

Tissue Interactions and Antlerogenesis: New Findings Revealed by a Xenograft Approach

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ABSTRACT Tissue interactions play a pivotal role in organogenesis. Here we describe a xenograft approach to investigate how heterotypic tissue interactions control antler formation in deer. Deciduous antlers grow from the apices of permanent protuberances, called pedicles. Histogenesis of pedicles depends on the antlerogenic periosteum (AP). Pedicles and growing antlers are made up of interior osseocartilaginous tissue (a mixture of bone and cartilaginous tissue) and exterior skin. In a previous study we hypothesised that pedicle growth may result from mechanical interactions between the interior and exterior components whereas antler generation from a pedicle would involve molecules communicating between the interior and exterior components. To test this hypothesis, we subcutaneously transplanted AP of red deer (*Cervus elaphus*), either alone or with future pedicle skin, onto nude mice. The results showed that under the nude mouse skin, subcutaneously xenografted AP alone not only could form pedicle-shaped protuberances but also could differentiate into well-organised pedicle-like structures. The overlying mouse skin accommodated the expansion of the grafted AP by initial mechanical stretching and subsequent formation of new skin. Nude mouse skin was not capable of participating in antler tissue formation. However, grafted deer skin together with AP may have successfully rescued this failure after wounding, which highlights the necessity of the specificity of the overlying skin for antler tissue generation. Therefore, we conclude that it is the interaction between the antlerogenic tissue and the overlying skin that results in antlerogenesis: reciprocal mechanical interactions cause pedicle formation, whereas reciprocal instructive interactions induce first antler generation. *J. Exp. Zool.* 290:18–30, 2001. © 2001 Wiley-Liss, Inc.

Deer antlers are male secondary sexual characters which are cast and fully regenerated each year. Antlers do not grow directly from the head of the deer, instead they form from the apices of permanent bony protuberances, called pedicles. Deer are not born with pedicles. These start to develop when deer approach puberty (Fennessy and Suttie, '85). When pedicles grow to their species-specific height (about 5–6 cm high in red deer), first antlers generate spontaneously from the apices of these pedicles. Both in vivo (Suttie et al., '91, '98) and in vitro (Li et al., '99) studies have shown that pedicle initiation is triggered by high levels of androgen hormones. The transformation from pedicle to antler is independent of androgen hormones, and antler growth is associated with low androgen levels.

Pedicles form from antlerogenic cells within the periosteum overlying the lateral crests present on deer frontal bone (Hartwig and Schrudde, '74; Goss and Powel, '85). Removal of this periosteum prior to pedicle initiation will prevent future pedicle growth and hence antler formation. How-

ever, transplantation of this periosteum elsewhere on the deer body will cause an ectopic pedicle and antler to develop. This special periosteum has been called antlerogenic periosteum (AP) (Goss and Powel, '85). AP consists of two layers: an inner cellular layer and an outer fibrous layer, both of which are much thicker than their adjacent somatic counterparts (Li and Suttie, '94).

Pedicles and growing antlers are made up of interior osseocartilaginous tissue and exterior skin. Li and Suttie ('94) reported that osseocartilaginous tissue of a pedicle and first antler develops from AP through three distinct processes. Firstly, trabecular bone is laid down via intramembranous ossification. Osseocartilaginous tissue is then formed through an intermediate process called transitional ossification. Finally, vascularised cartilage is added on,

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and this process is associated with modified endochondral ossification.

Exterior pedicle skin formation proceeds through three histologically distinguishable stages (Li and Suttie, 2000). Initially the subcutaneous loose connective tissue (SLCT) is compressed at the transitional ossification stage. Secondly, the overlying undulated epidermis is expanded and stretched at the early pedicle endochondral ossification stage. Thirdly, the skin and its associated appendages are formed at the mid-stage of pedicle endochondral ossification *de novo*. Li and Suttie (2000) inferred that the change in ossification type and the alteration in skin structure during pedicle formation are caused by reciprocal mechanical interactions between the two tissues. The change in ossification type may be caused by the mechanical pressure derived from the stretched overlying skin, if the differentiation pathway of antlerogenic cells, like that of somatic osteoprogenitor cells, can be altered by different mechanical pressure. The alteration in skin type may be induced by the mechanical tension created by the expansion of the interior antlerogenic tissue. If this hypothesis is correct, pedicle formation should take place independent of skin type as long as the appropriate mechanical interactions can be established between the antlerogenic tissue and the overlying skin. However, this hypothesis has not been tested experimentally.

First antler generation from a pedicle is considered to be a unique zoological phenomenon (Goss, '83), because, although pedicles are permanent, antlers are deciduous. Thus far, the process of the transformation from pedicle to antler is not well understood. The transplantation experiments of AP (Goss, '87) demonstrated that ectopic antlers could not generate unless the antlerogenic tissue derived from AP was closely associated with the overlying skin. Therefore, Goss ('90) concluded that this close association was indispensable for antler formation. The results from the histological studies of normal pedicle formation and transformation to first antlers (Li and Suttie, 2000) support this claim. The indispensability of tissue association for antler formation has been compared to the close association process involved in the formation of some embryonic organs (Goss, '90). Formation of these organs depends on the operation of inductive interactions between tissues derived from mesoderm and ectoderm. For the interactions to occur in embryos, the communicating tissue must be in extremely intimate contact. The reason that wounding is effective in inducing

antler generation (Lincoln and Fletcher, '76; Jaczewski, '82; Goss and Powel, '85) has been attributed to wounding facilitating the establishment of the close association and subsequent interaction between antlerogenic tissue and the overlying skin (Goss, '90). Thus far, the question whether these interactions during first antler generation belong to instructive or permissive induction (see Discussion) has not been asked. Induction from antlerogenic tissue to the overlying skin is likely to be an instructive one. As evidenced, only subcutaneous transplantation of AP (Hartwig and Schrudde, '74), but no other tissue, such as pedicle skin (Goss, '72), can cause ectopic antlers to form and subsequently induce the overlying deer skin to transform into antler velvet. However, the nature of the feedback induction from the overlying skin to the antlerogenic tissue is not known.

Nude mice, which are congenitally athymic animals, have been widely used for biomedical research in recent years principally for their ability to accept xenografts (Demarchez et al., '92; Debiec et al., '94; Scott et al., '95). Goss ('83) reported a demonstration that a piece of AP implanted beneath the scalp skin of a nude mouse caused a relatively large osseous mass to develop at the graft site. No visible velvet-like skin developed. This demonstration clearly showed that AP could grow in the milieu provided by the nude mouse. The system that allows grafting of AP into nude mice offers a unique opportunity to investigate the underlying mechanism of antlerogenesis.

The overall aim was to take a xenograft approach using nude mice to test the hypothesis that pedicle formation is simply the result of mechanical interactions between antlerogenic tissue and the overlying skin and to determine whether the feedback induction from the overlying skin to antlerogenic tissue is instructive or permissive.

MATERIALS AND METHODS

1. *Experimental animals and objectives*

Animals

This study consisted of four experiments. In these experiments, red deer (*Cervus elaphus*) stag calves were selected prior to pedicle initiation to provide antlerogenic periosteum (AP), non-antlerogenic frontal periosteum (FP), and/or scalp skin (Table 1) for grafts. The deer were maintained outside on pasture from birth to the biopsy surgery. Nude mice (individually kept in a pathogen-free environment in a filtered cage) were recipients for transplantation of the biopsied deer xenografts (Table 2).

TABLE 1. Allocation of the experimental deer (tissue donor)¹

Experiment	Animal number	LW (kg) (mean)	Age (month)	Tissue xenograft/deer		
				AP	FP	Skin
1	2	60.2	6	12	4	–
2	3	49.2	6	3	1	–
3	3	60–70	8	12	4	–
4	1	52.0	8	9	–	12

¹AP, antlerogenic periosteum; FP, normal frontal periosteum; LW, live weight; –, does not apply.

Experiment objectives

Experiment 1. To determine (1) whether subcutaneous AP xenografts could grow into pedicle-like protuberances under nude mouse skin; (2) which sex and age of nude mouse was best for supporting AP growth; (3) whether AP xenografts could fuse to the underlying mouse skull and, if so, whether fused grafts could grow bigger protuberances than unfused ones. FP was used as a control tissue.

Experiment 2. To investigate the effects of both castration of the nude mice and wounding the apices of the protuberances formed from AP xenografts when these protuberances had stopped growing. FP was used as control tissue. And in addition, to determine whether the overlying mouse skin could accommodate expanding deer xenografts, apart from mechanical stretching, by synthesising new skin from localising mitotic cells during the rapid growing period of the protuberances.

Experiment 3. Similar to Experiment 2, but castration and/or wounding were carried out during the period of maximum growth of the protuber-

ances. And in addition, to test whether AP xenografts from different parts of the presumptive pedicle growth region (lateral, medial, anterior, and posterior) had differential growth potential in forming a pedicle-shaped protuberance.

Experiment 4. To determine whether future pedicle skin when transplanted together with AP could induce antler-like tissue formation in the milieu provided by nude mice. Future pedicle skin alone was used as a control tissue.

2. Biopsy and transplantation

Common methods among the experiments are described together to improve clarity and brevity.

Biopsy

Biopsy of deer tissue was approved by the Animal Ethics Committee, AgResearch Invermay, New Zealand.

Antlerogenic periosteum (AP). The detailed procedure for biopsying AP has been reported elsewhere (Li and Suttie, '94). Briefly, under anaesthesia, a crescent-shaped incision was made in

TABLE 2. Allocation of the experimental nude mice (tissue recipient) and treatments¹

Expt no.	Group	LW (g) (mean)	Implantation		Early sampling		Castration		Wounding		Late sampling	
			n	Age ²	n	Age ²	n	Age ²	n	Age ²	n	Age ²
1	AP	25	6	29	–	–	–	–	–	–	6	102
	FP	25	4	29	–	–	–	–	–	–	4	102
	AP	35	6	116–149	–	–	–	–	–	–	6	220
	FP	35	4	116–149	–	–	–	–	–	–	4	220
	AP ³	25	3	30	–	–	–	–	–	–	3	101
2	AP	25	9	25–27	3	80	6	149	6	159	6	201
	FP	25	3	25–27	1	80	2	149	2	199	2	201
3	AP	30	36	35–59	12 ⁴	86	18	75	12 ⁵	86	22 ⁶	150
	FP	30	12	35–59	12	86	6	75	–	–	–	–
4	AP + deer skin	35	9 ⁷	56–60	2	80	–	–	6	85	6	210
	Deer skin	35	3	56–60	1	80	–	–	2	85	2	210

¹–, absent or does not apply; AP, antlerogenic periosteum; FP, normal frontal periosteum; LW, live weight.

²Age in days.

³Female mice.

^{4,5}Six from intact mice and 6 from castrated mice.

⁶Two mice died before late sampling.

⁷One mouse died after surgery.

the deer scalp medial to the frontal lateral crest. After the overlying skin was lifted up, an incision was made in the periosteum along the midline of each crest. Starting from the posterior end, AP xenografts (varying in numbers among experiments; 4×5 mm/piece for all four experiments) were biopsied either lateral or medial to the incision line of each crest (Fig. 1). Pieces of AP were pooled within each experiment of Experiments 1, 2, and 4 and randomly allocated for the transplantation. For Experiment 3, AP pieces were individually numbered for testing differential growth potential (Fig. 1).

Nonantlerogenic frontal periosteum (FP). After the AP xenografts were removed, the crescent-shaped incision was extended from its two ends, medial to the sagittal suture, to expose the frontal bone. FP xenografts (4×5 mm/piece, numbers as required for each experiment) were taken at a site near the sagittal suture as a control for Experiments 1, 2, and 3.

Deer scalp skin. Deer scalp skin xenografts (4×5 mm/piece) were taken from either side of the margin of the laterally reflected skin overlying the lateral crest as a control for Experiment 4.

All biopsied deer xenografts were kept in a culture medium (BGJb + F12 nutrient, Sigma Chemical Co., St. Louis, MO) during transfer from the biopsy surgery room to the transplantation surgery room.

Transplantation

Transplantation experiments were approved by the Animal Ethics Committee, Medical School of Otago University, New Zealand.

The surgery was carried out in a laminar flow hood with aseptic precautions. The mice were anaesthetised by intraperitoneal injection of a mixture (300–400 μ l/mouse) of ketamine (5 mg/ml, Parnell Laboratories New Zealand Limited) and xylazine (0.25 mg/ml, Rompun, Bayer Ltd.). A 4–5 mm-long incision was made coronally on the scalp at the shortest distance between the ears, and the skin was lifted to form a pocket anterior to the incision. Deer xenografts were then inserted into the pockets, and the wounds were closed with silk sutures. The mice were monitored until they fully recovered from anaesthesia.

In order to facilitate fusion of the deer xenografts onto the underlying mouse skull, the connective tissue and periosteum overlying the skulls of the mice at the implantation sites were scraped away in four young (29 days) and four old (140 days) mice in Experiment 1 and every mouse in Experiments 2 and 3. An attempt was made to position each xenograft on insertion into a pocket, so that the cellular layer surface was resting directly on the mouse skull.

In Experiment 4 for the treated group, each piece of AP and each piece of skin were sutured together before they were inserted into the recipient pocket. There was no predetermined xenograft position for this experiment.

3. Observation and measurement

The mice were observed biweekly and photographed when necessary after transplantation surgery. The final shape of each protuberance formed on a mouse head was defined according to the following rules: a protuberance with a hemispherical shape is called a “dome-shaped” protuberance; a flatter protuberance is called a “dish-shaped” protuberance; a cylindrical protuberance with a hemispherical tip is called a “pedicle-shaped” protuberance.

The final size of the protuberances formed on the nude mice was determined after sampling (see section “Sampling and histology”) by measuring their height and diameter on the tissue sections cut from histologically processed blocks. The height of a protuberance was measured from the junction between the tissue derived from AP or FP and the underlying mouse skull to the top of the overlying mouse skin. Diameter was measured

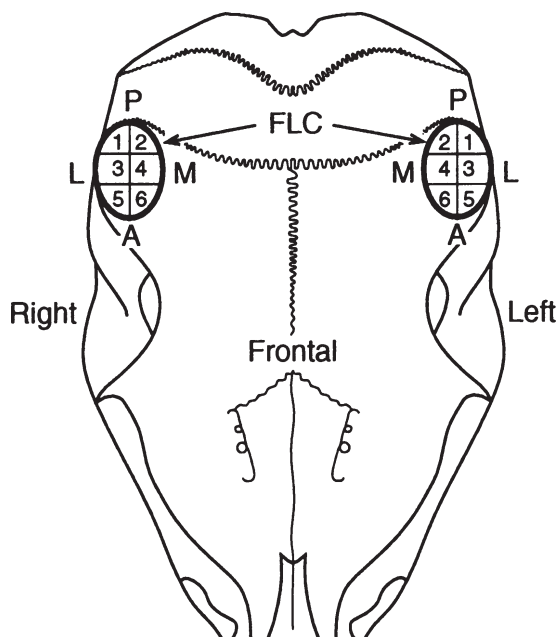


Fig. 1. Illustration of the sampling sites on a deer head in Experiment 3. FLC, frontal lateral crest; A, anterior; L, lateral; M, medial; P, posterior.

at the base of each dome-shaped protuberance or at the narrowest site of the shaft of a pedicle-shaped protuberance.

4. Treatment

Social facilitation

To facilitate AP xenografts to form pedicle-shaped protuberances, an internal environment in which AP naturally develops into a pedicle in the deer, i.e., a high level of androgen hormone, was simulated. A young female mouse was introduced into each cage within which a recipient male nude mouse was contained after the wound had healed from the transplantation surgery (about 10 days) (Walkden-Brown et al., '99).

Castration

To induce the protuberances formed on the mouse heads to form antler-like tissue, castration was undertaken. The timing of castration was either at the termination of growth of the protuberances in Experiment 2 or at the maximum growth period of the protuberances in Experiment 3 (Table 2). This action simulates the internal environment in which a pedicle naturally gives rise to an antler (a low level of androgen hormone) (Table 2).

The castration was conducted following the same anaesthesia procedure for transplantation surgery. A 3–4 mm-long incision was made at the base of the scrotum. Each testis and epididymis was squeezed out through the incision and removed by tearing to separate. The wound was left open to allow drainage.

Wounding

Wounding to the apices of the protuberances was carried out either at the termination of growth of these protuberances in Experiment 2 or at the maximum growth period of the protuberances in Experiments 3 and 4 (Table 2).

Following the same procedure of anaesthesia for tissue transplantation, the tips of protuberances were wounded using a scalpel. The wounding was deep enough to ensure the underlying bony tissue of the protuberance was included, which was shown to be crucial in the induction of antler growth in deer castrated and later treated with testosterone (Jaczewski, '82).

5. Sampling and histology

Sampling

The protuberances formed on the mouse heads were sampled either at the termination of their growth (late) in Experiments 1–4 for histological

examination or at the maximum growth period (early) of the protuberances in Experiments 2–4 for localisation of mitotic cells (see below) (Table 2).

To sample a protuberance, a mouse was decapitated, a circular incision was made around the protuberance on the mouse scalp, and the protuberance was then removed together with the underlying mouse skull. Care was taken not to dislocate or detach the overlying mouse skin from the bony core of the protuberance and surrounding mouse skull. In addition, a small piece (about $3 \times 4 \text{ mm}^2$) of facial skin remote from the transplantation site anteriorly was also taken for BrdU localisation (see below).

Histology

The removed protuberances were preserved in 10% buffered Formalin immediately after removal. The tissue samples were decalcified and then neutralised. Decalcification was considered to be complete when no evidence of mineralisation was observed on radiographs. Each neutralised protuberance was divided sagittally into two even parts. The trimmed tissue blocks were dehydrated, embedded in paraplast wax, sectioned at $5 \mu\text{m}$ (five consecutive sections), and stained with either haematoxylin (H)/eosin (E) or alcian blue/HE. The mouse skin was fixed, embedded, and sectioned following the same procedure described above.

6. BrdU injection and mitotic cell localisation

BrdU injection

BrdU injection was carried out at the maximum growth period of the protuberances in Experiments 2–4 for localisation of mitotic cells in the protuberances (Table 2). Four hours before a mouse was killed, BrdU (5'-bromo-2'-deoxyuridine, Sigma Chemical Co.; $25 \mu\text{g/g}$), a synthetic thymidine analogue, was injected intraperitoneally in the mice.

Mitotic cell localisation

After the samples were obtained (see above), the protuberances were histologically processed (see above) and sectioned at $5 \mu\text{m}$. The sections were collected onto either conventional slides for histological evaluation (see above for procedures) or gelatin-coated slides for BrdU localisation. The manufacturer's recommended immunohistochemical technique was used to localise BrdU labelled cells. Anti-BrdU antibody was purchased from Becton-Dickinson (Rutherford, NJ), and anti-

mouse IgG was purchased from Amersham (Arlington Heights, IL). After localisation, the sections were examined using appropriate fluorescence optics on a Zeiss fluorescence microscope.

7. Statistical methods

Height and area of protuberances were analysed by least squares, fitting age followed by a factor for whether or not the bony core had fused to the skull for male mice in experiments and fitting some region for Experiment 3. Classification of protuberance type and fusion and xenograft ossification were pooled across experiments.

RESULTS

1. Morphology

The morphological results from each of the four experiments are shown in Table 3. In male mice from all experiments, 4.3% of AP xenografts formed dish-shaped protuberances (all in the old mouse group), 21.7% formed dome-shaped protuberances (Fig. 2A), and 74% formed pedicle-shaped protuberances (Fig. 2B) including a tumour-like protuberance (Fig. 2C). Of these protuberances, 47.8% had their bony cores fused with the underlying mouse skull. Only one FP xenograft out of 10 from all experiments formed a dome-shaped protuberance, with the rest forming dish-shaped protuberances. None of the protuberances from this type of xenograft fused with its underlying mouse skull. In female mice in Experiment 1, all three AP xenografts formed dome-shaped protuberances and did not fuse with their underlying

mouse skull. Deer skin xenografts in Experiment 4 formed dish-shaped protuberances that were barely detectable.

In Experiment 1, the protuberances formed by young male mice were significantly larger than those formed by old male mice in both mean height (5.46 vs. 3.54, SED 0.45 mm; $P < 0.001$) and area (34.7 vs. 21.3, SED 3.49 mm²; $P < 0.001$). The bony core-fused protuberances were significantly larger than the unfused ones in both mean height (5.06 vs. 3.97, SED 0.50 mm²; $P < 0.05$) and area (32.8 vs. 22.8, SED 3.89 mm²; $P < 0.05$). No visible antler tissue was formed on any protuberance derived from the deer xenografts.

Neither castration nor wounding carried out at the termination of growth of the protuberances in Experiment 2 or at the maximum growth period of the protuberances in Experiment 3 induced visible antler-like tissue formation from the dome-shaped or pedicle-shaped protuberances (Fig. 2D,E). After wounding, the wounds healed with scar-like tissue formed on the top of these protuberances. The surrounding mouse skin in some cases became swollen, as does normal pedicle skin just before an antler starts to grow (Fig. 2D). In Experiment 3, no significant difference was found in either height or area of the protuberances formed from the AP derived from lateral, medial, anterior, or posterior part within a presumptive antler growth region ($P > 0.05$).

After wounding at the apical sites, 50% of the AP + deer skin xenograft-derived protuberances (three out of six) formed antler-like tissue (dead

TABLE 3. Morphology of the protuberances formed by deer xenografts¹

Expt no.	Group	Dish-shaped	Dome-shaped	Pedicle-shaped	
				Only	A-L ² on top
1	AP	0	1	5 (6 × 4.5) ³	0
	FP	4	0	0	0
	AP	2	0	4 (5 × 4) ³	0
	FP	4	0	0	0
	AP ⁴	0	3	0	0
2	AP	0	3	3 (8.5 × 5.5) ³	0
	FP	1	1	0	0
3	AP	0	3	19 (10 × 5.5) ³	0
	FP	—	—	—	—
4	AP + Deer skin	0	3	0	3 (6.5 × 2.5) ³
	Deer skin	2	0	0	0

¹AP, antlerogenic periosteum; FP, normal frontal periosteum.

²A-L, antler-like tissue.

³Size of the largest protuberance formed in each experiment (height × width, mm).

⁴Female mice.



Fig. 2. Differently shaped protuberances formed on the nude mouse heads. **A.** Dome-shaped protuberance in Experiment 1. **B.** Pedicle-shaped protuberance in Experiment 1. **C.** Tumour-shaped protuberance in Experiment 2. **D.** Pedicle-shaped protuberance with scar-like tissue formed on its top in Experiment 3. Notice that the surrounding mouse skin showed obvi-

ous swelling around the wounded edge (arrow). **E.** Pedicle-shaped protuberance with scar-like tissue (arrow) formed on its top in Experiment 2. **F.** Pedicle-shaped protuberance with a piece of antler-like bony tissue (arrow) on its top in Experiment 4.

bare bone) (Fig. 2F), or 100% of the pedicle-shaped protuberances derived from this type of xenograft formed antler-like tissue in Experiment 4.

2. Histology

The histological results from each of the four experiments are shown in Table 4.

In male mice from all experiments, 52.2% of AP xenografts formed bone, 43.5% formed osseocartilage (Fig. 3A), and 4.3% formed fibrous tissue (only in old mouse group). Only one FP xenograft out of 10 formed bony tissue, and rest formed fibrous tissue. In female mice in Experiment 1, all three AP xenografts formed bone tissue. Skin xenografts in Experiment 4 only formed skin.

In Experiment 1, the gross histological structure of the fused osseocartilaginous protuberances (Fig. 3A) could be divided into four portions. Distal-proximally, these are apical perichondrium, cartilaginous tissue, osseocartilaginous tissue, and osseous tissue. Interestingly, the cartilaginous tissue formed in the protuberances was avascular. The cellular layers of the protuberances sampled at this stage were thin. The osseocartilaginous and osseous portions were well organised, although osteoclasts and chondroclasts were frequently encountered within these portions. Within the osseocartilaginous portion, cartilaginous cells were only located in the central part of the trabeculae. In the osseous protuberances, the well-organised cancellous trabecular bone component was surrounded by a very thin layer of AP.

At this stage, remodelling had taken place within the bone component.

The mouse skin enveloping the protuberances could be divided into three regions: surrounding, shaft, and apical (Fig. 3A). In the surrounding region, the epidermis was extremely undulated and the subcutaneous loose connective tissue was very loose. In comparison to the surrounding region, the width of subcutaneous loose connective tissue in the shaft region was much narrower and the amplitude of the epidermal undulations was substantially smaller. In the apical region, the epidermis was essentially flat but was thicker than the other two regions. Subcutaneous loose connective tissue was dense and merged into the fibrous layer of underlying apical perichondrium. However, a visible gap could still be detected between the tissue derived from the AP xenografts and apical mouse skin (Fig. 3B).

In Experiments 2 and 3, the histological structure of the pedicle-shaped protuberances that had their bony core fused with the underlying mouse skull was comparable to the counterpart of the protuberances in Experiment 1. However, after wounding, the two main tissue components (AP-derived tissue and its overlying mouse skin) at the apices had grown together, and the gap between them was no longer detectable (Fig. 3C,D).

In Experiment 4, three out of four osseous protuberances (pedicle-shaped) formed dead antler-like bone on top. These protuberances were composed of two portions: distal naked dead bone and proximal living tissues enveloped in mouse skin (Fig. 3E). The proximal portion made up of living bone, AP, and mouse fibrous tissue disto-proximally. Healthy deer skin was found in the vicinity of the living bone tissue within the narrow living bone part. Healthy osteocytes were located in most lacunae (Fig. 3F). At the junction of the living bone and the cellular layer of the AP, most of the bone surfaces were lined with active osteoblasts (Fig. 3F), although osteoclasts were occasionally encountered (Fig. 3G). Proximally the fibrous layer of AP had closely grown together with mouse fibrous tissue. The distal dead bony portion comprised the main part of the protuberances and was of laminar character. Nearly all the lacunae in that portion were devoid of osteocytes (Fig. 3H).

3. Mitotic cell localisation

AP xenograft-derived interior tissue

Histological examination showed that at the maximum growth period, AP xenografts formed

TABLE 4. Histology of the protuberances formed by deer xenografts¹

Expt. no.	Group	Fuse/ not fuse	Bone	OC	FT	Skin
1	AP	2/4	1	5	0	—
	FP	0/4	1	0	3	—
	AP	2/4	0	4	2	—
	FP	0/4	0	0	4	—
	AP ²	0/3	3	—	—	—
2	AP	3/3	3	3	—	—
	FP	0/2	—	—	2	—
3	AP	15/7	16	6	0	—
	FP	—	—	—	—	—
4	AP + deer skin	0/6	4	2	—	—
	Deer skin	0/2	—	—	—	2

¹OC, osseocartilaginous tissue; FT, fibrous tissue; AP, antlerogenic periosteum; FP, normal frontal periosteum; —, absent or does not apply.

²Female mice.

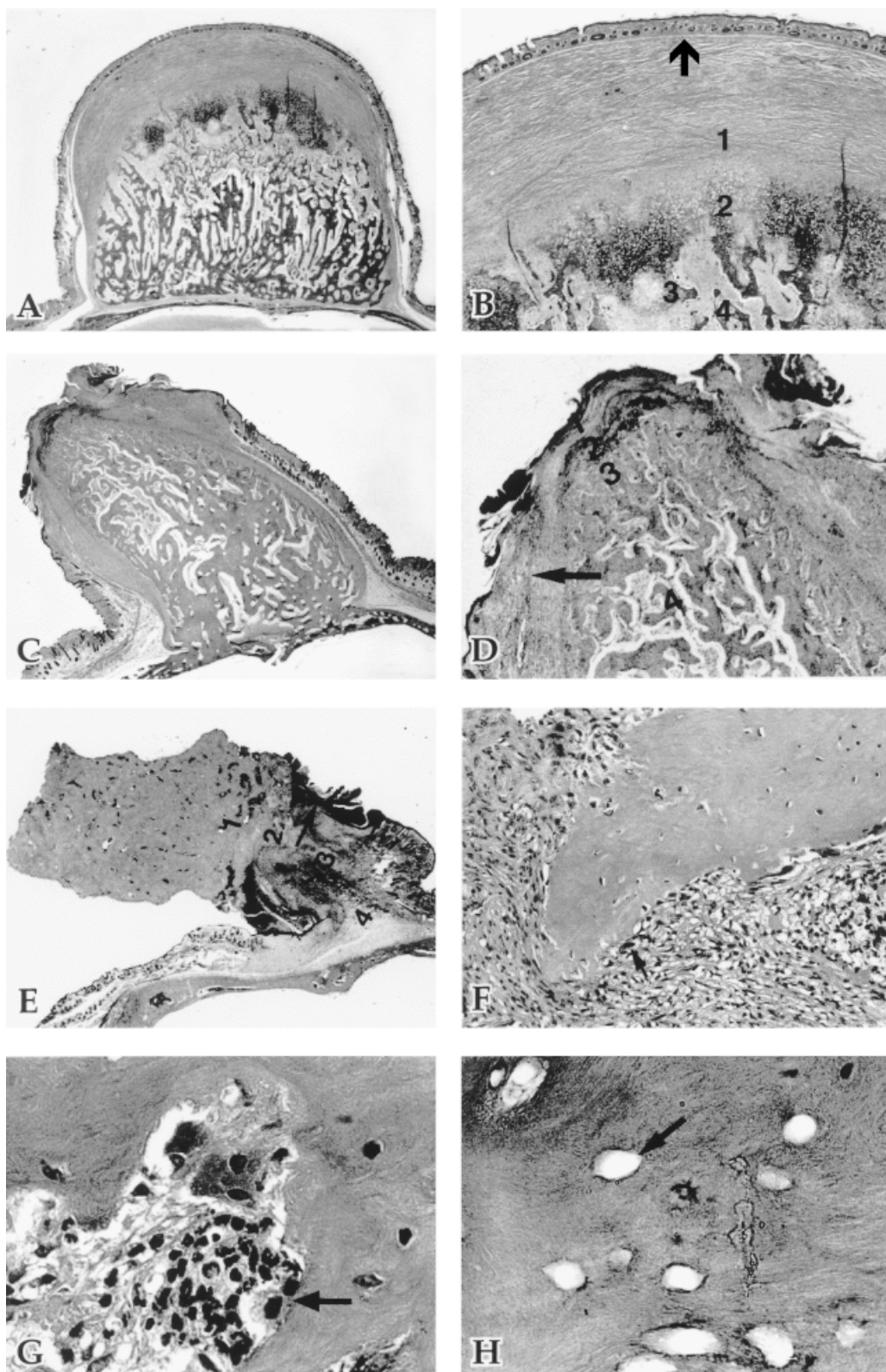


Fig. 3.

either osseous, osseocartilaginous, or cartilaginous protuberances. Compared to the protuberances sampled at the time of growth termination, these tissues were overlaid or enveloped by thicker hyperplastic AP. More active osteoblasts lined the surfaces of well-organised cancellous trabeculae and the spicules.

Immunohistochemical localisation showed that BrdU-labelled cells in the AP tissue of the protuberances were mainly located in the inner part of the cellular layer (Fig. 4A). However, BrdU-labelled cells were only occasionally encountered in the outer part of the cellular layer (Fig. 4B) or in the fibrous layer (Fig. 4C).

Overlying mouse skin

Histology of the overlying mouse skin sampled at this stage is essentially similar to those sampled at the time of growth termination (see above).

BrdU-labelled cells were found mainly in sebaceous glands (Fig. 4E), hair follicles (Fig. 4F), and

epidermis (Fig. 4D), but sparsely in dermis, in apical mouse skin. BrdU-labelled cells were nearly devoid in the protuberances derived from the FP xenografts (dish- or dome-shaped) and were not encountered in the facial skin.

DISCUSSION

Deer antler development consists of major two phases: pedicle formation from antlerogenic periosteum (AP) and transformation to antler at the apex of a pedicle. The present study showed that AP, when subcutaneously xenografted onto nude mouse heads, can not only form pedicle-shaped protuberances which are proportional to the size of mouse body but also differentiate into well-organised histological structures which are comparable to those of a normally formed pedicle [distoproximally: apical perichondrium, cartilaginous tissue, osseocartilaginous tissue, and osseous tissue (Li and Suttie, '94)]. These well-organised tissues are essentially formed by the inner part of the cellular layer of AP xenografts, as mitotic cells are mainly located in this layer. The overlying mouse skin releases the mechanical force derived from the underneath expansion of AP xenografts initially by mechanical stretch and subsequently by forming new skin as mitotic cells are located in the apical skin and its associated sebaceous glands and hair follicles, but not in the facial skin which is remote to the grafted site. Therefore, we conclude that (1) pedicle growth does not need any stimulus (endocrine or paracrine factors) specific to deer and (2) skin type is not critical for pedicle formation. In other words, pedicle formation does not require competent skin to participate. Consequently these results favour the notion (Li and Suttie, 2000) that pedicle formation may not involve specific molecules interacting between the antlerogenic tissue and the overlying skin, but rather that it is the result of mechanical interactions between these two tissue components. Mechanical pressure derived from the stretched overlying skin causes changes in ossification type, which has been demonstrated by our previous study (Li et al., '95), and the mechanical tension created by the expanding antlerogenic tissue results in pedicle skin formation through both mechanical stretch and skin neogenesis.

The process leading from a permanent pedicle to a deciduous antler is less well understood than is pedicle formation. As an organ, antler generation must depend on interactions between mesenchyme (antlerogenic tissue) and epithelium (epidermis of the overlying skin) (Goss, '95). Epi-

Fig. 3. Histological sections cut sagittally through three protuberances. Haematoxylin and eosin (HE)/alcian blue (AB) counterstaining. **A.** Micrograph showing the whole section of an intact pedicle-shaped protuberance from Experiment 1. Note that the bony core is histologically well-organised tissue and fused onto the mouse skull (original magnification $\times 8$). **B.** Higher magnification micrograph of the top part of A, showing that a gap between the AP derived apical perichondrium and the overlying mouse skin still exists (arrow). Four portions can be clearly distinguished from distal to proximal: perichondrium (1), cartilaginous (2), osseocartilaginous (3), and osseous (4) portions (original magnification $\times 110$). **C.** Micrograph showing the whole section of a wounded pedicle-shaped protuberance from Experiment 2. Note that the bony core is histologically comparable to that of A (for portion labeling, see D) and is also fused onto the mouse skull (original magnification $\times 7.5$). **D.** Higher magnification micrograph of the top part of C, showing that the tissue derived from an AP xenograft and the overlying mouse skin had grown together (arrow). The labels 1–4 are the same as shown in B (original magnification $\times 104.5$). **E.** Micrograph showing the whole section of a pedicle-antler-shaped protuberance in Experiment 4. Four portions can be clearly distinguished from distal to proximal: naked dead bone (1), mouse skin wrapped living bone (2), AP (3), and mouse fibrous tissue (4). The tissue derived from an AP xenograft and the enveloping mouse skin had grown together (arrow) (original magnification $\times 8.3$). **F.** Higher magnification micrograph from a part of E, showing that most of the bone surfaces were lined with active osteoblasts (arrow) at the junction of living bone and the cellular layer of the AP (original magnification $\times 30.5$). **G.** Higher magnification micrograph from a part of E, showing an osteoclast with multinuclei (arrow) eroding the bone surface (original magnification $\times 63.5$). **H.** Higher magnification micrograph from a part of E, showing that nearly all the lacunae in the dead bone portion were devoid of osteocytes (arrow) (original magnification $\times 63.5$).

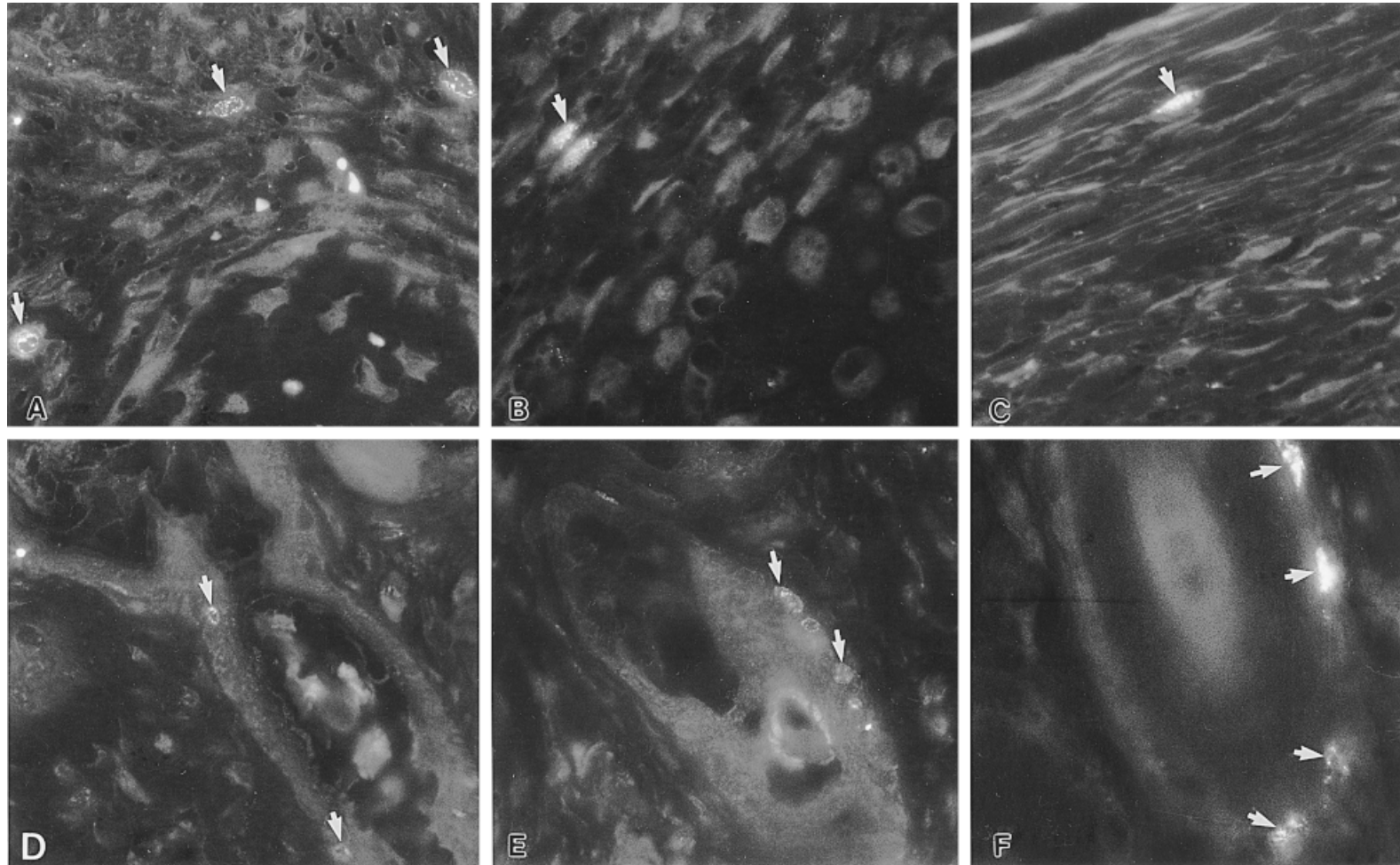


Fig. 4. BrdU-labelled cells (arrows) localised in the tissue derived from AP xenografts (A–C) or in the overlying mouse skin (D–F) (original magnification $\times 636$). **A.** Three BrdU-labelled cells in the inner part of the AP cellular layer surrounding a bony spicule. **B.** Two BrdU-labelled cells in

the inner part of the AP cellular layer surrounding a cartilaginous column. **C.** BrdU-labelled cell in the AP fibrous layer. **D.** Two BrdU-labelled cells in the epidermis of the mouse skin. **E.** Four BrdU-labelled cells in a mouse sebaceous gland. **F.** Four BrdU-labelled cells in a mouse hair follicle.

thelial-mesenchymal interactions are known to consist of two categories: instructive induction and permissive induction (Hardy, '83). Instructive induction results in the cells of the tissue(s) developing patterns that they would not otherwise have followed. The determination of the responding tissue is therefore set by the specific tissue interaction. For example, prostatic morphogenesis in epithelium can be only elicited by urogenital mesenchyme (Cunha and Chung, '81). Permissive induction results in the realisation of the prospective fate of already-determined tissue through tissue interactions. For example, the specific secretory function of salivary epithelium (Cunha et al., '83) or hepatocytes (Bhatia et al., '99) can be realised by recombination with any type of mesenchymal tissue. In the case of antler generation, it is readily acceptable that the induction from antlerogenic tissue to the overlying skin is an instructive one, as only AP can induce ectopic antler formation. However, it is not known whether feedback induction from the overlying skin to antlerogenic tissue is instructive induction or permissive induction. It has been found that the skin of deer ventral tail, back, or nose, unlike the skin elsewhere on the deer body, cannot successfully participate in antler formation, and this observation favours the view that the feedback induction from the overlying skin is likely to be instructive. The incompetence of these skins has been attributed to their inability to become intimately associated rather than to their interaction with the underlying antlerogenic tissue (Goss, '87). Therefore, the results do not shed any light on the feature of this feedback induction.

In the present study, xenografted nude mouse skin failed to participate in antler tissue formation. This is still true even when an internal milieu for normal antler generation (low androgen hormone level) was created by castration of the nude mice or when the indispensable close association between the tissue derived from AP xenografts and the overlying mouse skin was induced by wounding the apices of the protuberances.

Interestingly, the co-xenografted AP and deer skin in Experiment 4 formed antler-like tissue on top of the pedicle-shaped protuberances after wounding. However, one may argue that the antler-like tissue formed by the AP + deer skin xenograft may not be true antler tissue. This is because (1) skin shedding from the bony protuberances was not recorded, so it cannot be ruled out that the bare bone may have been pushed out of the pedicle-shaped protuberances after wound-

ing, as growth was taking place at their base. The nude mice were left unobserved between the period of wounding and the late sampling (about 80 days). However, the height of these protuberances was measured at these two time points, and the results showed that the heights at wounding and at late sampling were similar, except that at the late sampling the distal bare bony portion accounted for about 65% of the full length of these protuberances. In addition, the antler-like tissue is organised laminar bone. It is hard to imagine that there was sufficient time for the initially formed cancellous trabecular bone to go through the whole remodelling procedure to become well-organised compact laminar bone. Therefore the results of the height measurement and the feature of the bare bone favour the notion that the antler-like tissue is formed by skin shedding. (2) It is not known whether the antler-like tissue can be artificially induced to cast by castration of the nude mice. Nude mice were not castrated to artificially induce the antler-like tissue to cast is because of their limited life span.

Nonetheless, the fact that only AP + deer skin xenografts formed antler-like tissue in the whole study highlights the necessity of the specificity of the overlying skin for antler tissue generation. Consequently, the feedback induction from the overlying skin to the antlerogenic tissue during antler generation has to be instructive. The instructive induction from antlerogenic tissue transforms the overlying pedicle skin into antler velvet and in return the instructive feedback from the overlying skin create a milieu permitting antlerogenic tissue elongation and ramification.

Overall, the present study clearly demonstrated that under the mechanical pressure applied by the overlying mouse skin, subcutaneously xenografted AP alone could not only form pedicle-shaped protuberances but also could differentiate into well-organised pedicle-like structure. The overlying mouse skin released the mechanical tension formed from expansion of AP xenografts by initial mechanical stretching and subsequent formation of new skin. Nude mouse skin is incompetent to participate in antler tissue formation. However, the grafted deer skin together with AP may have successfully rescued this failure, which highlights the necessity of the specificity of the overlying skin for antler tissue generation. Therefore, we conclude that it is the interactions between the antlerogenic tissue and the overlying skin that results in antlerogenesis: reciprocal mechanical interactions cause pedicle formation, whereas re-

ciprocal instructive interactions induce first antler generation.

Transplantation of AP into nude mice offers a powerful xenograft approach for investigating antlerogenesis. Through this approach, some important findings are made in this study that increase our understanding of pedicle formation and its transformation to first antlers. In the future, this approach will undoubtedly facilitate the identification of the interacting molecules during first antler generation and the pathway (diffusion, extracellular matrix, or cell-cell contacts) whereby the interactions are realised.

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LITERATURE CITED

- Bhatia SN, Balis UJ, Yarmush ML, Toner M. 1999. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB J* 13:1883-900.
- Cunha GR, Chung LW. 1981. Stromal-epithelial interactions. I. Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice. *J Steroid Biochem* 14:1317-1324.
- Cunha GR, Shannon JM, Taguchi O, Fujii H, Meloy BA. 1983. Epithelial-mesenchymal interactions in hormone-induced development. In: Sawyer RH, Fallon JF, editors. *Epithelial-mesenchymal interactions in development*. New York: Praeger Publishers. p 51-74.
- Debiec RM, Asgari K, Jones RF, Grignon DJ, Haas GP, Wang CY. 1994. Maintenance of human benign prostatic hyperplasia tissues in the nude mouse. *Anticancer Res* 14:943-946.
- Demarchez M, Hartmann DJ, Regnier M, Asselineau D. 1992. A nude mouse xenograft model of fetal intestine development and differentiation. *Development* 114:67-73.
- Fennessy PF, Suttie JM. 1985. Antler growth: nutritional and endocrine factors. In: Fennessy PF, Drew KR, editors. *Biology of deer production*. Royal Society of New Zealand Bulletin 22. Wellington, New Zealand: Royal Society of New Zealand. p 239-250.
- Goss RJ. 1972. Wound healing and antler regeneration. In: Maibachand HI, Rovee DT, editors. *Epidermal wound healing*. Chicago, IL: Yearbook Medical Publishers. p 219-228.
- Goss RJ. 1983. Deer antlers. Regeneration, function and evolution. New York: Academic Press.
- Goss RJ. 1987. Induction of deer antlers by transplanted periosteum. II. Regional competence for velvet transformation in ectopic skin. *J Exp Zool* 244:101-111.
- Goss RJ. 1990. Of antlers and embryos. In: Bubenik G, Bubenik A, editors. *Horns, pronghorns, and antlers*. New York: Springer-Verlag. p 299-312.
- Goss RJ. 1995. Future directions in antler research. *Anat Rec* 241:291-302.
- Goss RJ, Powel RS. 1985. Induction of deer antlers by transplanted periosteum. I. Graft size and shape. *J Exp Zool* 235:359-373.
- Hardy MH. 1983. Vitamin A and the epithelial-mesenchymal interactions in skin differentiation. In: Sawyer RH, Fallon JF, editors. *Epithelial-mesenchymal interactions in development*. New York: Praeger Publishers. p 163-188.
- Hartwig H, Schrüdde J. 1974. Experimentelle Untersuchungen zur Bildung der primären Stirnauswüchse beim Reh (*Capreolus capreolus* L.). *Z Jagdwiss* 20:1-13.
- Jaczewski Z. 1982. The artificial induction of antler growth in deer. In: Brown RD, editor. *Antler development in Cervidae*. Kingsville, TX: Caesar Kleberg Wildlife Research Institute. p 143-162.
- Li C, Suttie JM. 1994. Light microscopic studies of pedicle and early first antler development in red deer (*Cervus elaphus*). *Anat Rec* 239:198-215.
- Li C, Suttie JM. 2000. Histological studies of pedicle skin formation and its transformation to antler velvet in red deer (*Cervus elaphus*). *Anat Rec* 239:62-71.
- Li C, Littlejohn RP, Suttie JM. 1999. Effects of insulin-like growth factor 1 and testosterone on the proliferation of antlerogenic cells in vitro. *J Exp Zool* 284:82-90.
- Li C, Waldrup KA, Corson ID, Littlejohn RP, Suttie JM. 1995. Histogenesis of antlerogenic tissues cultivated in diffusion chambers in vivo in red deer (*Cervus elaphus*). *J Exp Zool* 272:345-355.
- Lincoln GA, Fletcher TJ. 1976. Induction of antler growth in a congenitally polled Scottish red deer stag. *J Exp Zool* 195:247-252.
- Scott ER, Phipps JB, White HS. 1995. Direct imaging of molecular transport through skin. *J Invest Dermatol* 104:142-145.
- Suttie JM, Fennessy PF, Crosbie SF, Corson ID, Laas FJ, Elgar HJ, Lapwood KR. 1991. Temporal changes in LH and testosterone and their relationship with the first antler in red deer (*Cervus elaphus*) stags from 3 to 15 months of age. *J Endocrinol* 131:467-474.
- Suttie J, Li C, Sadighi M, Gunn J, Fleming J. 1998. Physiological control of antler growth. In: Milne J, editor. *Recent developments in deer biology*. Aberdeen: Macaulay Land Use Research Institute. p 189-196.
- Walkden-Brown SW, Martin GB, Restall BJ. 1999. Role of male-female interaction in regulating reproduction in sheep and goats. *J Reprod Fertil Suppl* 52:243-257.