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Mapping the morphogenetic potential of antler fields through deleting and transplanting subregions of antlerogenic periosteum in sika deer (*Cervus nippon*)

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Abstract

Morphogenetic fields are a localised and regionally regulated group of cells capable of responding to signals leading to the development of organs. In this study, we sought to determine if antlers develop from such a field. We divided antler fields into four subregions: anterior, posterior, medial and lateral. The antlerogenic periosteum (AP) in each subregion (half of the AP) was deleted and then transplanted into an ectopic site. Antlers form from the cells exclusively residing in the AP, which is located in an antler field. The morphogenetic potential of each subregion was assessed by the antler growth from both the defective field and the transplantation site. The results showed that when the AP anterior half was absent, the fields formed antlers missing the first tine, whereas when the anterior half was present, the ectopic sites regenerated antlers containing the first tine. When the medial half was deleted, the fields could only grow spike antlers, and when the medial half was present, the ectopic sites developed branched antlers. In contrast, the antler fields were able to compensate the defects caused by ablation of the posterior or the lateral half to form relatively normal antlers; and the ectopic sites containing these grafted halves only formed spike antlers. Therefore, antler morphogenetic information was primarily held in the AP anterior-medial halves. This study substantiates the presence of morphogenetic fields in regulating the distinct pattern of antler growth, and demonstrates that antler development is a useful model for the study of morphogenetic fields.

Key words: antler; antlerogenic periosteum; deletion; morphogenetic field; pedicle; transplantation.

Introduction

Deer antlers are unique mammalian organs that hold the potential to be developed into valuable biomedical models. The annual renewal of antlers offers the only opportunity to explore how nature has bestowed full organ regeneration in mammals (Goss, 1983; Bubenik & Bubenik, 1990). The unprecedented growth rate of antlers (up to 2.75 cm per day) provides a rare system where fast cell proliferation is elegantly regulated without becoming cancerous (Goss, 1995; Li, 2003). The atypical features of antler stem cells, such as expression of key embryonic stem cell markers (i.e. Oct4, Nanog and SOX2) and multi- or pluri-potency, serve as an invaluable model for understanding how embryonic stem cell attributes are retained in the postnatal animal cell populations (Price et al. 2005; Rolf et al. 2008; Kierdorf et al. 2009; Li et al. 2009). The remarkable ability of self-differentiation and regulation of AP (also including pedicles), within which antler stem cells reside, offers a novel system for the exploration of morphogenetic fields in organogenesis in mammals (Goss, 1961; Hartwig & Schrudde, 1974; Li & Suttie, 2001b).

Morphogenetic fields within an embryo, such as a limb field, are generally defined as a group of cells able to respond to discrete, localised biochemical signals leading to the development of specific morphological structures or organs (Hall, 2005). A field is bound to a particular substratum from which a dynamic pattern arises. It is heteroaxial and heteropolar, has recognizable distinct patterns, and can maintain its pattern when its mass is either reduced or increased. The concept of the fields was postulated in 1910 by Boveri (Gilbert, 2006) and was extensively explored in the 1920s and 1930s. The research intensity of this was eclipsed in the 1960s because the molecular techniques to analyze them had not been invented and because the rise

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of genetics was proposing an alternative program for development (Gilbert et al. 1996). In recent years there has been renewed interest in morphogenetic fields after molecular techniques have established these as functional entities and because they are now regarded as being fundamentally important for providing an understanding not only of ontogenetic, but also of phylogenetic mechanisms regulating the development of organs (Gilbert, 2006). Currently, the mechanisms underlying the establishment, maintenance and activation of the morphogenetic fields are not fully understood. We believe that the mechanism regulating the growth of deer antlers may offer a valuable insight into how the morphogenetic fields are maintained. The antler morphogenetic field is initially established at an early stage of embryo development in male deer (60–100 days of gestation, based on the morphological observation), remains quiescent during rest of pregnancy (over 180 days) and the first half a year of postnatal life, then is activated at puberty for pedicle and antler formation (Li & Suttie, 2001a). Therefore the antler fields are effectively maintained for at least a year.

The antler field was first exploited by Goss (1961). In that study he divided the pedicle (the permanent bony protuberance from which an antler is grown, cast and regenerated annually), into four halves – anterior, posterior, medial and lateral – and found that the greatest antlerogenic potential of the pedicle was concentrated in the lateral half because, without this, no antler grew. In contrast, removal of the other three halves did not significantly affect antler growth. However, Jaczewski (1982) argued that lack of antler development following deletion of the lateral part may be caused by damage to the major nerves and blood vessels supplying the antler, as they reside in this region (Wislocki, 1942).

Taking advantage of the discovery of the AP, the tissue that holds the exclusive potential for antler formation (Hartwig & Schrudde, 1974), Goss & Powel (1985) further explored the morphogenetic potential of the antler fields through evaluating the resultant antler morphology from each of the autologously transplanted halves of the AP (anterior, posterior, medial and lateral) on forelegs of deer. Using this approach they were able to avoid the confounding influence of nerves and blood vessels. However, the results were inconclusive, as only diminutive (0.4–4 cm) and spike-shaped antlers (which lack the hallmark tines to enable identification of the anterior-posterior and medial-lateral axes) were formed on the grafted sites. The failure to form branched antlers could be attributed to the foreleg being unsuitable as a graft site for the purpose, more time (years) being required for the full expression of antler morphology, or a combination of the two.

When mapping the competency of deer skin that is capable of velvet skin transformation under the influence of grafted AP, Goss (1987) found that the forehead region is the optimal site for supporting the elaboration of big and branched antler growth in fallow deer. This finding was subsequently confirmed in red deer (Li & Suttie, 2001a) and sika deer (Li et al. 2010). Furthermore, Goss (1991) conducted a series of experiments to determine the axis and polarity of the antler field by observing the antlers grown after rotating, inverting, or mincing the AP. The results from these experiments not only greatly enhanced our knowledge of antler development, but also laid the foundation for the establishment of the antler, the unique mammalian organ, as a novel model for the investigation of morphogenetic fields.

To qualify as a plausible model for the study of morphogenetic fields, the antler field must fulfil certain criteria (Gilbert, 2006). Properly mapping out each subregion of the antler field through deletion and transplantation of the AP resident in the subregion is essential to establish their contribution to the morphogenetic field. At present, the information for this is lacking, largely due to the inadequate description of the anatomical boundaries that delineate antler fields, and division of the four subregions (Goss & Powel, 1985). This detailed description is a requisite for further study.

To establish a standardised procedure of AP sampling for antler stem cell research, Li & Suttie (2003) carefully studied the topographic landmarks within an antler field in relation to the position of a pedicle, and identified that the centre of pedicle formation is located at the apex of the frontal crest in an antler field. In that study, the authors also delineated the boundary between AP and non-AP based on the transition in periosteum thickness, as the AP is around three times thicker than the surrounding periosteum (Li & Suttie, 1994). Combining the standardised AP sampling procedure with the identified optimal ectopic sites for antler formation has undoubtedly offered the opportunity to re-investigate the morphogenetic potential of each subregion of an antler field.

Using these standardised AP sampling procedures and optimal graft sites on the forehead, the aims of the present study were to map the antlerogenic potential for each of the four subregions (anterior, posterior, medial or lateral) of antler fields by comparing the final size and morphology of their derived antlers between the different individuals (in Group I and III, see 'Materials and methods') and within the same individuals (in Group II, see 'Materials and methods') for four consecutive years. At the same time, deer age and bodyweight (important factors influencing antler development) were also included in the study to determine when antler fields acquire the full potential or competency to initiate pedicle/antler growth.

Materials and methods

Animals

The study was conducted in the Institute of Wild Economic Animals and Plants, China. Twelve male sika deer calves

Table 1 Allocation of animals and side of AP deletion and transplantation.

	Group I (date: 14 April)			Group II (date: 17 April)			Group III (date: 21 April)		
AP half	Deer	BW (kg)	Treat side	Deer	BW (kg)	Treat side	Deer	BW (kg)	Treat side
A	1	43.5	Right	5	31.0	Left	9	27.1	Left
P	2	37.0	Left	6	38.3	Right	10	24.5	Right
M	3	32.6	Right	7	41.8	Right	11	26.8	Left
L	4	31.4	Right	8	38.8	Left	12	24.3	Right
Mean ± SD		36.1 ± 5.5			37.5 ± 4.6			25.7 ± 1.5	

AP, antlerogenic periosteum; BW, bodyweight; A, anterior; P, posterior; M, medial; L, lateral; SD, standard deviation.

Table 2 AP deletion from the control sides in Group II.

Deer	Side	AP deletion	Purpose
5	Right	Total	Impacts on contralateral and ectopic antler growth
6	Left	Anterior	cf. antler growth from one side without anterior and the other without posterior
7	Right	Lateral	cf. antler growth from one side without lateral and the other without medial
8	Right	Medial	cf. antler growth from one side without medial and the other without lateral

AP, antlerogenic periosteum.

were selected during the initiation period of pedicle growth and allocated into one of the three groups (Table 1): *Group I* (deer born in the normal season, mean bodyweight 36 kg, around 11 months of age). Each half of the AP was removed from one side of the two antler fields selected at random (treated side) and transplanted to the forehead region to determine the antlerogenic potential for each of the four subregions of the two antler fields. AP in the contra-lateral side was kept intact to serve as controls. *Group II* (born in the normal season, mean bodyweight 37 kg, around 11 months of age). The manipulation on a treated side was as per Group I. However, on the control side, removal of the AP tissue (Table 2) was also conducted to allow comparisons of the antlerogenic potential between the subregions on the same individuals. *Group III*. The manipulation on a treated side was carried out as per Group I but these deer were born late (6 weeks) and had lighter bodyweights (mean bodyweight 25 kg, around 9.5 months of age) and were selected to determine the influence of age and bodyweight on the timing of frontal crest/pedicle/antler initiation (AP maturation).

Delineation of the four subregions of antler fields

Based on the topographical location of the antlerogenic centre (the apex of a frontal crest in an antler field), the four subregions were divided using the following procedure. For delineation of the anterior and posterior halves, a line was drawn perpendicular to the frontal crest ridge, across the apex of the crest and terminating at the junctions between thicker and thinner periostea on each side of the crest. For the medial and lateral halves of the field, a line was drawn along the ridge of a frontal crest starting from the lowest point between the orbit of the eye and the crest, through the apex of the crest ridge to terminate at a point posterior to the crest and where the thicker and thinner periostea meet on the skull. The width and length of the medial half were similar to those of the lateral half (8–10 and 26–29 mm, respectively), those of a posterior half were 17–19 and 12–14 mm, respectively, and those of an anterior half were roughly equal (square) (around 17–19 mm) (Fig. 1A).

Deletion and transplantation of each half of the AP

All surgery was approved by the local Institutional Animal Ethics Committee and carried out under general anaesthesia. The side from which the AP half to be deleted was selected at random (Table 1). A detailed procedure for removal and transplantation of the AP tissue is reported elsewhere (Li & Suttie, 2003; Li et al. 2010). Briefly, an incision was made in the skin around 2 cm medial to the frontal crest and the skin was then reflected laterally to expose the underlying frontal crest. Following the guidelines described above, incisions were made in the periosteum to delineate an antler field and to divide the AP into the respective subregions. The AP half from each subregion (anterior, Fig. 1B; posterior; medial, Fig. 1C; and lateral) was peeled off to create an AP deletion side (Original side-subregion AP^-) using rat-tooth forceps. For transplantation of the AP half, an incision was made in the skin (2 cm) along a line drawn between the two eye orbits. A pocket was made in the skin anteriorly by blunt dissection, and each AP half was then inserted into the pocket (Fig. 1D) with no particular attention paid to its orientation (but dorsal and ventral axes were registered). The incision was closed with sutures after the grafted piece of AP was carefully inserted into the pocket to create an ectopic AP transplantation site (Ectopic site–subregion AP^+). No particular attention was paid to the orientation.

While removing the AP halves from the late-born deer (Group III), we noticed that the AP in this group was thinner and more adherent to the underlying bone than that from the deer born in the normal season (Groups I and II), which supports the

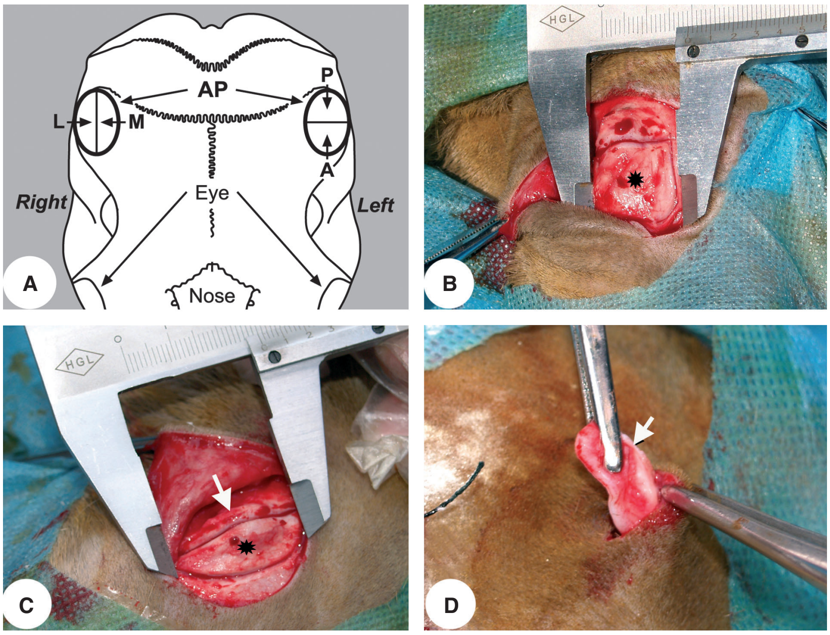

Fig. 1 Antlerogenic periosteum (AP) deletion and transplantation surgery. (A) Illustration of the subregions of antler fields in a prepubertal male deer. A, anterior; P, posterior; M, medial; and L, lateral. (B) Deletion of the AP anterior subregion (asterisk). (C) Deletion of the AP medial subregion (asterisk). Note that the AP lateral subregion is located on the steep slope of the frontal crest (arrow). (D) Transplantation of an AP subregion (Arrow).

histological finding that development of the AP region was delayed in late-born deer (Li & Suttie, 1994).

Observations and photographic recording

Deer were observed daily after the surgery for a week, then once a week thereafter and photographed when necessary. The sutures were taken out 30 days after surgery. Measurements for the resulting pedicles and antlers were carried out using a calliper whenever the deer were immobilised using general anaesthesia for close examination or for velveting. All antlers were removed and weighed at the end of the second antler growth season.

Results

The growth (length, weight and shape) of antlers are presented in Tables 3 and 4 for the two cycles of antler growth. In addition, a brief description of the pedicle and antler development from each of the AP halves is given below.

First antler growth cycle

Original side-anterior AP$^-$

All three treated antler fields formed pedicles and spike antlers. Deer 1 formed a similar-sized antler as the control (intact) side, but was slightly deformed with a clearly visible coronet (Fig. 2A), a feature for subsequent regenerating antlers. Removal of the entire AP from the control side in Deer 5 did not affect antler formation on the treated side (Fig. 2B). The late-born Deer 9 formed two very morphologically similar antlers from both the treated and the control (intact) sides (Fig. 2C), although the timing of antler initiation was significantly delayed (about 3 months) compared with deer born in the normal season.

Ectopic site–anterior AP$^+$

The ectopic bulge of Deer 1 reached 3 cm in height and acquired the typical appearance of shiny velvet skin (Fig. 2A, inset 1) but did not grow further or transform into antler tissue by the end of the growth season (Fig. 2A, inset 2). Interestingly, an almost species-specific sized pedicle (4 cm in height) and a spike antler (Fig. 2B, inset) were formed by Deer 5 at the ectopic site. The late-born Deer 9 only formed a palpable ectopic bulge, which did not transform into antler tissue (Fig. 2C).

Original side-posterior AP$^-$

Deer 2 formed a shorter pedicle and somewhat curved antler (Fig. 2D) from the treated side. Deer 6 formed a longer and more laterally oriented pedicle and antler compared to the one developed from the control (anterior AP$^-$) side (Fig. 2E). The late-born Deer 10 did not develop pedicles or antlers on either side of the antler fields (Fig. 2F).

Ectopic site-posterior AP$^+$

The ectopic bulges of both Deer 2 and 6 were transformed into antler tissue (Fig. 2D, inset 1 and 2; Fig. 2E, inset), but the grafted AP-posterior-half in Deer 10 failed to form a bulge (Fig. 2F).

Table 3 Pedicle and antler formation from the defective antler fields and the ectopic sites in the first antler growth season.

		Pedicle and antler growth side/site								
		Control			Treated			Ectopic		
		Pedicle/antler			Pedicle/antler			Bulge		
Group	Deer	Initiation date	Length (cm)	AP del	Initiation date	Length (cm)	AP del/tran	Initiation date	Height (cm)	Antler
I	1	15 May/3 July	5.0/8.0	0	27 April/3 July	5.0/9.0	A	10 May	3.0	No
	2	17 May/12 July	5.0/9.0	0	17 May/3 July	3.5/6.0	P	17 May	2.5	Yes
	3	7 June/8 September	5.0/6.0	0	7 June/8 September	3.0/3.0	M	26 July	2.5	Yes
	4	28 June/20 August	4.5/6.0	0	28 June/20 August	4.5/2.0	L	26 July	1.5	No
II	5	0/0	0/0	T	28 June/15 August	5.0/13.0	A	25 May	4.0	Yes
	6	12 July/30 August	2.5/4.0	A	17 May/26 July	5.0/5.0	P	17 May	3.0	Yes
	7	25 May/19 July	5.0/8.0	L	25 May/19 July	4.0/7.0	M	29 May	2.5	No
	8	25 May/28 June	4.5/8.0	M	3 July/20 August	4.0/5.0	L	3 July	2.0	No
III	9	5 August/ 28 September	4.0/5.0	0	10 August/28 September	4.0/5.0	A	26 July	0	No
	10*	–	0	0	–	0	P	–	0	No
	11	10 August/ 1 November	3.5/5.0	0	5 August/1 November	3.5/5.0	M	5 August	0	No
	12*	–	0	0	–	0	L	–	0	No

AP, antlerogenic periosteum; A, anterior; P, posterior; M, medial; L, lateral; Del, deletion; Tran, transplantation.
*Deer died before initiation of pedicle and antler formation.

Table 4 Pedicle and antler formation/regeneration from the defective antler fields and the ectopic sites in the second antler growth season.

		Pedicle and antler growth site												
		Control					Treated					Ectopic		
			Antler					Antler					Antler	
Group	Deer	Cast date	M/1st/2nd/ABC Length (cm)	Tine No	WT (g)	AP del	Cast date	M/1st/2nd/ABC Length (cm)	Tine No	WT (g)	AP del/tran	Cast date	Length (cm)	Weight (g)
I	1	16 June	32/15/6/17	3	545	0	16 June	31/9.5/3/17	3	510	A	–	–	–
	2	25 June	30/4/2.5/11	3	242	0	25 June	26/3.5/0/13	2	185	P	25 June	11	60
	3	20 June	28/4.5/5/13	3	260	0	20 June	27.5/0/0/10	1	170	M	26 June	13	62
	4	25 June	25/2.5/0/12.5	2	255	0	25 June	13.5/1.5/0/9.5	2	70	L	–	–	–
II	5	–	–	–	–	T	18 June	30.5/0/6/10.5	2	205	A	18 June	39	450
	6	20 June	34.5/0/4/10	2	285	A	20 June	26/4.5/0/13	3	195	P	21 June	13	83
	7	10 June	34/4/11/14	1	398	L	10 June	27/0/0/9	4	173	M	23 June*	3	–
	8	20 June	29/14/0/14	2	240	M	20 June	20/0/0/9	1	90	L	20 June	15	87
III	9	22 June	34/4.5/4.5/12	3	280	0	22 June	29/0/7.5/10	2	235	A	–	–	–
	10	–	–	–	–	0	–	–	–	–	P	–	–	–
	11	5 July	26/4.5/0/13	3	195	0	5 July	26/0/0/9	1	185	M	30 June*	3	–
	12	–	–		–	0	–	–		–	L	––	–	–

AP, antlerogenic periosteum; M, main beam; 1st, first tine; 2nd, second tine; ABC, antler base circumference; A, anterior; P, posterior; M, medial; L, lateral; Del, deletion; Tran, transplantation; WT, weight.
*Transformation from a pedicle.

Original side-medial AP⁻

All three treated antler fields formed pedicles and spike antlers. The pedicle and antler of Deer 3 were oriented more laterally (Fig. 2G). Deer 7 formed an almost normal pedicle and antler comparable to those developed from the control side (lateral AP⁻; Fig. 2H). The late-born Deer 11

Fig. 2 Pedicle/antler growth status in the first antler growth season. (A–C) Deletion and transplantation (D and T) of the anterior AP half. (A) Deer 1. A similar-sized pedicle and spike antlers were formed from both the treated and the control (intact) sides, although the treated-side antler was slightly deformed and had a clearly visible coronet (arrow). The AP graft formed a 3-cm-high bulge at the transplantation site. The bulge acquired shiny apical skin (inset 1) but did not transform into antler tissue (inset 2). (B) Deer 5. Normal pedicles and spike antlers were formed from both the treated and ectopic sites (inset) but the control side (total AP deletion) failed to give rise to pedicle or antler tissue (arrow). (C) Deer 9. Morphologically similar pedicles/antlers were formed from both the treated and the control (intact) sides, although the timing of antler initiation was significantly delayed (about 3 months; note the winter coat). Only a barely detectable bulge had formed at the ectopic site (arrow). (D–F) D and T of the posterior AP half. (D) Deer 2. A shorter pedicle and somewhat curved antler was formed from the treated side. The AP graft formed a 2.5-cm bulge with shiny apical skin (inset 1), and the bulge was transformed into antler tissue (inset 2) at the end of the growth season. (E) Deer 6. A longer but more laterally oriented pedicle/antler was formed from the treated side (arrow). The AP graft formed a 3-cm bulge, which was transformed into antler tissue within the season (inset). (F) Deer 10. No pedicle/antler tissue had developed. (G–I) D and T of the medial AP half. (G) Deer 3. A more laterally oriented pedicle/antler had formed on the treated side (arrow). The grafted AP formed a 2.5-cm bulge, which was then transformed into antler tissue (inset). (H) Deer 7. A nearly normal-looking pedicle/antler had formed on the treated side. The grafted AP formed a 2.5-cm bulge but the bulge did not transform into antler tissue (inset). (I) Deer 11. Morphologically similar pedicles and antlers were formed from both the treated and the control (intact) sides, although the timing of antler initiation was significantly delayed (about 3 months; note the winter coat). A barely detectable bulge was formed at the ectopic site (arrow). (J–L) D and T of the lateral AP half. (J) Deer 4. A normal pedicle but a shorter antler had formed on the treated side. The grafted AP formed a 1.5-cm bulge, but the bulge did not transform into antler tissue (inset, arrow). (K) Deer 8. A disoriented pedicle and antler had formed on the treated side, and the latter was also shorter than the one developed from the control (Medial AP$^-$) side (arrow). The grafted AP formed a 2.0-cm bulge, but the bulge did not transform into antler tissue (inset). (L) No pedicle and antler had developed at any site.

formed two very morphologically similar antlers from both the treated and the control (intact) sides (Fig. 2I) but the timing of antler initiation was significantly delayed (about 4 months) compared to the deer born in the normal season (Groups I and II).

Ectopic site-medial AP$^+$

The ectopic bulge of Deer 3 was transformed into antler tissue (Fig. 2G, inset), but that of Deer 7 failed to do so, although it reached 2.5 cm in height (Fig. 2H, inset). The grafted medial half of the AP in Deer 11 (late-born) formed a barely palpable bulge, which did not transform into antler tissue (Fig. 2I).

Original side-lateral AP$^-$

Deer 4 formed a normal pedicle but a shorter antler compared to the control (intact) side (Fig. 2J). Deer 8 developed a disoriented pedicle and antler, and the latter was also shorter than the one developed from the control (medial AP$^-$) side (Fig. 2K). The late-born Deer 12 did not give rise to any antler tissue from either side of the antler fields (Fig. 2L).

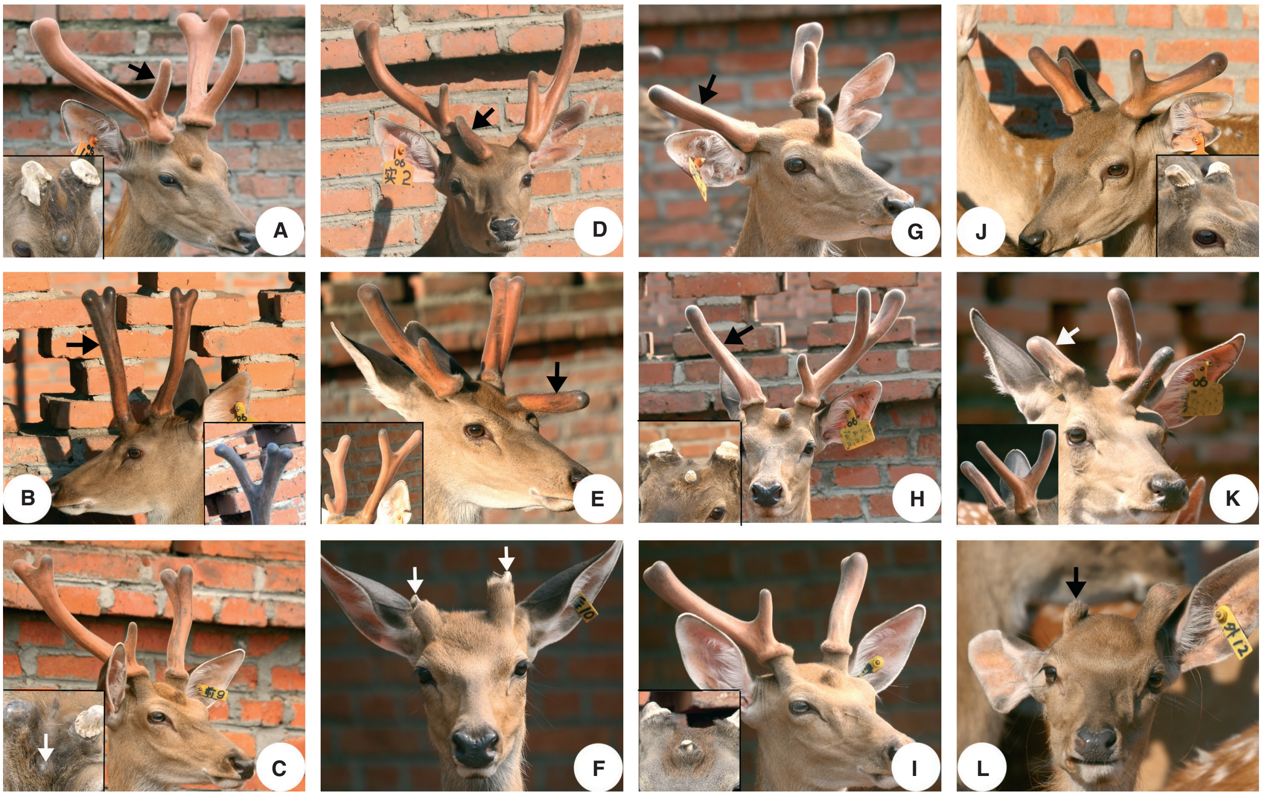

Fig. 3 Pedicle/antler growth status in the second antler growth season. (A–C) Deletion and transplantation (D and T) of the anterior AP half. (A) Deer 1. A three-branched antler with a smaller first tine (arrow) and a shorter second tine was regenerated. The bulge formed from the grafted AP again failed to transform into antler tissue (inset) in this season. (B) Deer 5. Antler regenerated in this deer was missing a typical first tine but had grown a short abnormal second tine. Surprisingly, a non-species-specific three-branched antler that was 39 cm long (arrow) was regenerated from the ectopic site (inset). (C) Deer 9. The regenerated antler was missing a typical first tine but had a relatively normal second tine. The bulge (arrow) formed from the grafted AP again failed to transform into antler tissue (inset) in this season. (D–F) D and T of the posterior AP half. (D) Deer 2. A three-branched antler with a normal first tine and a shorter second tine was regenerated. An antler was regenerated from the grafted site and was steeply inclined toward the control side (arrow). (E) Deer 6. The regenerated antler was species-specific and had normal first and second tines (inset). A spike antler (arrow) was regenerated from the grafted site and the antler was sharply inclined towards the deer's nose. (F) Deer 10. The hard but diminutive antlers (arrows) of the deer failed to cast in that growth season. No detectable bulge was formed from the grafted site. (G–I) D and T of the medial AP half. (G) Deer 3. A spike antler was regenerated (arrow) and it was inclined more laterally. A spike antler had formed from the ectopic site that was oriented vertically initially and then bent posteriorly at the distal end. (H) Deer 7. A spike antler was regenerated (arrow). The bulge formed from the grafted AP had transformed into antler tissue (inset). (I) Deer 11. A spike antler was regenerated from the treated side. The bulge formed from the grafted AP had transformed into antler tissue (inset). (J–L) D and T of the lateral AP half. (J) Deer 4. A two-branched antler was regenerated from the treated side. The bulge developed from the grafted AP did not transform into antler tissue (inset). (K) Deer 8. A two-branched antler was regenerated, whereas only a spike antler (arrow) had developed from the control (medial AP^{-}) side (inset). (L) Deer 12. Only an incomplete pedicle (2 cm high) had formed from the treated side. No detectable bulge was evident from the grafted AP.

Ectopic site-lateral AP^{+}

Neither of the ectopic bulges from Deers 4 and 8 was transformed into antler tissue (Fig. 2J and 2K, insets). The grafted posterior half of the AP in late-born Deer 12 failed to form a bulge (Fig. 2L).

Second antler growth cycle

Original side-anterior AP^{-}

Deer 1 regenerated a three-branched antler with a smaller first tine and a shorter second tine compared to the control (intact) side antler (Fig. 3A). The antler that regenerated in Deer 5 did not have the first tine, but did have a short and abnormal second tine (Fig. 3B). Deer 9 (late-born) did not have the first tine, but did have a relatively normal second tine compared to the control (Fig. 3C).

Ectopic site-anterior AP^{+}

The ectopic bulge of Deer 1 did not transform into antler tissue (Fig. 3A, inset). In contrast, Deer 5 regenerated a large (Table 4), but non-species-specific, three-branched ectopic antler (Fig. 3B, inset). The small pedicle that had developed in the previous year in late-born Deer 9 did not transform into antler tissue (Fig. 3C, inset).

Original side-posterior AP$^-$
Deer 2 regenerated a three-branched antler with a normal first tine and shorter second tine compared to the control (intact) side antler (Fig. 3D). The antler that regenerated in Deer 6 had a normal first tine, but the second tine was abnormal compared with the control (anterior AP$^-$) side (Fig. 3E and inset). Antlers developed in late-born Deer 10 but were small and failed to cast at the end of the season (Fig. 3F).

Ectopic site-posterior AP$^+$
Deer 2 regenerated an antler, which was oriented toward the control side (intact) antler (Fig. 3D). Deer 6 regenerated a spike antler that was oriented rostrally (Fig. 3E). The grafted posterior half of the AP in Deer 10 did not develop into a detectable bulge (Fig. 3F).

Original side-medial AP$^-$
Deer 3 regenerated a spike antler, which was oriented laterally (Fig. 3G). Likewise, Deer 7 regenerated a spike antler and a three-branched antler was regenerated from the control (lateral AP$^-$) pedicle (Fig. 3H). The late-born Deer 11 also regenerated a spike antler (Fig. 3I).

Ectopic site-medial AP$^+$
An antler was formed in Deer 3 from the ectopic site. This grew vertically initially and then bent posteriorly at the distal end (Fig. 3C). Both Deer 7 and 11 developed 3 cm antlers (Fig. 3H and inset; Fig. 3I).

Original side-lateral AP$^-$
Deer 4 regenerated a two-branched antler that was very similar to the one from the control (intact) side (Fig. 3J). Deer 8 regenerated a two-branched antler, whereas only a spike antler developed on the control (medial AP$^-$) side (Fig. 3K and inset). Deer 12 (late-born) only formed a small and incomplete pedicle (2 cm high) compared to the control (intact) side (Fig. 3L).

Ectopic site-lateral AP$^+$
The pedicle that developed in Deer 4 had grown no further from the previous year (Fig. 3J, inset), whereas a spike antler had developed from the pedicle (Fig. 3K) in Deer 8. A barely palpable pedicle formed from the grafted lateral half of the AP in late-born Deer 12 (Fig. 3L).

Third antler growth cycle

Deers 1, 4, 10 and 11 had regenerated antlers of similar dimensions to the previous year. Deers 7 and 12 died before the antler growth season had started.

AP anterior half
Antlers regenerated from both the defective original sides of Deera 5 and 9 with normal second tines, but without the first tines. Deer 5 was the only deer that developed an ectopic antler, and this had a first tine which branched laterally rather than anteriorly, possibly due to the random insertion of the AP during grafting (Fig. 4A).

AP lateral half
Deer 8 regenerated an almost normal three-branched antler from the defective original side, although the orientation was reversed, whereas only a small two-branched antler was formed from the defective control (medial AP$^-$) side (Fig 4B). Only Deer 8 developed an ectopic antler, which was grown as a spike antler with the distal part bending posteriorly (Fig. 4B).

AP posterior half
Both Deers 2 (Fig. 4C) and 6 regenerated nearly normal three-branched antlers from the defective original sides. The antlers that regenerated from both the treated and control sides in Deer 10 were similar to each other. Both Deers 2 (Fig. 4C) and 6 (Fig. 4D) developed long spike antlers (22 and 18 , respectively) on the ectopic sites and each was oriented at a sharp angle.

AP medial half
A spike antler was regenerated from the defective original side in Deer 3 (Fig. 4E). Both Deers 3 and 11 developed ectopic antlers. The ectopic antler of Deer 3 was bifurcated at the tip (Fig. 4E), whereas it was a spike in Deer 11 (Fig. 4F).

Fourth antler growth cycle

Deers 2, 3, 5, 6, 8 and 11 had regenerated antlers of similar dimensions to the previous year. The pedicle bulges from the grafted anterior halves of AP in both Deers 1 and 9 had transformed into antler tissue (Fig. 5A,B, respectively), whereas the lateral and posterior halves of the AP grafted in Deer 4 and 10, respectively, remained unchanged (Fig. 5C,D, respectively).

Discussion

In the present study we have convincingly demonstrated that morphogenetic fields exist in the future antler growth regions and that these have a remarkable degree of regulation and a distinct morphogenetic pattern. The AP half in the anterior subregion controls the formation of the first antler tine, and the potential for the development of all antler tines (first, second and third, etc.) resides in the AP half of the medial subregion. In contrast, neither the posterior nor the lateral subregions carry antler morphogenetic information (shape). Rather, these subregions carry the potential to grow. We also show that the competency of an antler morphogenetic field may be influenced by both the age and the bodyweight of deer. Therefore, we suggest that an antler field is a self-organising, spatially co-ordinated, and temporally synchronised morphogenetic field.

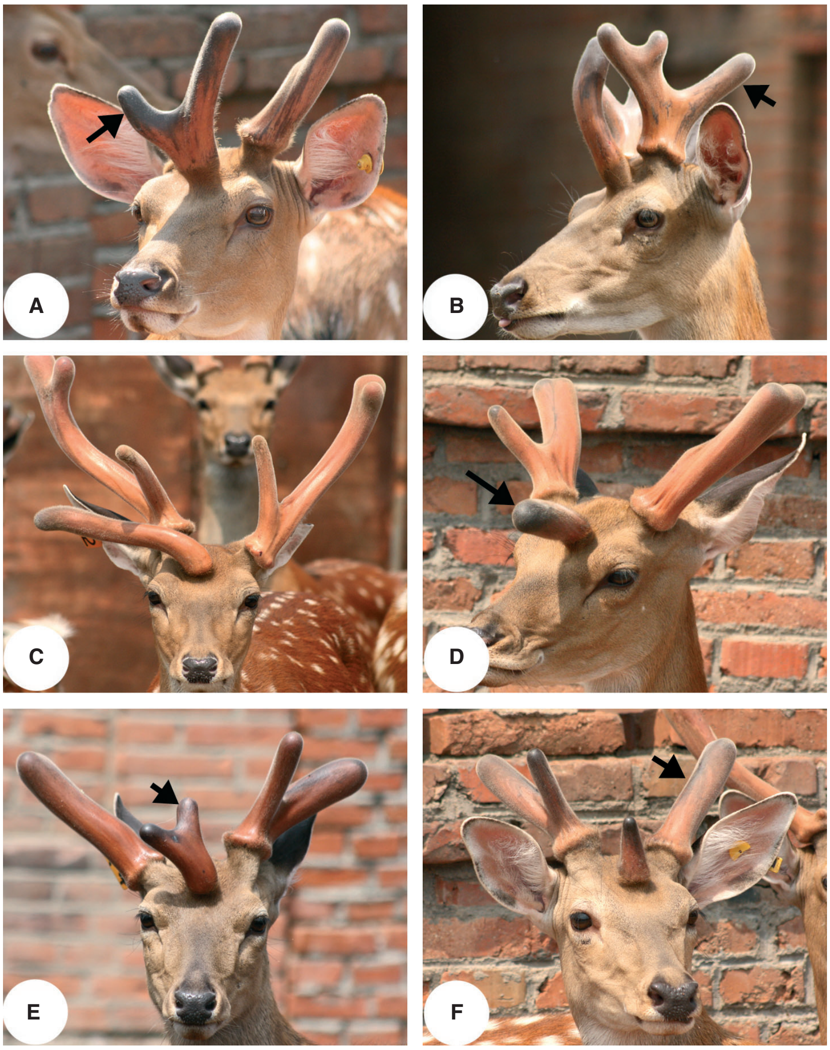

Fig. 4 Pedicle/antler growth status in the third antler growth season. (A) Deer 5. A spike antler had formed from the treated side (anterior AP$^-$), whereas a two-branched antler had regenerated from the grafted site (anterior AP$^+$). Note that this ectopic antler had a nearly normal first tine (arrow), although it had branched out laterally rather than anteriorly. (B) Deer 8. An almost normal three-branched antler had regenerated but with reversed polarity (arrow) after losing the lateral half of the AP. A spike antler had regenerated from the grafted site (lateral AP$^+$). The distal part of the ectopic antler had bent posteriorly. (C) Deer 2. A nearly normal three-branched antler had regenerated after losing the posterior half of the AP. A long (22 cm) spike antler had regenerated from the grafted site (posterior AP$^+$). Note that the ectopic antler had bent laterally. (D) Deer 6. Antlers regenerated from both the treated and ectopic sites resembled those of Deer 2 but the ectopic antler pointed anteriorly (arrow). (E) Deer 3. A spike antler had regenerated from the treated side (medial AP$^-$), whereas a two-branched antler (arrow) had regenerated from the grafted site (medial AP$^+$). (f) Deer 11. Antlers that regenerated from the treated (medial AP$^-$, arrow) and the grafted (medial AP$^+$) sites were all spiked in shape.

Anterior-medial AP halves vs. posterior-lateral AP halves

The morphogenetic information in an antler field of sika deer has a distinct pattern. Three of four original sides where AP halves were deleted from the anterior subregions formed antlers without first tines. The remainder (Deer 1) only formed an antler with a much smaller first tine compared to the control (Fig. 3A), possibly due to some residual AP cells remaining in the subregion after deletion of the AP half. Therefore, the anterior subregion could be considered an organisation centre containing information to form the first antler tine. Notably, all four original sides where the medial subregions were missing the AP halves, formed only spike antlers for four consecutive years, although the control sides all formed multi-branched antlers typical for sika deer from the second growth season onwards. Therefore, the medial subregion must carry the morphogenetic information for all antler tines (first, second and third, etc.). The morphology of antlers grown from the ectopic grafted sites further substantiates our view that the anterior and medial subregions carry morphogenetic information because ectopic antlers that developed from the AP halves of these subregions were all bifurcated (Fig. 4A,E).

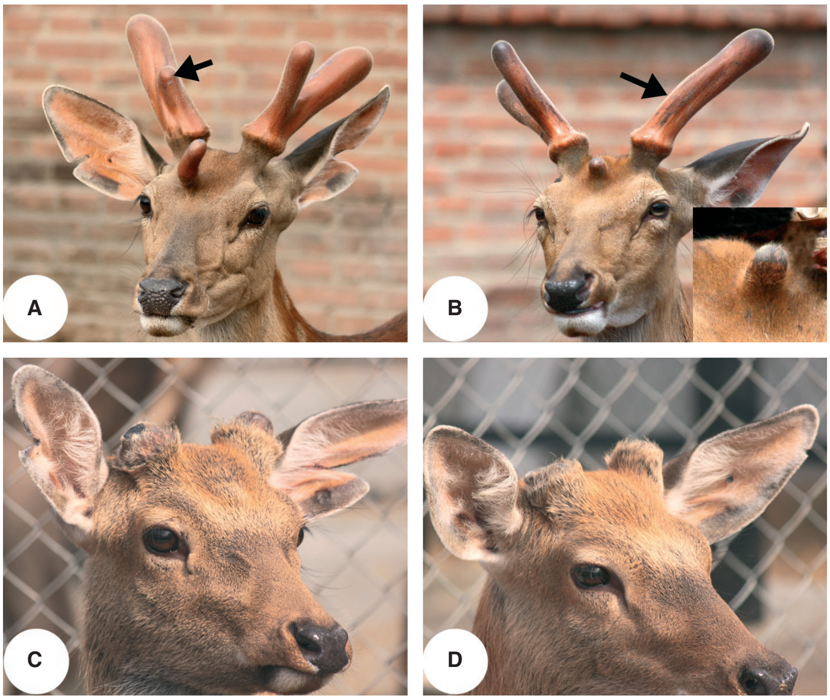

Fig. 5 Pedicle/antler growth status in the fourth antler growth season. (A) Deer 1. The regenerated antler from the treated side (anterior AP$^-$) formed a small first tine (arrow). The bulge that developed from the grafted site (anterior AP$^+$) was eventually transformed into antler tissue in this season. (B) Deer 9. The antlers grown were similar to Deer 1 but the antler (arrow) from the treated side did not form the first tine. (C) Deer 4. The bulge at the grafted site (lateral AP$^+$) failed to transform into antler tissue in this growth season. (D) Deer 10. The grafted AP (posterior AP$^+$) did not form a palpable bulge in this antler growth season.

In contrast, the antler fields seemed to be able to regulate the defects caused by ablation of the AP halves from the posterior or lateral subregions to grow nearly normal antlers. Furthermore, the AP halves of the posterior or lateral subregions could only give rise to spike antlers (Fig. 4B–D) when transplanted to ectopic sites, even if they were given sufficient time (four consecutive years) to express their morphogenetic potential fully. Indeed, one of these ectopic antlers grew up to 31 cm in length (Deer 2). As a consequence, we postulate that the posterior and lateral subregions do not carry morphogenetic information to grow antler tines, but only the information to form the main beam.

Unexpectedly, the results of the present study are at odds with those of Goss & Powel (1985), who reported that the antlerogenic potential was concentrated in the posterior-lateral subregions. We believe that the discrepancy between our results and those of Goss and Powel could be attributed to the differences in methodology, besides the species difference (sika vs. fallow). The methods used here and by Goss and Powel differ in two major respects. First, in the method used for the delineation of four subregions of antler fields, Goss & Powel (1985) did not provide explicit detail on how the AP halves were divided. Rather, they briefly stated that circular-shaped discs of AP were obtained using a cork borer of appropriate diameter. In contrast, after carefully examining an antler field, we (Li & Suttie, 2003) found that the territory of the AP is oval in shape rather than round (based on all the identifiable landmarks) and an antler field is not flat, because the lateral subregion is located on the relatively steep slope of a frontal crest (Fig. 1C). Secondly, the methods differ in the position of the incision made in the skin to expose the AP. Goss & Powel (1985) made an incision in the skin posterior to an antler field and reflected the skin anteriorly to reveal the underlying AP. In contrast, the skin incision in this study was made medial to an antler field and the skin was reflected laterally. In so doing, we effectively prevented any accidental damage to nerves and/or blood vessels that supply the field, as these are located at the lateral subregion of the field. Therefore, we avoided any confounding problems that may have been caused by damage to efferent nerves and blood vessels and could have influenced the resulting antler growth. Furthermore, the complex morphology of antlers that grew from the ectopic grafted sites (deer foreheads) in the present study provided another dimension of information to our conclusion, compared to Goss & Powel (1985), who selected sites (forelegs) that only support a spike antler to grow.

Deer age/bodyweight vs. antler field maturation

Antler fields are initially established in a male deer at about 60 days of gestation and they continue to grow to about 100 days (judged by the morphological evidence, but further research is needed to confirm the claim), after which they remain quiescent until after birth (Lincoln, 1973; Li & Suttie, 2001b). Antler fields are reactivated when a male deer approaches puberty (Suttie et al. 1991, 1995; Li et al. 2003) and reaches a species-specific bodyweight (Suttie & Kay, 1982; Li & Suttie, 1996). The results from the present study are consistent with these previous findings. In the late-born group, two deer (Deer 10 and 12) that had lighter bodyweights at the initial surgery (around 24 kg) failed to achieve the threshold bodyweight in the first growth cycle and hence did not initiate pedicle and antler formation. The other two (Deers 9 and 11) that had heavier bodyweights at the initial surgery (around 27 kg) were significantly delayed in reaching the threshold bodyweight in the first growth cycle, and hence the onset of pedicle and antler formation was pushed into mid-winter (12 December), the latest case of antler growth in a calendar year that has been recorded. From an evolutionary point of view, it is conceivable that deer preferentially utilise the available nutrients for survival, i.e. body mass building, rather than channelling them to the precocious development of pedicle/antlers, which are secondary sexual characteristics. It remains unclear, however, how age and bodyweight affect the maturation of antler morphogenetic fields.

In the present study, we found that the AP grafts from the late-born deer did not form a detectable pedicle bulge at the ectopic sites. In contrast, those from deer born in the normal season all formed sizable pedicle bulges (up to 3 cm high), although some of them failed to initiate ectopic antler formation in the first antler growth cycle. The size of these bulges is crucial for bringing the AP-derived tissue into close contact with the overlying skin – a prerequisite for triggering antler growth (Goss, 1990; Li et al. 2010). Therefore, it is likely that the grafted AP from the late-born deer did not give rise to antlers because the growth of the grafted tissue was insufficient to form a close association with the overlying skin.

The dormancy of the grafted AP from the late-born deer may be due to the immaturity of the original antler morphogenetic field from which the AP was obtained. This view is supported by our observation that the AP from the late-born deer, compared to that from the normal bodyweight deer, was thinner and more adherent to the underlying bone at the time of sampling. Although no further examination was carried out for the AP in the present study, we assessed the histology of the AP from both malnourished and castrated male red deer calves in a previous study and found that AP in the castrated deer was comparable to, but in the malnourished deer significantly thinner than, that of well nourished deer (Li & Suttie, 1994). Furthermore, the AP cells from malnourished deer were spindle-shaped with elongated nuclei and these cells were sparsely scattered throughout the thick bundles of collagen fibres. The surfaces of the underlying bony trabeculae were lined with discrete rows of resting osteoblasts. Collectively, it is malnutrition, but not androgen hormone, that inhibits activation of AP cells, hence delaying the initiation of frontal crest formation. In the present study, the late-born deer missed the season during which there is optimal nutritional biomass to assist their growth phase and did not achieve the threshold bodyweight to initiate growth of the frontal crest/early pedicle until the following winter. Therefore, the histological structure of the AP in the present study should be comparable to that of malnourished deer, and we speculate that delayed initiation of pedicle/antler formation would be mainly attributed to malnutrition. This speculation is also supported by the results of Gomez et al. (2006).

Ectopic antler formation vs. reestablishment of circulation

An interesting phenomenon noticed in the present study was that the timing of antler initiation and the final size of ectopic antlers from the same type of transplanted AP half varied considerably. For example, the AP halves from the anterior subregions from deer that were born in the normal season in one stag (deer 5) formed a 39-cm-long antler (with three branches) in the first growth cycle, and a 450 g antler in the second. In contrast, the other stag (Deer 1) initiated antler formation only in the fourth growth cycle and only formed a 5-cm-long spike antler. Although it is unclear why the growth was so variable, it is known that revascularisation is the single most important factor for a free graft to survive, grow and function (Stewart et al. 1984). Therefore, it is likely that variable growth rate can be attributed to differences in revascularisation to the transplanted AP tissue among deer.

The time and degree of spontaneous revascularisation of a graft depend on the vascularity of the site, the nature of the graft, and the way that a graft is placed (Burton et al. 1987). As the grafted tissue type (AP half from the anterior subregion) and site (deer forehead) in the present study are the same, the difference may be caused primarily by the orientation of the AP half that was inserted into the pocket, as we did this in a random manner. It is known that the initial vascular inosculation determines the quality of a graft revascularization. Inosculation involves the alignment of the donor vascular buds with graft capillaries and establishment of circulation (Zarem & Zweifach, 1965; Converse et al. 1975; Burton et al. 1987). If an AP graft in the present study was oriented in a way that vascular systems from the both sides could properly anastomose with minor or no adjustment, the graft would be more readily revascularised and give rise to earlier and bigger antler, and vice versa. In addition, the same type of AP half from different deer in the same treatment group may have different angiogenic potential. In a previous study we found that AP grafts have strong angiogenic activity that enables AP to survive and grow at the transplantation site. In contrast, the dimensions of the pedicle periosteum, within which progenitor cells for antler regeneration reside, did not expand, although it can survive at the ectopic site (Li et al. 2010). Therefore, there may be differences in angiogenic activity of the same type of AP half in different deer, which could explain the timing of growth and the final size of ectopic antlers.

The present study convincingly demonstrates that antler morphogenetic fields have a distinct pattern and that the competency of these fields is determined by the age and bodyweight of deer. We suggest that the antler growth system is a novel and readily accessible model for studying the regulation of morphogenetic fields of organogenesis.

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Author contributions

Conceived and designed the experiments: C.L., Z.G., and F.Y. Performed the experiments: Z.G., C.L., and C.M. Analyzed the data: Z.G., C.L., F.Y., and C.M. Wrote the paper: C.L., Z.G., C.M., and F.Y.

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