INTERNATIONAL RESEARCH PROMOTION COUNCIL

Announces

World Scientists Forum International Awards

EMINENT SCIENTIST OF THE YEAR 2003

Dr. CHUNYI LI

Dr. Chunyi Li, PhD, an internationally reputed scientist, is presently the Senior Scientist of the Bioactive Discovery Group, AgResearch Ltd, Invermay Agricultural Centre, Mosgiel, New Zealand. He was born on 11th August 1959 in Hebei Prov., P.R. China.

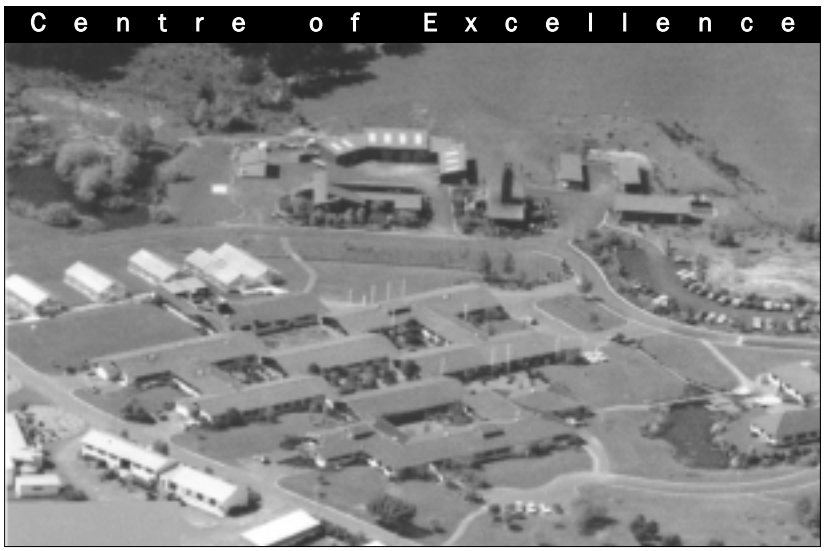
Dr. Li received his Master Degree of Animal Physiology from the Graduate School of Chinese Academy of Agricultural Sciences and Doctor Degree in Physiology from Medical School, Otago University, New Zealand. Dr. Li is one of the leading research scientists of deer antler biology in the world. He was introduced into this field in 1982 while doing his Master degree in the Institute of Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences. On completing his degree in 1985 he continued working in this area, until 1990 at which time he came to New Zealand as a visiting Chinese Scholar with the then MAF_Tech deer antler research team led by Dr. Jimmy Suttie. In 1993 under the supervision of Drs Suttie and Harris (University of Otago Medical School) he commenced PhD studies on the evaluation of initial antler stem tissue. On completion of his PhD in 1997 he was employed by AgResearch Ltd to further investigate antler biology. Through years of antler research he came to realise that antlers are a fascinating multiple biomedical research model. Annual renewal of antlers offers unique opportunities to explore just how nature solves the problem of mammalian epimorphic (blastema-based) regeneration. Un precedented growth rate (up to 2.75 cm/day!) of antlers provides a rare system where fast cell proliferation is elegantly regulated without becoming cancerous. The self-differentiation ability of initial antler stem tissue can serve as an invaluable model for stem cell research.

During the course of investigation of antler biology, Dr. Li has published approximately 50 papers in peer reviewed journals (the majority as principle author), attended approximately 20 relevant conferences, and written four books. A highlight of Dr Li's career was his delivery of the plenary talk in the antler science session at the 1st International Symposium on Antler Science and Product Technology (ASPT) in Banff, Canada in 2000. Since then he has been invited to give the plenary lecture in the antler science session at the 2nd ASPT, to be held in Queenstown, New Zealand, in 2004. The biggest contribution he has made through these 20 odd years of research is the promotion of the antler model to the biomedical research society. Dr Li believes his promotion of the antler model to biomedical research has the potential to greatly benefit mankind.

Dr. Li has received several awards like the Excellent student award in the Hebei Agriculture University in 1979, 1980 and 1981; Excellent student award in the Graduate School of C.A.A.S. in 1985; Excellent Young Scientist award from the Chinese Association of Science and Technology in 1991 etc. He is also the member of various Societies. Dr. Li is currently a Visiting Professor of Institute of Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences; and Jilin Agriculture University, P.R. China.

Dr. Li is married to Wenying Wang and has two children-Lu Li and Yang Li.

Dr. Chunyi Li was selected by the World Scientists Forum for "Eminent Scientist of the Year 2003" international award based on his research innovations and contributions in the field of Antler science.



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6

Development of deer antler model for biomedical research

Chunyi Li
AgResearch Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand

Specialised subjects have ramifications far beyond their own apparent limits, and implications sometimes of crucial importance to the solution of fundamental problems. Deer antlers are no exception.

Richard J. Goss

Deer antlers have the potential to become excellent biomedical research models. The annual renewal of antlers offers the unique opportunity to explore how nature solves the problem of mammalian epimorphic (blastema-based) regeneration. The unprecedented growth rate (1-2 cm/day!) of antlers provides a rare system where fast cellular proliferation is elegantly regulated without becoming cancerous. The self-renewal and differentiation ability of antlerogenic periosteum, the tissue that gives rise to antlers, can serve as a valuable model for stem cell research. Although these advantages have been appreciated for decades, the establishment of these models has been very slow. During the years of study on antler biology at Invermay New Zealand, we have been continuously developing and promoting antler models to the biomedical research field. This review consists of two parts: antler biology and antler as a model.

Key wards: antler, pedicle, antlerogenic periosteum, model, stem cells

ANTLER BIOLOGY

1. Morphogenesis

Deer antlers are bony organs which are cast and fully regenerate each year. Antlers do not directly grow from the deer head, instead they form from permanent bony projections, or pedicles. Deer are not born with pedicles, these develop from the frontal bone crests (Fig. 1A) when deer approach puberty (5-7 month old in red deer Cervus elaphus). When pedicles (Fig. 1B) grow to their species-specific height (about 5-6 cm high in red deer), first antlers generate spontaneously from the apices of these pedicles. This generation can be easily identified by a change in skin texture from typical scalp-like to velvet-like (Fig. 1C), which is called velvet skin. After initiation, first antlers enter a rapid growing period. First antlers normally form a single main beam and do not branch, hence they are called spikes. When the rutting season comes, spike antlers become fully calcified, nerve and blood supplies are terminated, and velvet is shed to expose bare bone (Fig. 1D). First antlers are cast in the next spring

Address for correspondence: Dr Chunyi Li, Ph. D AgResearch Invermay Agricultural Centre Puddle Alley, Private Bag 50034 Mosgiel, New Zealand Phone: (0064) 3 4899183 Fax: (0064) 3 4899038 E-mail: chunyi.li@agresearch.co.nz

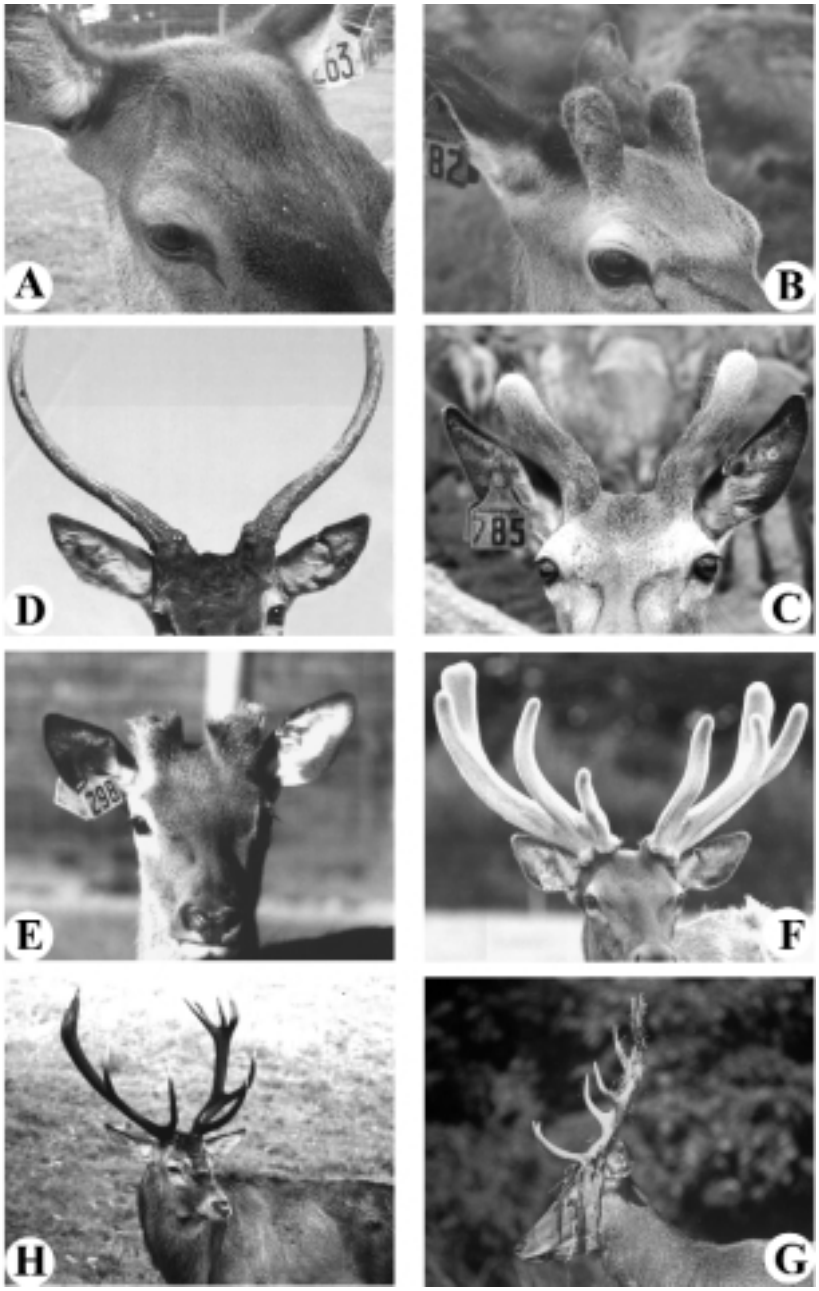

Figure 1. Ontogeny of deer antler (red deer)
A. Right side frontal lateral crest from a weaner stag. Pedicles develop from the crests when deer reach at a threshold body weight.
B. Late stage growth pedicles from a 7-month-old calf.
C. Rapid growing antlers from an 8.5-month-old young stag after transformation from pedicles.
D. Hard bare bone antlers are carried around by a yearling stag in rutting season.
E. Pedicle stumps from a 2-year-old stag immediately after previous hard antler casting (in late spring).
F. A pair of 60-day growing antlers (antler growth takes about 120 days) from an adult stag (in early summer).
G. Velvet stripping from the fully grown antlers of an adult stag (in early autumn).
H. Exposed hardened and polished antlers carried by adult stags (in late autumn and winter).

and regeneration of second set of antlers immediately follows (Fig. 1E and 1F). From then on, annual development of subsequent antlers enters a well-defined cycle: old antler casting and new antler regeneration in spring (Fig. 1E), rapid growth and maturation in summer (Fig. 1F), calcification and velvet shedding in autumn (Fig. 1G), and bare bony antlers in winter (Fig. 1H).

2. Histogenesis

The axis of a deer pedicle and antler consists of an interior component (osseocartilaginous tissue) and an exterior component (skin). The following are the descriptions of the histogenesis of both the interior and the exterior components of pedicles and first antlers in red deer.

1) Pedicle and first antler

The pedicle interior component, consisting of osseocartilaginous tissue (a mixture of bone and cartilage), builds up initially from the cellular layer of the periosteum overly the presumptive antler growth region, and proceeds

three ossification stages (Li and Suttie, 1994). These stages are firstly intramembranous ossification or IMO (only bone tissue is formed, Fig. 2A) up to 1.0 cm in pedicle height, followed by transitional ossification or TO (osseocartilaginous tissue, Fig. 2B, 2C and 2D) between 1.0-2.5 cm, and finally modified endochondral ossification or pECO (vascularized cartilage, Fig. 2E and 2F) over 2.5-3.0 cm in pedicle height. The interior component of first antler is, like late stage pedicle, also formed through modified endochondral ossification (aECO).

Formation of the exterior skin of a pedicle and an antler also proceeds through three histologically distinguishable stages (Li and Suttie, 2000). These stages are compression of the apical subcutaneous loose connective tissue when pedicles grow to about 1.5 cm high (in TO stage, Fig. 3A and 3B), stretching of the apical undulated epidermis when pedicles about 2.5 cm high (in early pECO stage, Fig. 3C, 3D and 3E), and neogenesis of the overlying skin and its associated appendages when pedicles are over 3.0 cm high (in mid pECO stage). Antler velvet transformation from pedicle skin does not occur until the apical pedicle skin becomes intimately associated with the underlying antlerogenic tissue when pedicles are over 3.5 cm high (in late pECO stage). This transformation includes the loss of arrector pili muscle and sweat glands, and the gain of large bi- or multi-lobed sebaceous glands (Fig. 3F, 3G and 3H). During this process,

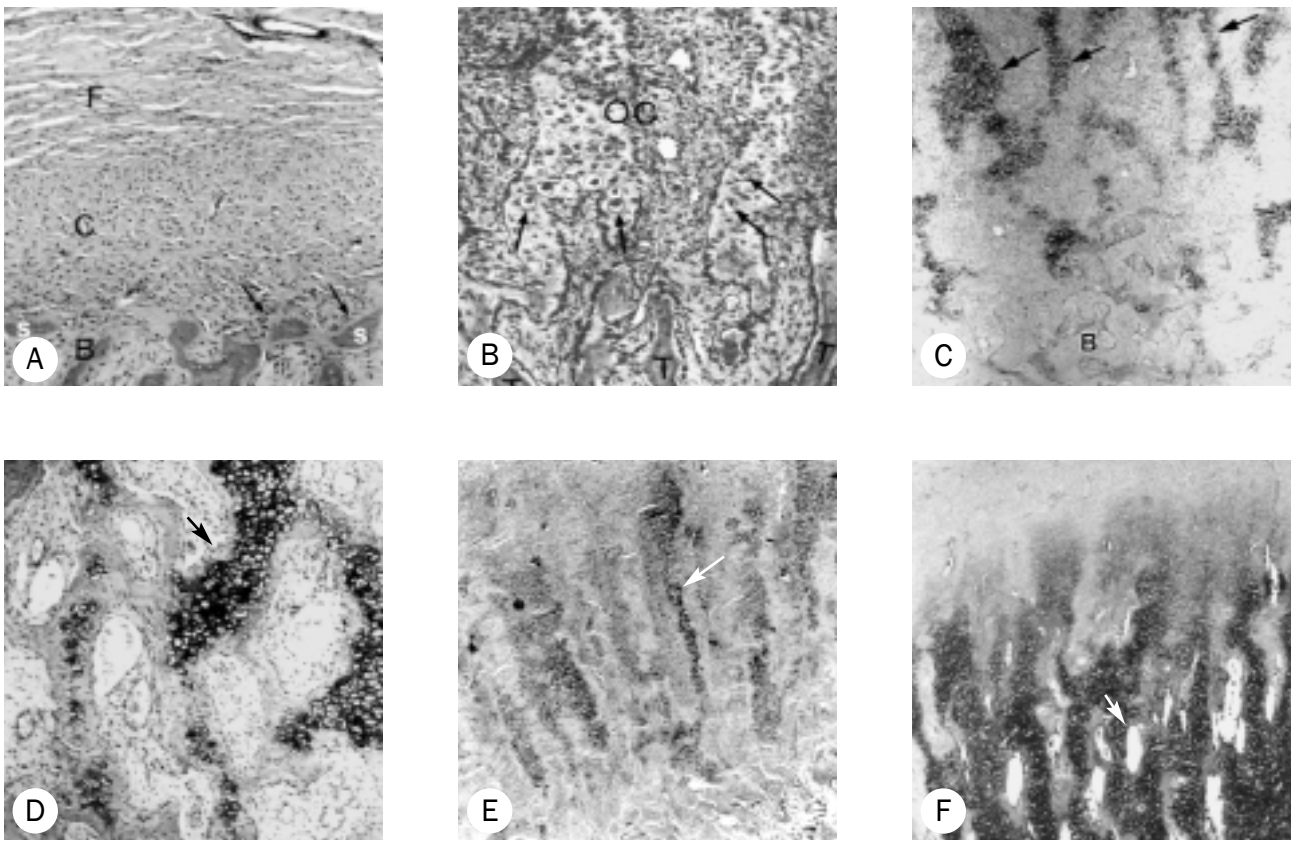

Figure 2. Vertical sections of the interior tissue from different developmental stages of pedicle/antler. Haematoxylin and eosin staining (HE) + alcian blue (AB) counter staining.

A. The periosteum overlying the presumptive antler growth region. The surfaces of the trabeculae and the spicules (S) were lined with active osteoblasts (arrows). F, C, and B are the fibrous layer, cellular layer and bone respectively.

B. Section of a palpable pedicle through osseocartilaginous tissue (OC) and cancellous bone from a 6-month-old calf. Note the discrete clusters of mature chondrocytes (arrows) appeared in the trabeculae (T).

C. Section of an incipient pedicle through osseocartilaginous tissue (arrows) and cancellous bone (B) from an 8-month-old young stag. Note that the proportion of the cartilaginous tissue increases towards the distal end (top).

D. Higher magnification of a part of Figure 2C, showing the mixture of cartilage (arrow) and bone tissue.

E. Section through the cartilaginous tissue from a 9-month-old young stag. Showing the continuous cartilaginous trabeculae (arrow).

F. Section through a pedicle and an antler junction which is distinguished by skin change. Showing that no demarcation can be detected as all the vascularized (arrow) cartilaginous columns are continuous between pedicle and antler.

the epithelial layer of the apical skin of growing pedicles and antlers becomes thicker and thicker (Fig. 3F, 3G and 3H). The evidence from this study suggests that pedicle skin formation results from mechanical stretch, and this mechanical force is derived from the fast expansion of the underlying osseocartilaginous tissue.

2) Second and subsequent antlers

Histogenesis of subsequent antlers, that is antler regeneration, can be classified into 2 phases: 1) antler casting and wound healing; 2) establishment of posterior and anterior growth centres. Once these growth centres are established, antler regeneration takes place in the same way as first antler generation (Li and Suttie, 1994). Histologically, the antler casting and wound healing phase consists of 3 stages. These stages are resorption of the pedicle and antler junction (Fig.4A-4B), casting of hard antler (Fig. 4C), and wound healing (Fig. 4D). Establishment of anterior and posterior growth centres consists of 2 stages. These stages are initiation of the growth centres through intramembranous ossification (Fig. 4E), and expansion of these centres via modified endochondral ossification (Fig. 4F, 4G and 4H).

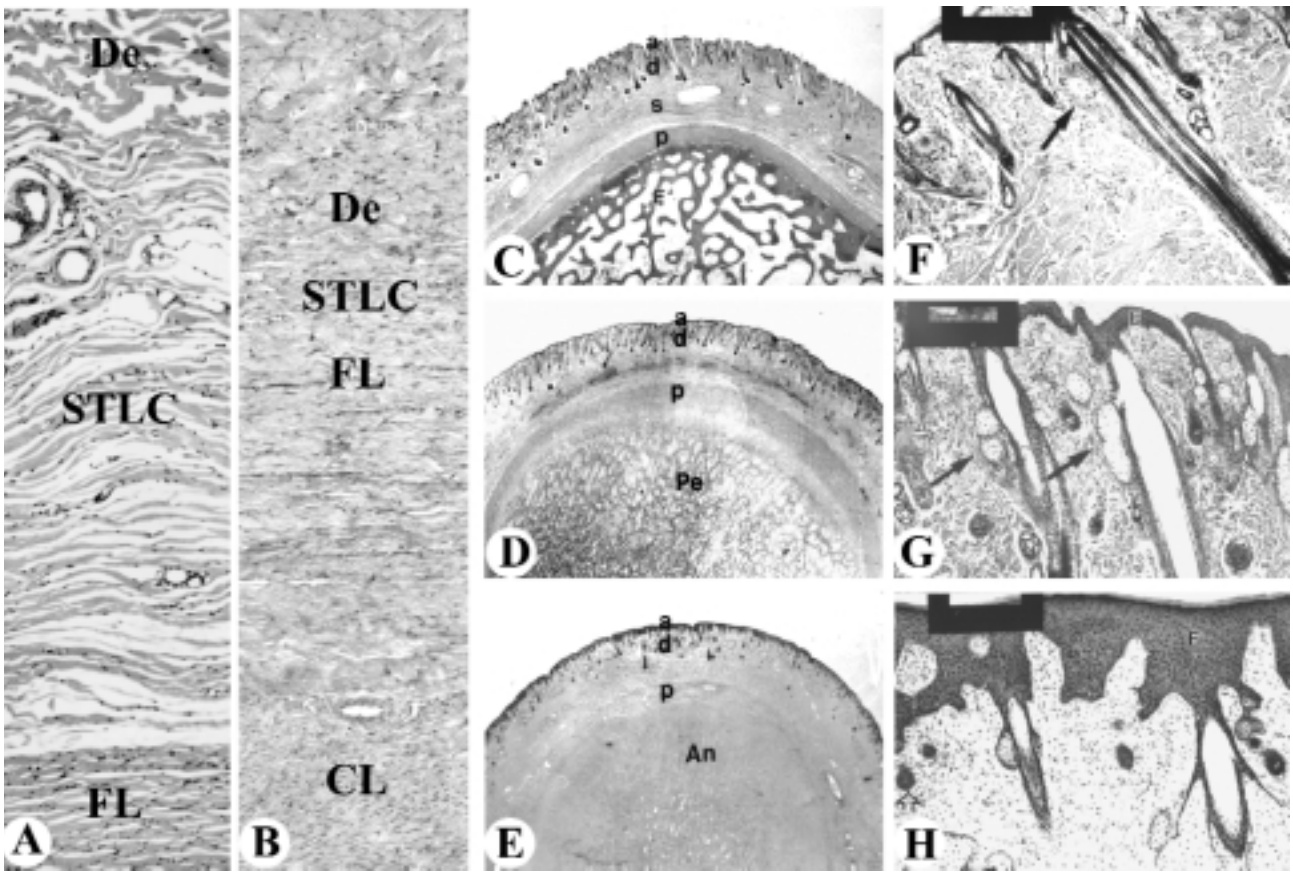

Figure 3. Vertical sections of the exterior tissue (skin) from different developmental stages of pedicle/antler. Haematoxylin and eosin (HE) staining.

A-B. subcutaneous loose connective tissue (SLCT). De, dermis, FL or CL, fibrous layer or cellular layer of periosteum/perichondrium. A. From frontal lateral crest stage. Notice that the SLCT is a very loose and thick layer. B. From early aECO stage. Notice that the SLCT layer has become a thin strip.

C-E. Epidermis (a). C. FLC from an 8-month-old male calf. Notice that the epidermis of the integument is undulated. D. Incipient pedicle at OPC stage from a 9-month-old male stag, showing the flat epidermis. E. Incipient antler from a 10-month-old male stag, showing the thick and flat epidermis. d, dermis; s, subcutaneous loose connective tissue; p, periosteum/perichondrium; Pe, pedicle; An, antler.

F-H. Apical epidermis and its associated appendages. F, From FLC stage. Notice that the epidermis (E) is thin and undulated; the sebaceous gland is small and monolobar with an obvious arrector pili muscle (arrow) attached. G, From OPC stage. The epidermis is thicker than at the FLC stage, but still undulated. Sebaceous glands are bigger than the FLC stage (arrow). H, From aECO stage. The epidermis is flat and thicker than at the OPC stage. The sebaceous glands are bigger and more complicated than at the OPC stage, and with no arrector pili muscle attached. Star, sweat gland.

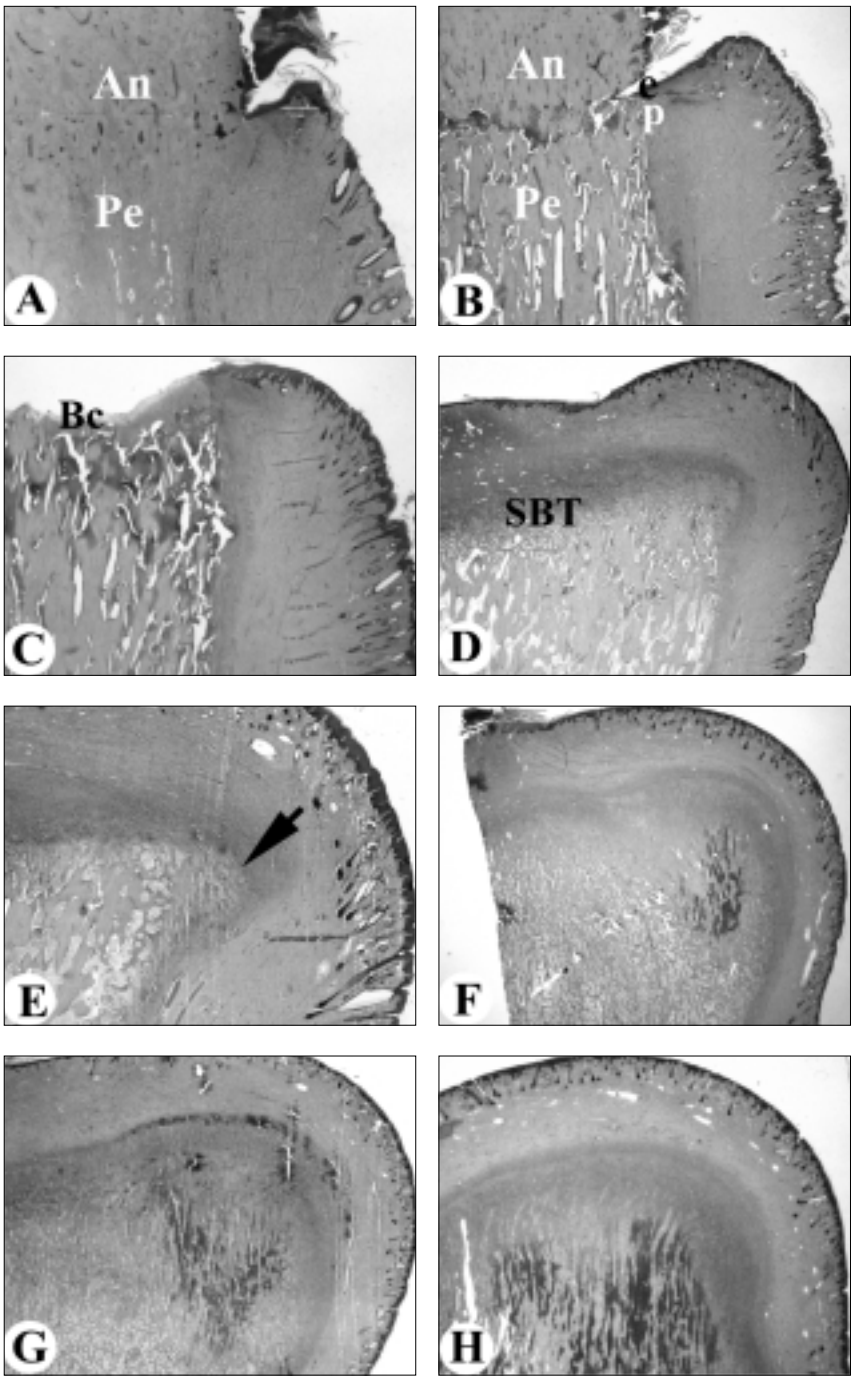

Figure 4. Vertical sections cut through the posterior ends of different regenerating stage antlers. HE + AB counter staining.

A. Pedicle (Pe) and antler (An) junction well before the date of antler casting. No sign of bone resorption along the junction can be identified. The distal tip of pedicle skin is wrapped by the thickened epidermis, which is closely attached to pedicle peripheral periosteum.
B. Pedicle (Pe) and antler (An) junction just before antler casting. A narrow, dark-reddish abscission line becomes apparent, which sharply delineates the plane of future separation. Notice that a peripheral circumferential cleft was formed at the junction. Both periosteum (p) and epidermis (e), which were closely associated with each other, start to grow into the cleft.
C. Pedicle stump just after antler casting. Notice the pedicle stump surface was covered with blood clot (Bc).
D. Healing stage pedicle stump. During the late stage of wound healing, bony trabeculae of the pedicle surface were surmounted by well vascularized connective tissue, from which new slender bony trabeculae (SBT) were formed over the existing bony trabeculae.
E. Posterior antler growth centre (arrow) becomeg apparent shortly after wound healing completion.
F-H Vascularized cartilage (dark colour) forms in the growth centre and continuously expands as the centre pushes disto-posteriorly to form antler main beam.

Histological examination shows that antler regeneration is a unique system, which allows full regeneration of mammalian appendages.

3. Stem tissue

The tissue that gives rise to initial pedicles and antlers was discovered by a combination of deletion and transplantation experiments. The presumptive pedicle growth region (Fig. 5A) consists of bone, periosteum, connective tissue, dermis and epidermis. Through deletion experiments, Goss et al. (1964) found that it was the bone, not the skin that was responsible for the initial development of pedicles and

antlers. Hartwig and Schrudde (1974) took a transplantation approach. They subcutaneously transplanted the periosteum from the presumptive pedicle growth region (Fig. 5A and 5B) of a roe deer to elsewhere on the deer body as an autograft. They found that an ectopic pedicle and an antler were formed from the grafted site, but the original site lost the potential to grow pedicle and antler (Fig. 5C). In contrast, grafted pedicle skin could not induce ectopic pedicle or antler formation (Fig.5d) These pioneer findings in roe deer were later confirmed by Goss and Powel (Goss and Powel 1985) on fallow deer, and by Li and Suttie (Li and Suttie 2001) on red deer. In these experiments, ectopic pedicles and antlers were induced when this periosteum was subcutaneously transplanted either on the deer nose (Fig. 5E) or on the foreleg

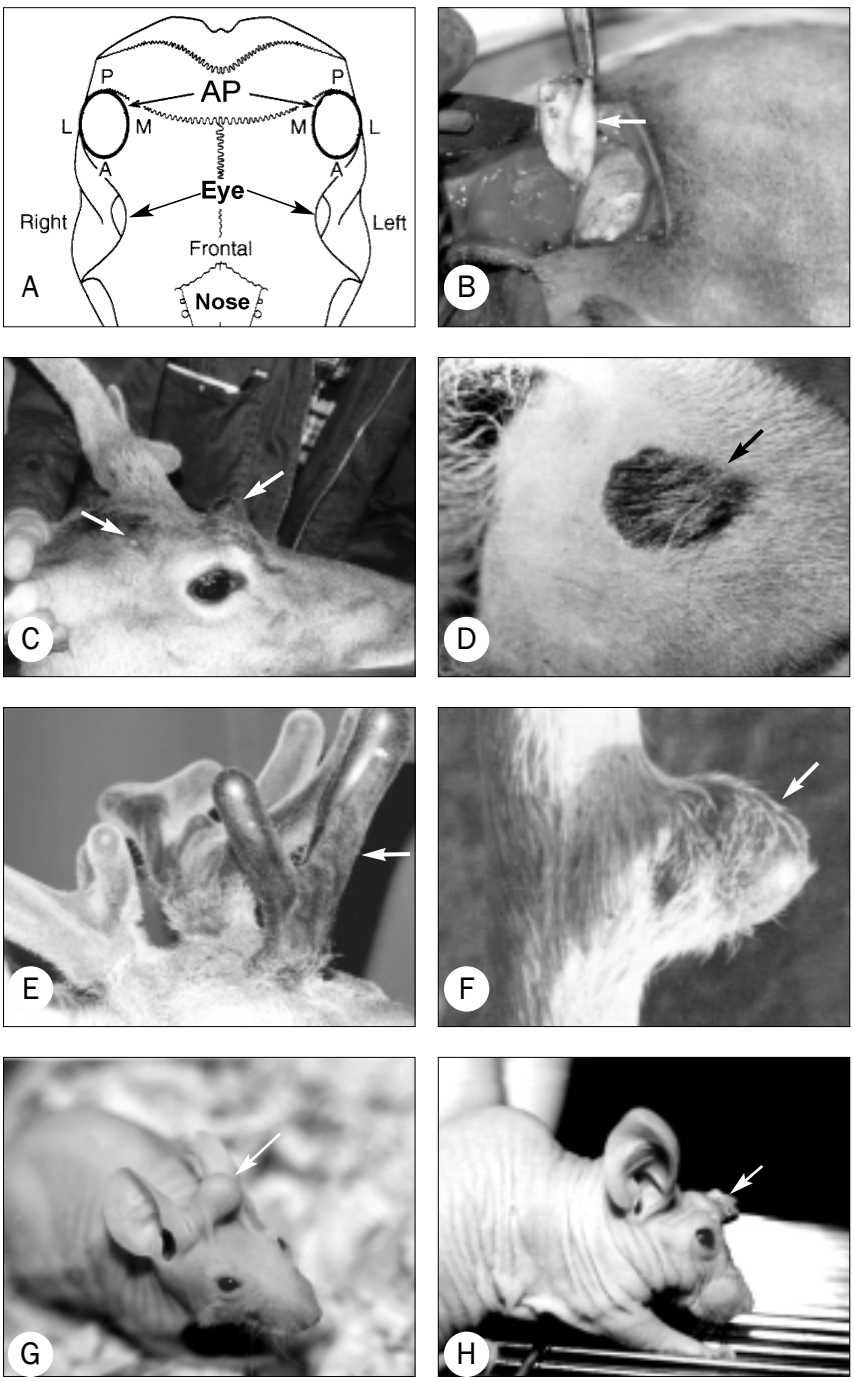

Figure 5. Deletion and transplantation of antlerogenic periosteum.

A. Illustration of the location of antlerogenic periosteum on a deer head. AP, antlerogenic periosteum; A, anterior; L, lateral; M, medial; P, posterior.
B. AP (arrow), which is being removed surgically for transplantation.
C. An ectopic antler formed on a male red deer nasal bone (arrow) by a piece of grafted AP from the right side presumptive pedicle growth region (arrow). Note that no pedicle or antler formed from the original region after the removal of AP.
D. Graft of a piece of pedicle skin (arrow) on the inner surface of a deer ear. Note that no pedicle or antler formed from the surviving pedicle skin (reproduced with permission from: Goss, 1983, Deer Antlers. Regeneration, Function and Evolution. Academic Press, New York, p143).
E. An ectopic branched-antler (arrow) formed on the midline of frontal bone of a 3-year-old red deer stag by the autologously grafted AP.
F. An ectopic antler (arrow) formed from the grafted AP on a foreleg of a fallow deer (reproduced with permission from: Goss, 1983, Deer Antlers. Regeneration, Function and Evolution. Academic Press, New York, p129).
G. A pedicle-shaped protuberance (arrow) formed on a nude mouse head by AP xenografts.
H. A pedicle-shaped protuberance with a piece of antler-like bony tissue (arrow) on its top formed by AP xenografts.

(Fig. 5F). Therefore, this periosteum is called antlerogenic periosteum (AP). Interestingly, pedicle-shaped (Fig. 5E) or even pedicle-antler-shaped (Fig. 5F) protuberances can be induced to grow if small pieces of AP (2 x 2 mm^2) are subcutaneously transplanted onto the heads of nude mice (Li et al., 2001).

Further research on AP reveals that histologically AP (Fig. 6A), like its somatic counterpart (Fig. 6B), consists of two layers: an inner cellular layer and an outer fibrous layer (Li and Suttie, 1994). However, these layers are much thicker than those of a somatic periosteum. In red deer, the cellular layer is 3.7 times thicker than that of the facial periosteum (Fig. 6A and 6B). Inasmuch as the cellular layer accounts for bone formation (Ham and Harris, 1971), the AP should possess a greater potential than facial periosteum to form bone. Ultrastructurally, the cellular layer cells are very rich in glycogen (Li and Suttie, 1998) (Fig. 6C and 6D). Thus, these cells closely resemble embryonic cells. The intracellular glycogen is known to be mainly used as a source of energy (Scott and Glimcher, 1971) and for intracellular synthesis of mucosubstances (Cabrini, 1961) in foetal osteoblasts. When these AP cells are left for an extended period in a culture medium in vitro, they form large bone nodules (Fig. E; Li and Suttie, 2001). Histological examination shows that these nodules have a well-organised structure (Fig. 6F; Li and Suttie, 2001) and resemble the bone trabeculae within a pedicle or a growing antler. That is: the more differentiated

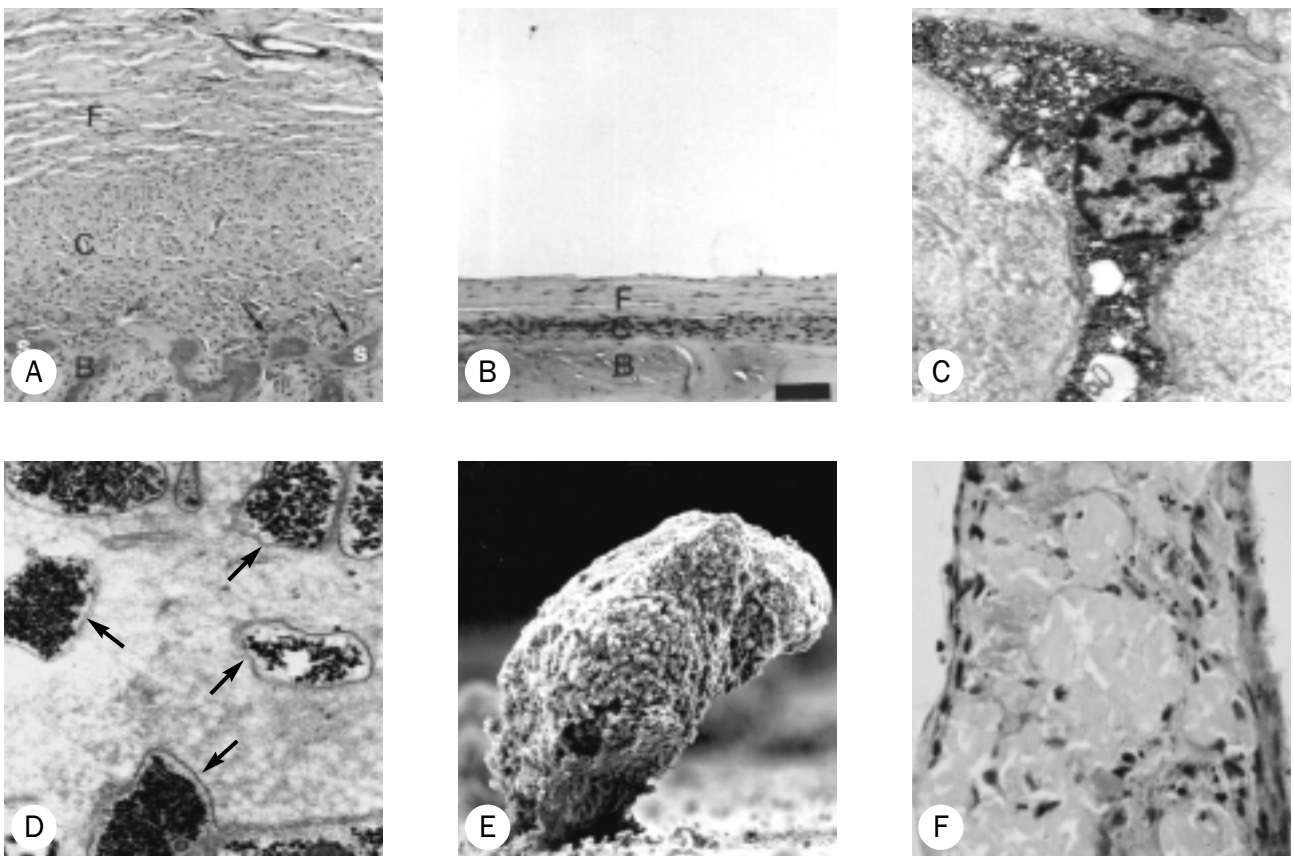

Figure 6. Antlerogenic periosteum and antlerogenic cells

A. AP and underlying bone from a 4-month-old male calf. F, fibrous layer; C, cellular layer; B, bone. The bony spicules (S) were covered with active osteoblasts (arrows).

B. Facial periosteum and the underlying bone from a 4-month-old male calf. F, C, B are the same as shown for Figure 6A.

C-D. Intracellular glycogen of antlerogenic cells from the cellular layer. C, the cytoplasm of a cell densely occupied by glycogen granules. D, a section of a group of glycogen-filled processes of a cell or cells.

E. A bone nodule formed in a culture dish from cultured antlerogenic cells.

F. Longitudinal section of a bone nodule formed in a culture dish from antlerogenic cells. Note that more differentiated cells are in the centre, whereas less differentiated cells are mainly found peripherally.

cells are located in the centre and actively secrete extracellular matrix including collagens, whereas the less differentiated cells are found peripherally. Some of these features of AP, such as astonishing growth potential and rich glycogen content, can only be found in embryonic cells. This astonishing self-differentiating ability shown by AP can only be paralleled, in mammals, by embryonic stem cells. Therefore, we conclude that AP cells are the true antler stem cells.

In order to confirm our histological findings that pedicles and antlers are the derivatives of AP, we recently carried out an experiment to trace AP cell lineage using a genetic marker. In this experiment, some of the AP cells were labelled using the LacZ gene in vivo during the pedicle initiation. The pedicle and early antler formed from the genetic-marker-labelled AP were biopsied, processed and stained using X-gal. The results clearly showed that all types of cells in the interior component of pedicle and antler are the progeny of AP cells (Fig. 7). Therefore, we have concluded that deer pedicles and antlers are the derivatives of AP, the antler stem cells (Li and Suttie, 2001).

4. Regulation

As male secondary sexual characters, formation of deer pedicles and antlers is controlled by androgen hormones. However, pedicles and antlers differ in response to androgen stimulation: a high level of androgen hormone triggers pedicle initiation and sustains pedicle growth, but causes antler calcification and death.

1) Pedicle

Pedicles develop when male deer approach puberty. Suttie et al (1984; 1991) reported that pedicle formation is associated with increasing plasma testosterone level. Castration prior to pedicle initiation will abolish future pedicle and antler formation, whereas administration of exogenous testosterone to the castrated deer can overcome this abnormality and initiate pedicle growth (Wislocki et al., 1947; Jaczewski, 1982; Li et al., 2003). Androgen hormone may have direct effects on pedicle initiation as antlerogenic periosteum possesses specific binding sites for testosterone (Li et al., 1990; Li et al., 1998; Li et al., 2001).

Autoradiographic localisation (Li et al., 1998) has demonstrated that AP contains specific binding sites for testosterone. Therefore, these results support the notion that pedicle initiation results from direct androgen stimulation on AP (Fennessy and Suttie 1985). However, in vitro studies (Li et al., 1999; Li et al., 2001) have shown that the primary cultured AP cells do not proliferate in response to testosterone, although these cells do respond to IGF1 in a dose-dependent manner.

Besides of androgen hormones, nutrition is also involved in pedicle formation as pedicle initiation always tends to occur at a threshold body weight, irrespective of age or season (Suttie and Kay, 1982; Fennessy and Suttie, 1985). The results from our in vitro experiment showed that cultured antlerogenic cells react to testosterone in proliferation only when sufficient IGF1 is in presence (Li et al., 1999). Therefore, nutritional cues may act synergistically with androgens for the development of pedicle through growth factor pathways.

2) Antler

First antlers in red deer form from fully formed pedicles when the testosterone level decreases to a threshold level, which is however moderately high compared with that recorded for subsequent antler regeneration (Suttie, Lincoln et al. 1984). Antler growth fell in the period when testosterone levels were barely detectable. Antler calcification and velvet skin shedding were the consequence of high plasma levels of testosterone, and antler casting and subsequent antler regeneration were associated with very low or undetectable levels of testosterone (Bubenik 1982; Suttie et al., 1991).

i. First antler

By administering a single dose of exogenous testosterone, Wislocki et al (1947) induced not only pedicles, but also first antlers to grow from two ovariectomised white-tailed deer. However, attempts by Jaczewski et al (1974; 1976; 1981) on red deer, and Goss (1983) on sika deer were failed to achieve the same results using prepubertally castrated or ovariectomised deer, although incomplete pedicles (around 2 cm high, normal pedicle height in these deer species is about 5 to 6 cm) were induced. Jaczewski (1982) concluded that there must be a difference in the hormonal control of first antler generation between genus Cervus (red or sika deer) and genus Odocoileus (white-tailed deer). Thus, besides androgen hormones some other stimuli may be required for the completion of the process of first antler formation from the exogenous testosterone induced pedicles in genus Cervus. However, considering that in the case of red or sika deer might lay in the failure to produce complete pedicles in previous experiments, we treated prepubertally castrated male or young female red deer with repeated testosterone

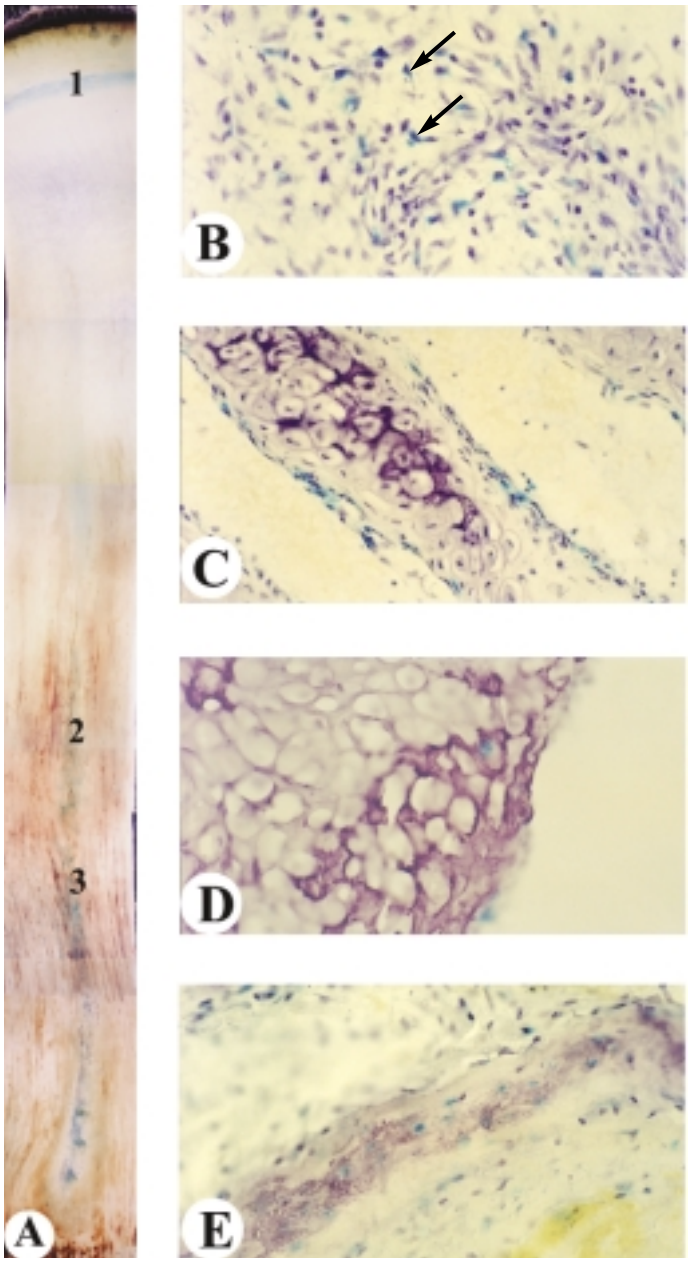

Figure 7. Antlerogenic cell lineage tracing using a genetic marker LacZ gene in vivo. Stained with X-gal.

A. A vertical section cut through a pedicle and growing antler, which was formed from a partially-LacZ-gene labelled antlerogenic periosteum. Notice the blue column located in the centre of the section was made up of LacZ gene expressing cells.
B. Cells from the area labelled 1 in Figure 7A. Notice that some of the cells (arrows) were expressing the LacZ gene.
C. Cartilaginous column, from the area labelled 2 in Figure 7A, containing the LacZ gene expressing cells. Notice these cells are mainly less-differentiated chondroblasts.
D. A part of a cartilaginous column from the area labelled 3 in Figure 7A. Notice that a chondrocyte was expressing the LacZ gene.
E. A part of lamellar bone. Note that most of the cells in the bone were expressing the LacZ gene.

injection (once every two weeks) until the induced pedicles reached approximately 5 cm high, then ceased the testosterone treatment. By so doing, we successfully induced first antler formation from castrated or normal female red deer. Consequently, we have concluded that testosterone itself is sufficient to stimulate both pedicle and first antler formation in genus Cervus (Li et al., 2003).

ii. Second and subsequent antlers

Although deer antler is a male secondary sexual character, the mechanism underlying androgen control of antler formation is complex and some phenomena have not been satisfactorily explained. Wislocki (1943) found that antlers could continue to grow after a deer was castrated and that antlers began their renewal at a time when the testes and seminal vesicles were most inactive. However, antlers became hard and velvet was shed when testes were rapidly enlarging and new antler regeneration took place when the deer testes had begun to decrease in size. These phenomena promoted Wislocki (1943) to think some nongonadal factor

must be involved in this control. Therefore, he advanced an "antler growth stimulus" (AGS) hypothesis. Since then, extensive work to identify the putative AGS has been carried out (Goss 1963; Hall et al., 1966; Bubenik 1982; Suttie et al., 1985)

Suttie et al. (Suttie et al., 1985) reported that the seasonal peak of insulin-like growth factor 1 (IGF1), a non-gonadal factor, almost perfectly coincided with the peak of antler growth rate. As IGF1 plays a growth-promoting role in cartilage formation and the antler growth centre is mainly composed of cartilage, they suggested that IGF1 might be the AGS. IGF1 receptors were subsequently located in the growing antler tissues (Elliott et al., 1992). More convincing results to support the notion that IGF1 might be the AGS were subsequently reported by Sadighi et al. (1994). They found that IGF1 increased the proliferation of the mesenchymal and cartilaginous cells derived from antler proliferation zone in a dose dependent manner. Taking these IGF1 results together with their extensive study on the role of steroids in antler growth, Suttie et al. (1995) concluded that antler growth cycle is under the control of androgen hormones, but antler growth per se, at least in red deer, does not appear to require testosterone. If a trophic role were to be considered, testosterone would exert a priming effect on the cells in the proliferation zone, which would then become more responsive to IGF1 during the antler growth phase. This conclusion was further supported by the study reported by Li et al. (1999), where testosterone alone in serum-free medium did not show any mitogenic effects on the mesenchymal cells from pedicles or first antlers.

DEVELOPMENT OF ANTLER MODELS

1. Stem tissue/cell

Stem cells, including embryonic and adult stem cells, are crucial for tissue repair and regeneration, as they have the ability for self-renewal and to differentiate into a variety of cell types. While models for research on embryonic stem cells have been well established and studied extensively, a comprehensive model for adult stem cell research is still under development.

Deer antler stem tissue/cell, antlerogenic periosteum (AP), provides an excellent model for this purpose. AP, as a piece of postnatal tissue, has an astonishing self-differentiation ability (refer to "Antler biology"). The self-differentiation ability of stem tissue can be studied using AP in vivo as autografts (on the same deer), allografts (within the same species using diffusion chambers) or xenografts (on nude mouse); or in vitro using disaggregated cells. Using the antler stem cell model coupled with recent advanced techniques, such as laser microdissection and microarray analysis, novel genes or specific regulatory pathways controlling the self-differentiation of stem cells may be identified.

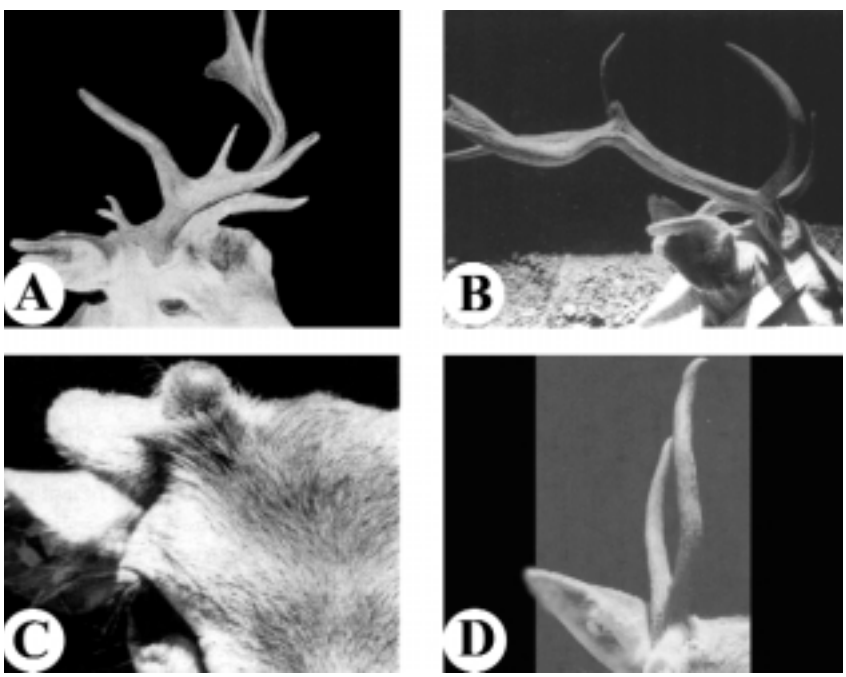

Figure 8. Regulative properties of antlerogenic periosteum

A. An antler with reversed anterior-posterior orientation formed on the antler growth region where AP was rotated 1800 (reproduced with permission from: Goss, 1991, J. Exp. Zool., 259: 246-251).
B. A reasonably normal antler formed from the antler growth region where the periosteal discs were doubled by grafting one on top of the other with their axes co-ordinated (reproduced with permission from: Goss, 1991, J. Exp. Zool. 259: 246-251).
C. An antler bud formed from the antler growth region where AP was removed, minced and grafted back.
D. Final form of the antlers formed from the minced AP of the same deer in this figure 8C. (Both C and D are reproduced with permission from: Goss, 1990, Of Antlers and Embryos. Springer-Verlag. New York, p304)

In addition to the self-differentiation ability described in "Antler biology", AP also holds patterning information for antler formation. This patterning information has been studied using AP in the following ways by Goss (1991). Prior to pedicle initiation, a piece of AP was surgically rotated 180°, subsequent antler formed from the rotated AP was in the reversed anterior-posterior orientation (Fig. 8A). When the left-side AP was grafted onto the right side AP, a normal antler formed from the right side region (Fig. 8B). After a piece of AP was cut into small pieces and then transplanted back to the sampling site, a well-organised antler (Fig. 8C and 8D) formed from the minced AP grafted site. One wonders how such a small piece of periosteal tissue (1 mm in thickness and 15 mm in diameter) can hold all the patterning information for antler formation and at the same time this patterning information is also highly regulative in nature.

In the case of limb formation, proximodistal, anteroposterior and dorsoventral axes are controlled by three separate signalling centres. Each centre expresses its own specific signalling molecules, such as the FGF family in the proximodistal signalling centre, SHH in the anteroposterior signalling centre, and the WNT family in the dorsoventral signalling centre. Most of these signalling genes have been identified in the growing antler tips by our group (de Alwis et al., 1996; Ashery, 1999). It would be very fruitful to use the antler stem tissue model to study how these signalling centres are initiated and regulated from the stem tissues.

2. Organogenesis

Although the phenomenon that appendages, such as deer antlers, develop from postnatal animals is unique to mammals, we have convincingly demonstrated that the initial antler generation resembles the formation of some organs during embryo development, such as limb development (Li and Suttie, 2001). However, in that study we did not provide experimental data to discuss whether elongation of first antler and limb uses the same or similar mechanism.

In limb development, the elongation is driven by the interactions between the apical ectodermal ridge (epithelium) and the subridge mesenchyme through diffusible molecules. In this case, the epithelium and the mesenchyme are the contiguous tissue layers. In first antler formation, the apical epidermis and the apical cellular perichondrial layer of pedicles are the corresponding epithelium and mesenchyme. If the force driving antler growth is also derived from epithelial mesenchymal interactions, the diffusible molecules must traverse the fibrous perichondrial layer, subcutaneous connective tissue and dermis, or vice versa, to reach and affect the corresponding target tissues. This represents an interaction across a distance of over 1 mm, exceedingly long-range in histological terms. It is critical to find out whether epithelial-mesenchymal interactions are involved in first antler formation, if this model is to be used for the study of limb development or the formation of other similar organs.

In lieu of this, we firstly carried out an electron microscopic study to detect the integrity of the basement membrane between epidermis and dermis (Li and Suttie, 2001). It is known that an intact basement membrane inhibits epithelial-mesenchymal interactions (Neufeld and Aulthouse 1986). The results from this study showed that at the pedicle growth stage, the basement membrane of apical skin was kept well-intact (Fig. 9A). However, at the early antler generation stage, the basement membrane of apical skin was seriously disrupted (Fig. 9B). Consequently, the results from this experiment support the notion that epithelial-mesenchymal interactions are involved in antler formation. However, this experiment does not shed any light on the issue as to whether this epithelial-mesenchymal interaction is via diffusible molecules, or other pathways (like cell-cell or cell-extracellular matrix)? In order to answer this question, we conducted the following experiment.

A group of red deer male calves at the early pedicle developmental stage ((Fig. 9C) were selected for the experiment. A piece of permeable (0.45 mm pore size) hydrophilised Teflon membrane (Millipore Comp) was surgically inserted between the apical pedicle skin and the underlying perichondrium (Fig. 9D) on one pedicle. The other pedicle was sham-operated to serve as a control. The results showed that insertion of a piece of permeable membrane did not stop pedicle growth (Fig. 9E), antler transformation or antler growth (Fig. 9F), although these events were substantially delayed compared to the control side. This experiment clearly demonstrates that the interactions between epidermis of apical pedicle skin and the underlying perichondrium take place through diffusible molecules, as the pore size (0.45 mm) of the inserted membrane can effectively preclude the contacts of cell-cell or cell-extracellular matrix, but allow the passage of molecules. The eventual identification and isolation of these putative diffusible molecules would have very important medical applications.

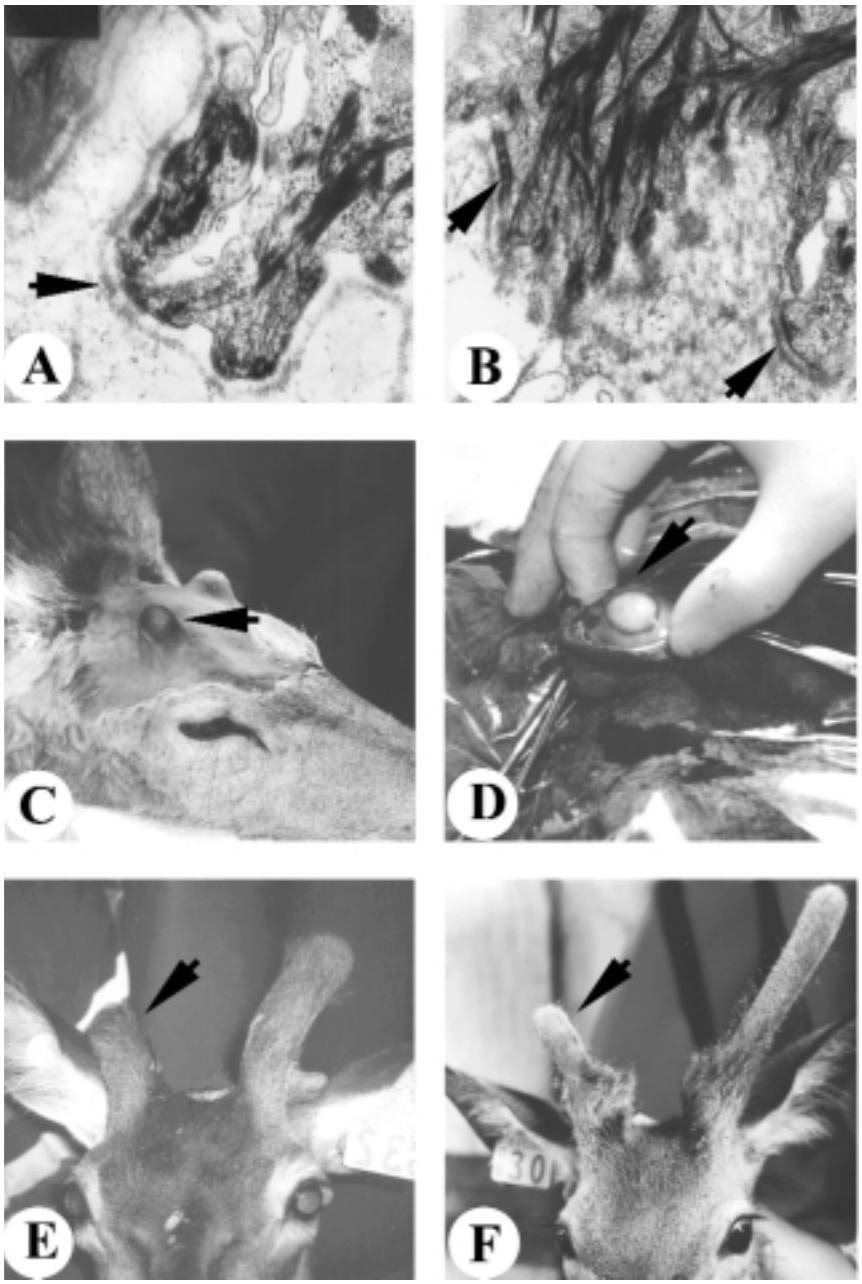

Figure 9 Tissue interactions and antler formation

A-B. Ultrastructure of the apical skin basement membrane. A. from an OPC stage pedicle. Showing the intact basement membrane (arrow). B. From an early antler. Note that the basement membrane was fragmented (arrows).

C-F. Permeable membrane insertion experiment. C. incipient pedicles (arrow) from a 7-month-old stag. D. a piece of permeable (0.45 mm) hydrophilised Teflon membrane (arrow) was surgically inserted between apical skin and the underlying periosteum of the right side pedicle. E-F. The inserted permeable membrane significantly delayed antler transformation (E, arrow) and growth (F arrow) compared to the left side controls.

The experiments described above provide further evidence to support use of the first antler formation model for research into organogenesis. Using the antler model for organogenesis research, embryologists may reveal the underlying mechanism of limbless syndrome, where early limb development is normal, but later the apical ectoderm ridge of the limb disappears and further development stops. In antler development, pedicles transiently develop, but then cease this development prenatally. This process is reactivated postnatally to form antlers. If we can understand the mechanism underlying the postnatal reactivation of antler formation, we may be able to trigger the limbless syndrome to grow new arms or legs postnatally.

3. Epimorphic (blastema-based) regeneration

During the course of evolution mammals have lost the ability to regenerate their missing appendages, although lower vertebrates, like newts, do grow back their missing

limbs. Anatomically, the failure of mammalian regeneration is caused by the dominance of scar formation over blastema formation during the healing process that occurs over an amputated stump. Blastemas are rounded masses of cells endowed with the capacity to develop into a replacement structure, whereas scar is regenerated dermal tissue lacking this ability. During newt limb regeneration, the epidermal tissue (E) migrates in first over the stump surface and sets up the interactions with the underlying stump mesenchyme (M), which results in blastema formation and subsequent regeneration. In mammals, however, the dermal tissue leads the way to heal the stump surface, which causes scar formation. The scar tissue constitutes a barrier and effectively blocks the E-M interactions, thus inhibiting regeneration.

In defiance of this general rule, deer antlers, as complex mammalian appendages, are cast and fully regenerate each year from their pedicles. Histological examinations (refer to "Antler biology") in our lab revealed that the healing process over a pedicle stump resembles that of newt stumps. Subsequently, instead of forming a scar, an antler blastema develops across the pedicle stump. One wonders why a scar does not form on a pedicle stump, as it does so readily elsewhere on the deer body.

In order to reveal the mechanism underlying antler regeneration, recently we have been setting up a series of experiments in our lab. One approach is to study the known genes that are involved in epimorphic regeneration in lower vertebrates, like newts, using in situ and Northern blot analysis. By so doing, we would be able to know whether antler regeneration uses similar molecular mechanism to that of lower forms.

The second approach is to explore novel or unique genes expressed during antler regeneration using the techniques of 2-dimensional electrophoresis (2DE) and a subtracted antler cDNA library. For carrying out these studies, the selected male deer will be allocated into two groups. Group one will be castrated to induce hard antler casting and new regeneration. The distal portions (about 1 cm in length) of the pedicles from these two groups will be biopsied. The tissue samples will, then, be processed and used for 2DE and making a subtracted cDNA library.

The identified unknown molecules or genes will be analysed in vivo using our nude mouse model, or in vitro using our co-culture techniques. If we can define the molecular mechanism for antler regeneration, we would be in a better position to promote partial or even full regeneration of human legs and arms.

4. Rapid growing tissue

Deer antler is a prime example of rapid growth with rates during late spring and early summer reaching 12.5 mm/day in sika deer (Gao and Li, 1988) to 27.5 mm/day in elk (Goss, 1970). Therefore, the antler model provides the unique opportunity to identify novel growth factors or unique regulatory pathway in a system where the growth rate is accentuated. It is known that the antler growth centre locates in the antler tip. Histologically, antler tip from distal to proximal consists of the zones of proliferation, maturation, hypertrophy and calcification (Banks and Newbrey, 1982a). Rapid growth of antler is mainly achieved through the proliferation of the cells residing in the proliferation zone. The proliferation zone consists of three layers. These layers are distoproximally: reserve mesenchyme, precartilage and cartilage (Banks and Newbrey, 1982b).

To undertake the study of revealing the underlying mechanism of rapid antler growth at the molecular level, different tissue types from the proliferation zone must be precisely sampled. Although the tissue layers in the zone are well classified (see above) and readily identified after histological processing, we recognised a need to locate and sample these layers from a piece of unstained antler tip tissue. Any process designed to facilitate identification of functionally distinct layers would need to be rapid, thus ensuring timely and effective processing of labile substances in the tissue, and must also result in the tissue being suitable for gene expression studies.

In order to establish the antler model for rapid tissue research, we have developed a generally applicable and standardised procedure to meet the sampling requirements for gene expression and in vitro studies. This procedure was confirmed by histological assessment (stitches, see below) and the probing of velvet antler tip ESTs to virtual northern blots (Li et al., 2002).

Four red deer antlers were collected at 60 days of growth and the tips (top 5 cm) were then removed (Fig. 10A). The tips were then sectioned sagittally into 5-mm-thick slices along the longitudinal axis (Fig, 10B and 10C). Stitches were sewn into the points identified by morphological markers under a dissecting microscope (Fig. 10C). The following observable

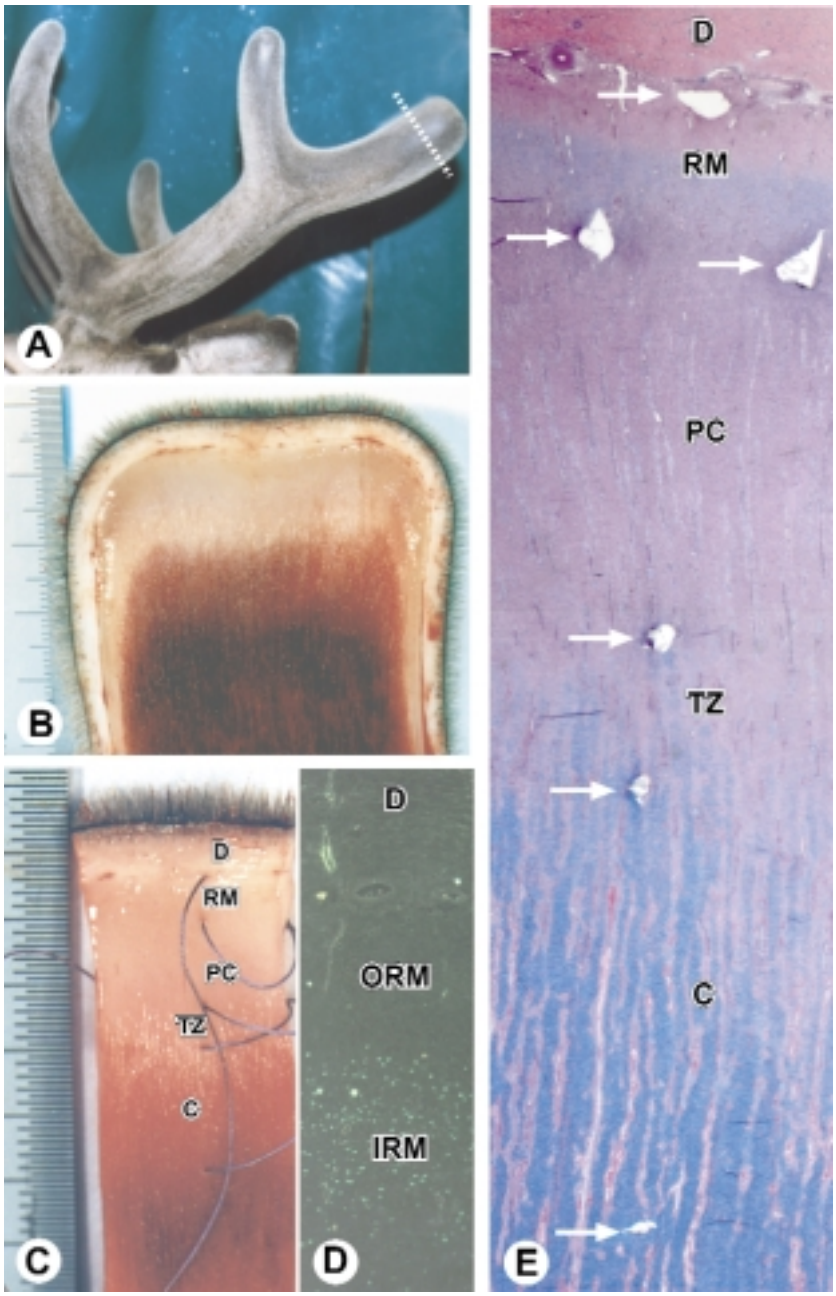

Figure 10. Antler tip layer dissection
A. Antler ready to be harvested and line indicating amount of tip removed.
B. Tip after being cut sagittally.
C. Layers as identified by the distinct morphological markers and marked by the stitches.
D. BrdU incorporation in the dermis (D), outer reserve mesenchyme (ORM) and inner reserve mesenchyme (IRM).
E. Histological section of the antler tip with holes from the stitches evident (arrows). D. Dermis, RM. reserve mesenchyme, PC. precartilage, TZ. transition zone, C. cartilage.

markers were identified distoproximally: the dermis (4.86mm), the subdermal bulge (2.90 mm), the discrete columns (6.50 mm), the transition zone (a mixture of discrete and continuous columns; 3.22 mm), and the continuous columns (8.00 mm). Histological examination showed that these markers corresponded to the dermis, reserve mesenchyme, precartilage, transitional tissue from precartilage to cartilage, and cartilage respectively. Both our histological observation (Fig. 10E) and the gene expression studies (refer to Li et al, 2002) revealed that these morphologically identified layers were distinct tissue types. Consequently, our morphological marker approach can be confidently used in the investigation of rapid antler growth at molecular level.

Overall, deer antlers can be developed into a variety of novel biomedical research models. I believe that the full appreciation of antler models by the biomedical research

community will greatly benefit mankind.

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