration [26]. Importantly, the eyes of the animal must be carefully protected while doing LN2 spray.

6 In vitro identification of the interactive molecules between AnSCs and niche cell populations

- **Cell co-culture system**

6.1 Purpose

We know now that interactions between the AnSCs in the AP or PP and the epidermal cells in the overlying skin result in antler formation. There is evidence to suggest that dermal papilla cells (DPCs) in the hair follicle bulbs in the overlying skin are involved in the process via mediation of interactions between the AnSCs and epidermal cells. As described previously, it is only skin adorned with hair follicles that is competent to interact with AnSCs for antler generation, and importantly, the DPCs are the only dermal-derived cells in a hair follicle and are also the closest part of a hair follicle to the AP tissue. Hair shafts are significantly thinned after transformation to velvet skin, and

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the thickness of hair shafts depends on the size of DPC aggregates [28]. Therefore, the unknown molecules involved in these interactions between AnSCs, DPCs and epidermal cells must be identified and isolated if the mechanisms of the initial generation and the annual renewal of antlers are to be elucidated, with the ultimate goal being the contribution to the fields of organogenesis and regenerative medicine.

Due to the complexity of the AnSC niche and the likely low abundance of interactive molecules, it would be a formidable, if not impossible, task to identify and isolate these interactive molecules in vivo. Therefore, we have recently established an in vitro co-culture system in which all the interactive cell types that have so far been identified are placed together in a way that seeks to mimic the in vivo situation. In so doing, the process of identification of the putative interactive molecules is effectively simplified. In addition, if downstream analysis is coupled with a powerful proteomic approach, such as stable isotope labeling of amino acids in culture (SILAC) or isotope-coded affinity tag (ICAT), to enable detection of proteins that are synthesized and secreted only during co-culture period.

6.2 Principle

We can define the topographical position of these interactive cell types of the future antlerogenic region, and so we can mimic the in vivo situation when placing these cell types together in a co-culture system. However, the critical challenge for a successful co-culture system is whether the system can mimic successfully the functionality of the in vivo situation. In this respect, we know that the hallmark of successful establishment of the interactions and thus antler formation in vivo is the transformation of epidermis from a scalp-type to a velvet-type; the former is with a thick hair shaft and the latter with a thin shaft.

Interestingly, formation of a hair follicle in vivo is induced by the underneath DPC aggregate and diameter of a hair shaft is in direct proportion to the size of the inductive DPC aggregate [28]. Therefore, if the co-cultured DPCs with AP or PP cells can form a significantly smaller nodule size compared to that of the DPCs in singular culture, then we would know that the system has worked at the morphological level. Furthermore, we know that both AP and PP cells have stem cell attributes. If on co-culture with DPCs, AP or PP cells have significantly down-regulated the key stem cell marker genes expressed in the AnSCs in singular culture, then we would know that co-culture has stimulated AnSCs to start to differentiate.

A tissue culture inert system (such as 6 well inserts) is preferred as the backbone of the co-culture system. The permeable membrane of an insert can act as the interposing tissue layer in vivo, as the membrane allows molecules to pass through, but blocks the passage of cells. Therefore, when the AnSCs (AP or PP cells) and the niche cell types (DPCs and/or

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epidermal cells) are cultured at each side of the membrane, one would know the two key interactive cell types have been unambiguously separated without compromising the interaction of the cells through diffusion of active molecules.

6.3 Procedure

Detailed procedures for isolation of AnSCs [29,30] and for DPCs [28,31]have been published. Briefly, for isolation of DPCs from the deer scalp, the skin is cut into thin slices (around 0.7 mm in thickness) from the epidermis to the SLCT direction and along the longitudinal orientation of resident hair shafts using our patented tissue cutter (Patent No: ZL2014 2 0335401.8).The dermal tissue above (including epidermis) and under the hair bulb zone is removed using a scalpel, while keeping the mid zone, where the dermal papillae reside, intact (Figure 6A). Each hair follicle is isolated; the bulb located and cut off with a short piece of hair root (Figure 6B). The isolated follicle bulbs are then transferred into a 10 cm dish containing the digestion medium. After 2–3 h digestion, each follicle bulb is isolated and the DPCs removed (Figure 6C) using a sharp scalpel or a needle tip under a stereomicroscope. The dermal papillae are then cultured in a 3.5 cm dish, and the DPCs trypsinized; when most of the cells have migrated out of the papilla, the cells are ready for co-culture.

AnSCs and DPCs are suspended in the culture medium at a density of around 1.0×10^5 cells/ml and 1.2×10^6 cells/ml respectively. For one co-culture style, AnSC suspension (0.5 ml) is firstly seeded onto the membrane of each inverted insert of a 6-well-plate in the cell culture incubator for 3 h. Thereafter, the insert is put back into the original well containing 2.6 ml culture medium/well. The DPC suspension (1.5 ml) is then seeded into each insert, the cells allowed to precipitate onto the inner surface of the membrane, and the plates are returned to the incubator for 7–12 days. The AnSCs or DPCs singular culture are used as a control.

Different combinations of cell co-culture approaches can be exploited depending on the application, which might include: culture of AnSCs and DPCs at each side of the insert membrane (Figure 6D); or addition of epidermal cells onto the top of the DPCs in the insert (Figure 6E); the culture AnSCs on the insert membrane and DPCs in the well (Figure 6F); or addition of epidermal cells onto the top of the DPCs in the well (Figure 6G), etc.

Compared to the nodule size in DPC singular culture (Figure 6H), the DPCs co-cultured either with APCs (Figure 6I) or PPCs (Figure 6J) at each side of the membrane resulted in significantly smaller DPC nodules (Figure 6K). Gene expression profiling results also confirmed this finding. Three stem cell marker genes, Nanog, klf4, DKK3, were significantly down-regulated in the co-cultured ASCs compared to singular-cultured ASCs (Fig, 6L). It is reported that expression of these three marker

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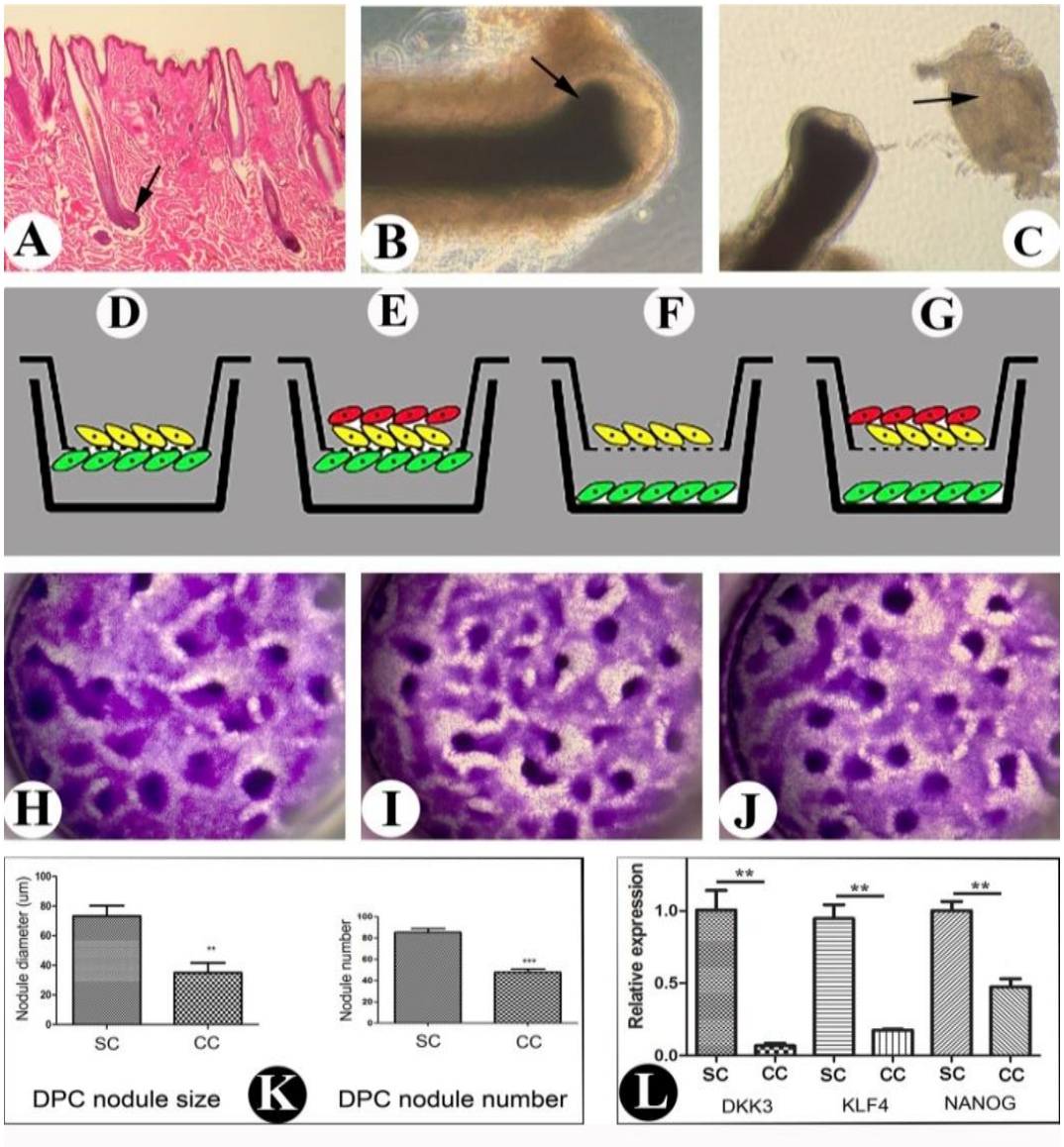
genes is closely correlated with the degree of stemness of AnSCs [7]. Therefore, the evidence is that our tissue-culture-insert co-culture system has sufficiently mimicked the in vivo situation.

Figure 6: Cell co-culture system. **A:** A histological section of deer scalp skin to show the region where hair bulbs reside (arrow). **B:** An isolated partial hair follicle containing the bulb (arrow). **C:** Removal of a dermal papilla (arrow). **D-G**. Schematic drawings to depict the different styles of cell co-culture. **D:** AnSCs (outer surface, green) and DPCs (inner surface, yellow) are cultured on each side of the insert membrane; E. Epidermal cells (red) are added on top of the DPCs in Figure 5D. **F:** AnSCs are cultured in the well and DPCs cultured in the insert; **G**. Epidermal cells (red) are added on top of the DPCs in Figure 5F. **H:** DPC aggregates formed in singular culture. **I:** DPC aggregates formed in co-culture with AP cells. **J:** DPC aggregates formed in co-culture with PP cells. **K:** Quantification of the DPC nodule size and number; note that both the nodule size and number in the co-culture are significantly smaller/fewer than those of singular culture. **L:** Evidence of significant down-regulation of expression of three stem cell marker genes, DKK3, KLF4 and Nanog, in the AnSCs in our co-culture system, compared to the corresponding singular culture system.



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6.4 Comments

As mentioned above, if the downstream analysis of the co-culture system can be coupled with modern analytical techniques, it will make the identification and isolation of the putative interactive molecules much more powerful. In a previous study [31], we have applied SILAC technique to our co-culture system, and identified 128 candidate proteins. These proteins are enriched in 13 transduction pathways, and the two most significant are involved in interactions (ECM-receptor interaction) and proliferation (PI3K/AKT signalling pathway). However, the relevance of these identified proteins and signalling pathways to antler genesis must be ultimately tested in an appropriate in vivo model (refer to Technique 4).

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