

Differential effects of the PI3K/AKT pathway on antler stem cells for generation and regeneration of antlers *in vitro*

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1. ABSTRACT

Understanding the role of the PI3K/AKT pathway in regulating basic antler stem cell parameters and angiogenesis may provide an insight into the mechanisms underlying mammalian appendage development. The present study took multiple approaches *in vitro* to investigate the effects of the PI3K/AKT pathway on antler stem cells. By addition of LY294002, proliferation rate of the antlerogenic periosteum (AP) cells was decreased significantly ($p < 0.01$), while the proliferation rate of the pedicle periosteum (PP) cells decreased to a lesser extent; the cytoskeleton of the AP cells was essentially collapsed; and the PP cells significantly shrunken. By addition of LY294002 or KU-0063794, formation

of networking tubular structures from HUVECs in the AP or PP cell conditioned medium was significantly inhibited; whereas, expression level of VEGF-B mRNA in the AP or PP cells was decreased by the former, and increased by the latter significantly. Therefore, the results suggest that the PI3K/AKT pathway is involved in proliferation and differentiation of the AP and the PP cells, and plays a more important role in the former than in the latter.

2. INTRODUCTION

Organ regeneration is the “Holy Grail” of modern regenerative biology and medicine (1).

Deer antlers are the only mammalian organs that are capable of complete renewal, hence they offer a unique opportunity to explore how nature has enabled regeneration to occur in these specialized tissues, while not allowing in all other mammals (2). Antlers renew annually from the permanent cranial bony protuberances, called pedicles (2-5). The PP is the key tissue type for antler regeneration because deletion of the PP renders the pedicle incapable of regenerating an antler (6). This unique attribute of the PP has been considered to arise from their developmental origin, i.e. AP (2,7). The AP is located in the presumptive pedicle growth region in male deer calves and gives rise to the entire pedicle and first antler under the stimulation of androgen hormones when concentrations of these increase as a male deer approaches puberty (8). The AP disappears once the pedicle and first antler form. From then on, antler regeneration is initiated from the PP annually (8, 9). Recent studies show that both the AP and the PP cells possess the attributes of stem cells, and hence are called antler stem cells (ASCs) (2, 10-12). However, these two types of ASCs are not identical, the former can induce ectopic antler growth when subcutaneously transplanted elsewhere on the deer body, such as on the forehead or a foreleg, whereas the latter is incapable of doing so (7, 13,14). Further studies show that the failure to induce ectopic antler growth by the PP may be due to an inability to attract or generate major blood vessels to the transplantation site to sustain the growth after being differentiated from the AP (15).

With the advent of the molecular biology era, antler researchers from different labs have been attempting to identify the molecular mechanisms underlying antler generation and regeneration, via the construction of cDNA libraries (16), sequencing of transcriptomes (17, 18) and analyzing proteomes (19). Through the proteomic approach, Li *et al.* (19) identified some differentially expressed proteins in the AP cells (such as S100A4, SPARC and ANXA5) and in the PP cells (such as galectin-1, IL8 and ANXA2) vs the adjacent tissue, facial periosteum (FP). Analysis of these differentially expressed proteins, using IPA software, identified a few dominant signaling pathways in ASCs. Specifically, in the AP cells, the predominant pathways were (order based on the level of significance) PI3K/AKT signaling ($p<0.001$), 14-3-3 signaling ($p=0.002$), Rho signaling ($p=0.015$) and ERK/MAPK signaling ($p=0.02$), and in the PP cells, the predominant pathways were ERK/MAPK signaling ($p<0.001$), actin cytoskeleton signaling ($p<0.001$), and PI3K/AKT signaling ($p=0.004$) (19). These results indicate that PI3K/AKT signaling pathway is common to both the AP and PP cells, although from the level of significance, may be more important in the AP cells. It remains unclear how the PI3K/AKT pathway in the ASCs regulates the generation or regeneration of antlers.

The PI3K/AKT pathway is known to regulate multiple cellular activities, including cell proliferation, differentiation, survival, adhesion, migration and angiogenesis (20-24). Phosphoinositol-3-kinase consists of two subunits: the catalytic P110 and the regulatory P85. Once activated, PI3K catalyzes the production of phosphatidylinositol-3, 4, 5-triphosphate, which then activates AKT through phosphorylation (25). The activation of AKT causes a cascade of responses involving downstream targets that regulate cellular functions (26). In the present study, we evaluated the effects of the PI3K/AKT pathway on the ASCs based on four parameters: proliferation, cell cycle, cell cytoskeleton changes and cellular adhesion. We also assessed the effect of the specific inhibitor LY294002, which targets the P110 catalytic subunit.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that acts, at least in part, through the PI3K/AKT pathway (27). Activation of the PI3K/AKT/mTOR pathway can increase VEGF secretion (28). The VEGF family includes five subtypes, namely VEGF A-D, and placental growth factor (PlGF) (29). VEGF-A is considered to be the prototype angiogenic factor (30). However, VEGF-A protein production was not detectable in either the AP or PP cells (19). VEGF-C and VEGF-D are mainly involved in lymphangiogenesis (31, 32). PlGF, which is expressed primarily in the placenta, is now generally believed to play an important role in angiogenesis (33, 34). VEGF-B was reported to be angiogenic in some studies (35-37), but lacked this function in others (38-40). Although VEGF-B shares a high degree of sequence homology to VEGF-A and PlGF, its contribution to angiogenesis in antler development remains to be elucidated.

Our results provide the first evidence that the PI3K/AKT pathway plays an important role in regulating the activity of ASCs, and more so in the AP cells than in the PP cells. Our results also suggest that VEGF-B may not be required for angiogenesis during antler generation.

3. MATERIALS AND METHODS

3.1. Tissue collection and cell culture

The AP, PP and FP were obtained from the frontal crests (1-year-old male sika deer, 4 deer), pedicles (2-year-old male sika deer, 3 deer) and heads (1-year-old male sika deer, 3 deer) as previously described (41, 42). Cells released from these tissue types were cultured in the culture medium (DMEM (Life Technologies, USA) containing 10% FBS (Gibco, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, USA)); incubated at 37 °C in a 5% CO₂ incubator; and stored in freezing medium (FBS + 10% DMSO) in liquid nitrogen. When required, the

AP, PP, and FP cells were thawed and cultured in T75 flasks (NEST, USA). Deer cells had reached the fourth passage when used in the assays. SP2/0 cells (mouse myeloma cells, Shanghai Fumeng Gene Bio-technology Co, China) were used as a reference tumor cell line for the study of cell cycle and cellular adhesion.

3.2. MTT assay

The AP, PP and FP cells were seeded in 24-well plates at 1.25×10^4 cells/cm², cultured until reaching about 40% confluence in the culture medium, and then transferred to the control medium (DMEM + 1% DMSO) or the control medium containing LY294002 (Sigma, USA, final concentrations: 15, 25, 35 or 50 μ M) dissolved in DMSO for 12, 24 or 48 h. At each predetermined time point, an MTT (Sigma) assay was carried out to measure cell proliferation rates using a Spectrophotometer (Tecan, Switzerland) at 570 nm of OD value. Analyses were performed with the use of SAS statistical software, version 9.3. (SAS Institute). Results were expressed as mean $\pm$ SEM.

3.3. Cytoskeleton staining

The AP, PP and FP cells were seeded at a density of 1×10^4 cells/cm² on cover glasses (NEST, USA) in 6-well plates containing the culture medium until they reached about 50% confluence. These cells were further cultured overnight in DMEM and then transferred to the control medium or the control medium containing LY294002 (35 or 50 μ M) for 12 h. The cytoskeleton was stained using the method reported elsewhere (43). In brief, cells grown on the cover glasses were fixed with 4% neutral buffered paraformaldehyde (Shanghai Yuanyelnc., China) for 5 sec, permeabilized with 1% Triton-X100 (Amresco, USA) for 30 min, fixed with 4% paraformaldehyde for 15 min, and stained with Coomassie brilliant blue R250 (0.2%, Sigma, USA) for 40 min. All incubation steps were carried out at room-temperature and between the steps cells were rinsed three times with PBS (PH 7.4) for 5 min/wash. After being stained, cells on the cover glasses were dipped into xylene for 15 min to differentiate the cytoskeleton from the background.

3.4. Cell cycle determination

The AP, PP and SP2/0 cells were grown in the culture medium until they reached about 80% confluence in 10-cm-dishes, and then transferred to the control medium or the control medium containing LY294002 (50 μ M). Cell cycle analysis was conducted when cells were nearing confluence: Briefly, each cell type was trypsinized, washed twice gently in pre-cooled PBS, fixed in 75% ethanol at -20 °C overnight, centrifuged at 500 g, re-suspended in

PBS containing RNase (Sigma, USA) for 30 min at 37 °C, and then labeled with Propidium Iodide (PI, Shanghai Shenggong, China) in the dark for 30 min. The distribution of cells in each phase (G0/G1, S, and G2/M) of a cell cycle was calculated based on the data of flow cytometry (channel: FL2, CV $\leq$ 8%; BD FACS Calibur, USA). This experiment consisted of three biological repeats and each biological repeat had three technical repeats.

3.5. Cellular adhesion testing

The AP, PP and SP2/0 cells were grown in the culture medium until they reached about 70% confluence in 6-well plates and then transferred to the control medium, the control medium containing LY294002 (50 μ M) or insulin (10^{-6} M) overnight. These cells were then trypsinized and re-seeded at a density of 3×10^4 cells/cm² in 96-well plates, which were pre-coated with Growth Factor Reduced (GFR) Matrigel (20 μ l/well, 354263, BD, USA) and cultured for a further 4h. Cells were then transferred to DMEM containing MTT (20 μ l/well) for 4h, and washed twice with PBS (to eliminate non-adhered cells). DMSO was added to each well (150 μ l/well) to dissolve the formazan salts. The OD₅₇₀ values acquired using a photospectrometer had a positive linear correlation with the numbers of adhered cells.

3.6. Tube formation assay

The AP and PP cells were grown in the culture medium until they reached ~ 60% confluence in T75 flasks and then transferred to DMEM overnight. The AP or PP cells were then allocated into three groups: the negative control (NC, the control medium), the treatment 1 (T1, the control medium containing 50 μ M LY294002), the treatment 2 (T2, the control medium containing 30 μ M KU-0063794). The cells in the three groups were cultured for 24h before collection of the conditioned medium from each group, and the conditioned media were then frozen at -20 °C. The tube formation assay was performed as previously described (44) with the following modifications: the GFR Matrigel was thawed at 4 °C overnight and diluted 2.5 times with DMEM. Each well of a 96-well plate was coated with the diluted GFR Matrigel (50 μ l/well) and the plates were then incubated at 37 °C in a 5% CO₂ incubator for 1h. HUVECs (human umbilical vein endothelial cells; 2.5×10^4 cells, Jilin University, China) were suspended in 0.2 ml of the conditioned medium, the control medium containing 50 μ M LY294002, or the control medium containing 30 μ M KU-0063794 or 20 nM VEGF, and then seeded into each well of a plate. Photographs were taken 6h after incubation using a Life EVOS® FL Imaging System (Thermo Fisher, USA). The number of tubes was counted using the Image J 1.4.8 software (National Institutes of Health, USA).

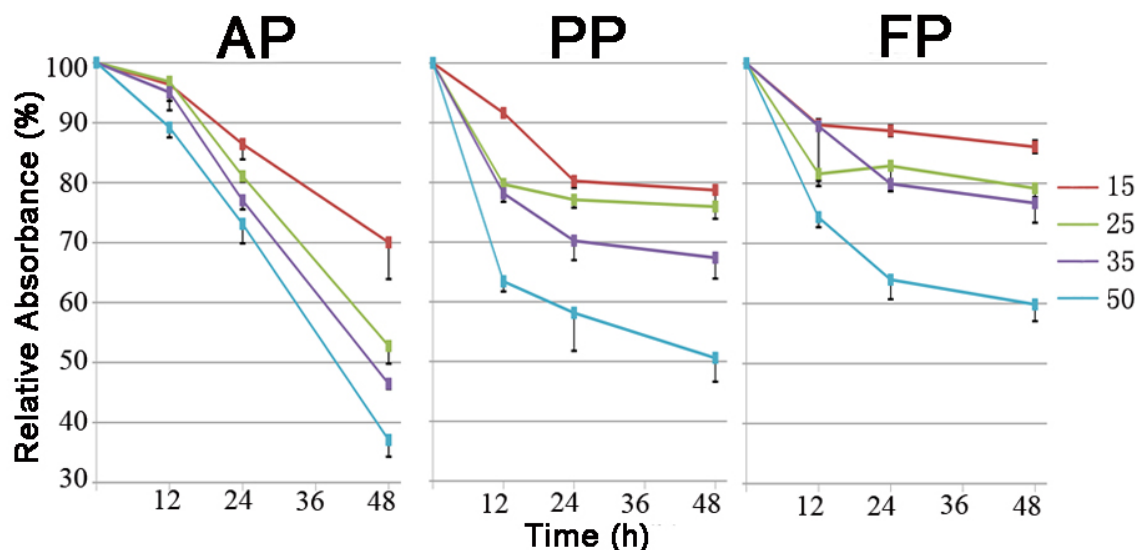


Figure 1. Effects of LY294002 on proliferation rates of the AP, PP and FP cells. Time points of the treatment: 12, 24 or 48h, and doses of the treatment: 0, 15, 25, 35, or 50 μ M. Relative absorbance (%) = $(A_{\text{treated group}} / A_{\text{control group}}) \times 100\%$. Mean $\pm$ S.D (n=3). AP, antlerogenic periosteum; FP, facial periosteum; PP, pedicle periosteum.

3.7. Quantitative PCR

To inhibit the PI3K/AKT and mTOR pathways, the AP, PP and FP cells were treated, respectively, with the control medium, or the control medium containing LY294002 (50 μ M) or KU-0063794 (30 μ M) for 3h. After harvesting, total RNA was isolated from each cell line using PureLinkTM RNA Mini Kit (Life Technologies, USA) according to the manufacturer's recommendation, and reverse-transcribed into cDNA using the PrimeScriptTM 1st Strand cDNA Synthesis Kit (Takara, Japan). Quantitative (q) PCR was performed using an ABI 7500 qPCR System (ABI, USA) with FastStart Universal SYBR Green Master (ROX) (Roche, USA) and the cycling conditions were as follows: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95 °C for 5 s, annealing at 61 °C for 10 s and extension at 72 °C for 15 s. The expression level of GAPDH mRNA was used for normalization. The VEGF-B primers were: forward 5'-CCC CTG GTC ACC AGA AGA AA-3', reverse 5'-CGC ACT CCA GGC CAT CGT-3'. The GAPDH primers were: forward 5'-ATG TTT GTG ATG GGC GTG AAC-3', reverse 5'-CCA GTA GAA GCA GGG ATG ATG TT-3'. Each reaction was performed in triplicates. The relative expression level in each cell line was calculated using the $2^{-\Delta\Delta CT}$ method and presented as bar graphs.

3.8. Western blot analysis

The AP, PP and FP cells were plated in 10-cm dishes containing the culture medium, transferred to DMEM after reaching about 80% confluence, and then cultured in the control medium

or the control medium containing LY294002 (50 μ M) for 3 h. Proteins were extracted from each cell line based on the manufacturer's instructions (Best Bio, China), quantified by Western blot analysis (12% polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad, USA)). Antibodies used in this work were phosphorylated AKT (p-AKT, Ser473) Antibody (Cat. 5012, Cell Signaling, USA), Goat Anti-Rabbit IgG (Cat. ab6741, Abcam, UK), GAPDH Antibody (Cat. 10494-1-AP, Proteintech, USA), HRP-labeled Goat Anti-Mouse IgG (Cat. A0216, Beyotime, China). The expression level of p-AKT protein was evaluated using Image J 1.4.8 software (National Institutes of Health, USA).

4. RESULTS

4.1. Effects of LY294002 on four basic parameters of the ASCs

4.1.1. Proliferation

To investigate the effects of down-regulating the PI3K/AKT pathway on proliferation of the ASCs, the proliferation rates of cells were measured following the addition of LY294002 at different time points and with different concentrations. The results (Figure 1, Table 1) showed that all the AP, PP and FP cells reacted to the treatment of LY294002 in proliferation in a clear dose-dependent manner ($p < 0.01$), the reaction being most intense in the AP cells. Proliferation rates of both the PP and FP cells exhibited a similar characteristic trend over the entire culture period at 50 μ M: in the first 12h, dropped more sharply than the AP cells (PP: 36.59%↓; and FP: 25.64%↓; whereas in the

Table 1. Effects of LY294002 on proliferation rates of the AP, PP and FP cells

Items		AP cell	PP cell	FP cell
Times (h)	12	94.77±4.01C	78.22 ±10.54B	83.8±7.78B
	24	79.41±5.73B	71.42±9.39A	78.88±9.77B
	48	51.59±12.96A	68.16±11.76A	75.45±10.26A
Concentrations(μM)	15	84.27±12.03D	83.53±6.27C	88.20±1.85C
	25	76.93±19.50C	77.57±2.19B	81.22±2.38B
	35	73.36±21.95B	71.92±5.43A	82.03±7.54B
	50	66.47±23.30A	57.38±6.79A	66.05±6.85A
P-value	Time	P<0.0001	P=0.075	P=0.104
	Concentration	P<0.0001	P<0.0001	P<0.0001
	Time*Concentration	P<0.0001	P=0.095	P=0.013

^{A-D} Means in a column without a Concentration/Time differ (P<0.05)

AP: 10.83%↓). Thereafter, the rate of decline eased and gradually stabilized toward the final culture time (reaching 49.41%↓ and 40.15%↓, respectively, at 50 μM and at 48h, compared to 62.92%↓ in the AP cells). Additional pathways might have been activated at the later culture period (after 12 h time point) in the PP and FP cells to compensate for the impaired proliferation rate due to the loss of PI3K/AKT pathway.

4.1.2. Cytoskeleton

Different cytoskeleton arrangements give each cell type a unique shape and motility, and this is clearly evident in the AP, PP and FP cells cultured in the same conditions (Figure 2). The AP cells resembled a three-pointed star (Figure 2A1); the PP cells were cobble-stone like (Figure 2B1); and the FP cells were something in the between (Figure 2C1). Treatment with LY294002 (30 μM) had profound effects on cell morphology and caused severe deformation in shape to the ASCs and the FP cells, which was most dramatic in the AP cells (Figure 2A2), intermediate in the PP cells (Figure 2B2) and least in the FP cells (Figure 2C2). The AP cells were essentially collapsed (Figure 2A3); the PP cells were significantly shrunken (Figure 2B2 and 2B3); and the FP cells became very condensed (Figure 2C2 and 2C3) after effectively inhibiting the PI3K/ATK pathway (LY294002 final concentration: 50 μM). Interestingly, the AP cells were the only cell type in which the change in shape occurred in a dose-dependent manner to the treatment of LY294002. These results indicate that PI3K/AKT pathway plays a key role in the maintenance of the shape of the ASCs, and even more so for the AP cells than for the PP cells.

4.1.3. Cell cycle

Cell cycles of the AP, PP, FP and SP2/0 cells, which were cultured in the absence or presence of LY294002, were measured using flow cytometry. Cell

cycle distributions of each cell type are shown in Figure 3a (without LY294002) and 3b (with LY294002), and the percentage of cell numbers in cell cycle G0/G1, S and G2/M phases of each cell type is shown in Table 2. The results (Table 2) showed that under the normal culture conditions (without LY294002), 94% or above of the AP, PP and FP (data omitted) cells remained at G1 phase and no distinct G2 accumulation (typical feature for the cells that have the potential to initiate epimorphic regeneration) was observed for these cells. On the contrary, less than 30% of the SP2/0 cells remained in G1 phase and the rest of the cells were distributed either in S (61.63%) or G2 (10.74%) phases. Treatment with LY294002 (Figure 3b, Table 2) did not affect the status of cell cycle distribution in the ASCs, but significantly shifted the SP2/0 cells into G1 phase (72.44% from 27.63%; $p<0.01$) from both S and G2 phases. There were few cells in the G2 phase (reaching 0.06%). These results indicate that inhibition of PI3K/AKT pathway did not significantly alter the cell cycle distribution of the ASCs, but seriously affected that of the tumor cell line, SP2/0. Therefore, the inherited cell cycle distributions of the ASCs could be vital to these cells and cannot be readily altered to ensure the unique organ, antler, to be developed, and at the same time will not go cancerous.

4.1.4. Adhesion

Both PI3K/AKT signaling (45) and insulin (46) positively regulate cell focal adhesion. Hence, we tested how these factors affect the ASCs in this regard. Treatment with LY294002 or insulin significantly increased the adhesion of all the AP and PP cells ($p<0.01$) and the effect of insulin was greater than that of LY294002 in this regard (Table 3). Inhibition of the PI3K/AKT pathway using LY294002 had no significant effect on adhesion of the FP cells ($p>0.05$); whereas, addition of insulin significantly increased adhesion of the FP cells ($p<0.01$). In contrast, adhesion of

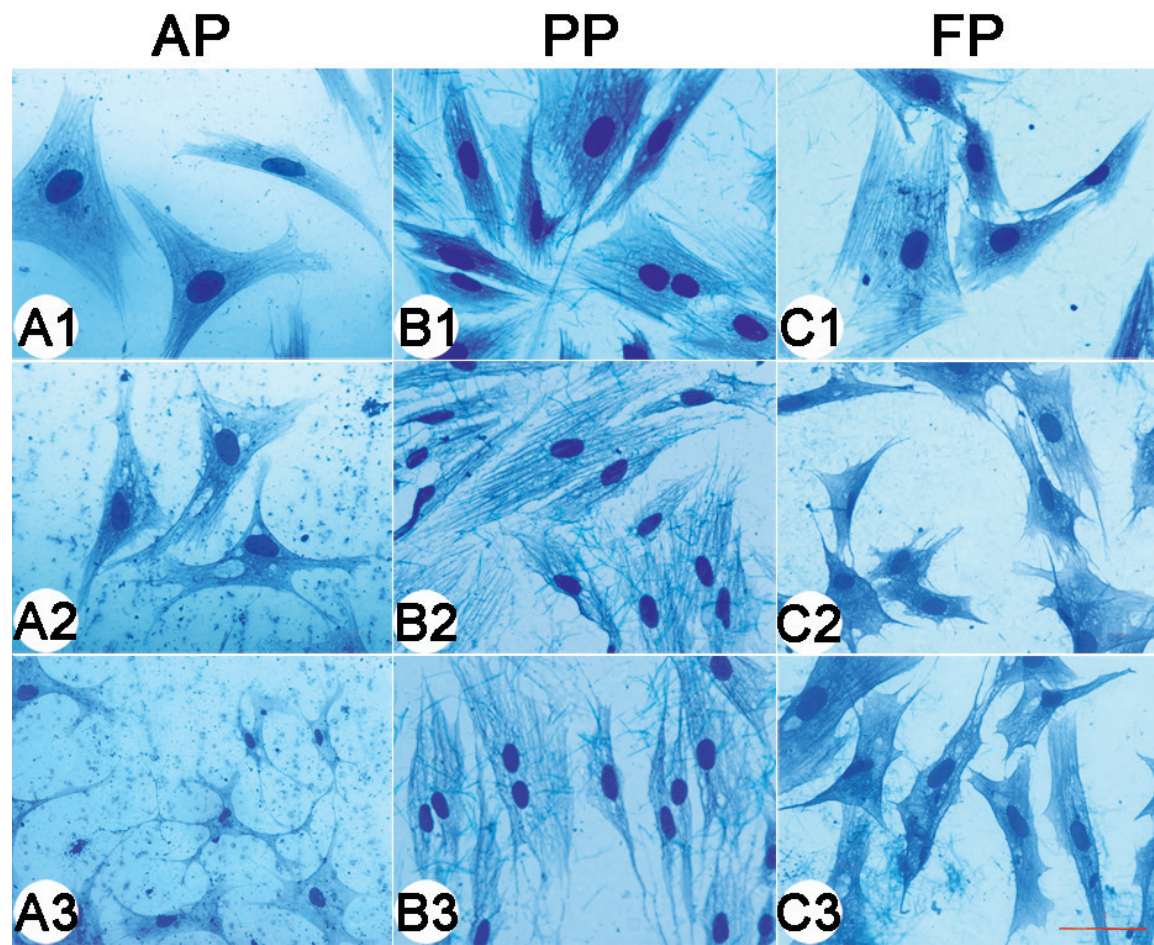


Figure 2. Effects of LY294002 on cytoskeleton of the AP, PP and FP cells. Coomassie brilliant blue staining. A1-A3 Cytoskeleton of the AP cells cultured in the control medium or the control medium containing LY294002 (35 μ M or 50 μ M). B1-B3 Cytoskeleton of the PP cells cultured in the control medium or the control medium containing LY294002 (35 μ M or 50 μ M). C1-C3 Cytoskeleton of the FP cells cultured in the control medium or the control medium containing LY294002 (35 μ M or 50 μ M). Bar= 400 μ m. For the detailed description of cell morphology, please refer to the text.

Table 2. Effects of LY294002on cell-cycle distribution of the AP, PP and SP2/0 cells

Type of cells	Treatment	G0/G1	S	G2/M
AP cells	Untreated	94.95 $\pm$ 2.36	1.69 $\pm$ 0.35	3.36 $\pm$ 0.41
	LY294002 (50 μ M)	96.8 $\pm$ 3.17	2.54 $\pm$ 0.48	0.66 $\pm$ 0.15
PP cells	Untreated	96.82 $\pm$ 1.83	1.2 $\pm$ 0.31	1.98 $\pm$ 0.42
	LY294002 (50 μ M)	95.97 $\pm$ 3.01	1.49 $\pm$ 0.27	2.54 $\pm$ 0.58
SP2/0 cells	Untreated	27.63 $\pm$ 4.75	61.63 $\pm$ 5.09	10.74 $\pm$ 2.59
	LY294002 (50 μ M)	72.44 $\pm$ 3.86	27.50 $\pm$ 3.23	0.06 $\pm$ 0.04

Data are presented as mean $\pm$ S.D.

the SP2/0 cells was either decreased significantly when treated with LY294002 ($p<0.01$), or increased significantly with insulin ($p<0.01$) (Table 3). Therefore, in contrast to the reported cell lines and the control cell lines in the present study, PI3K/AKT signaling seems to play a significant negative role in regulating adhesion of the ASCs.

4.2. Effects of the AP or PP conditioned medium on tube formation

In a previous study, we showed that transplanted AP could attract a large number of blood vessels to sustain the initiation of ectopic antler formation, but not the transplanted PP (47). We

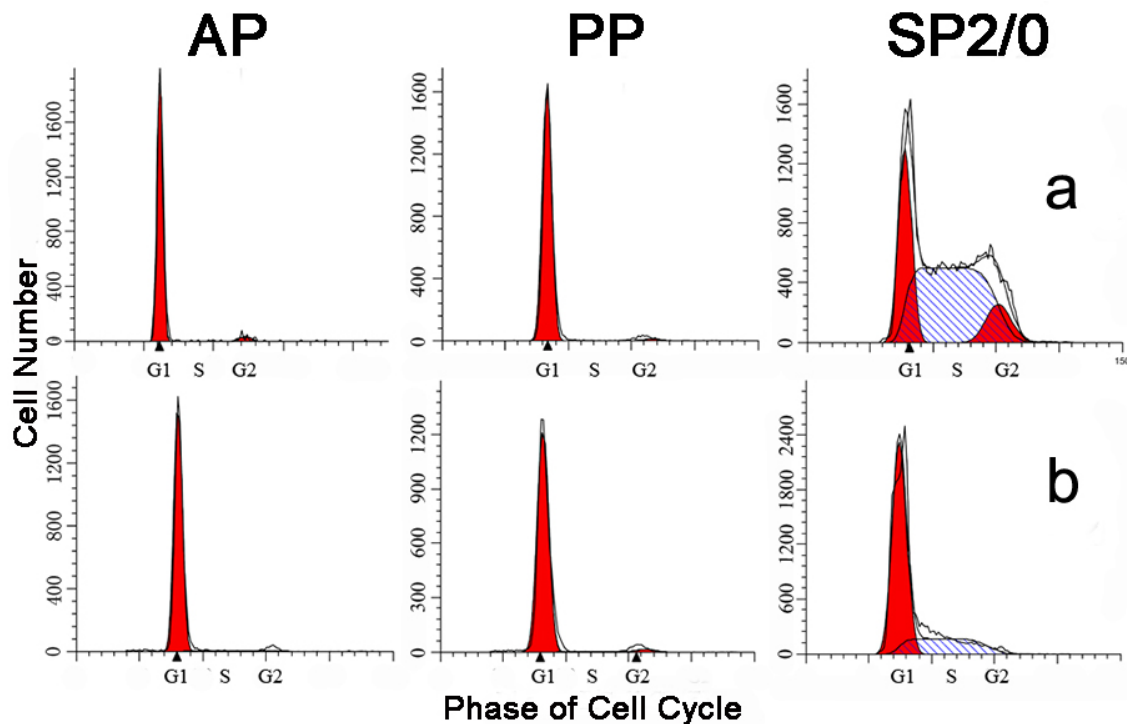


Figure 3. Effects of LY294002 on cell-cycle distribution of the AP, PP and SP2/0 cells. Data were analyzed using ModFit LT Software to estimate the percentages of cells at each phase of G0/G1, S and G2/M. **a** The AP, PP and SP2/0 cells were cultured in the control medium. **b** The AP, PP and SP2/0 cells were cultured in the control medium containing LY294002 (50 μ M).

Table 3. Effects of LY294002 or insulin on adhesion of the AP, PP, FP and SP2/0 cells

Treatment	AP cells	PP cells	FP cells	SP2/0 cells
Control	0.3098 $\pm$ 0.0135	0.2747 $\pm$ 0.0292	0.6035 $\pm$ 0.0181	0.2508 $\pm$ 0.0096
LY (5 $\times$ 10 ⁻⁵ M)	0.3638 $\pm$ 0.0078**	0.3689 $\pm$ 0.0135**	0.6186 $\pm$ 0.0029	0.1214 $\pm$ 0.0106**
Insulin (10 ⁻⁶ M)	0.4671 $\pm$ 0.0174**	0.4730 $\pm$ 0.0125**	0.9392 $\pm$ 0.0364**	0.3560 $\pm$ 0.0045**

Data are presented as mean $\pm$ S.D. from three experiments. T-test was carried out for pair-comparison between the control and each treatment for each cell line. ** p <0.01

wondered if this angiogenic effect could be achieved through diffusible substances from the AP. To test this, HUVECs were cultured in the conditioned medium from the AP or the PP cells, respectively. The results showed that HUVECs cultured in the conditioned medium from the AP cells clearly formed networking tubular structures (Figure 4A1), but failed to do so when in the presence of LY294002 (Figure 4A2) or KU-0063794 (Figure 4A3). The latter is an inhibitor of the downstream mTOR pathway. In contrast, HUVECs cultured in the PP-conditioned medium did not form proper cell aggregates (Figure 4B1) and the addition of LY294002 (Figure 4B2) or KU-0063794 (Figure 4B3) had no effect on cell aggregation. The HUVEC cells did not form tubular networks when cultured in the control medium the control medium containing 50 μ M LY294002 (Figure 4C1), or the control medium containing 30 μ M KU-0063794 (Figure 4C2), but did so when in the control medium containing 20 nM

VEGF (Figure 4C3). Overall, the results provide further experimental evidence for the previous conclusion that AP is angiogenic, but the PP is not.

4.3. Expression of VEGF-B mRNA in the AP or PP cells

Given that signaling via PI3K mediates the angiogenic activities of the AP (see above) and that VEGF is one of the key molecules in this pathway, we reckon VEGF may be involved in this function. In a previous study we found that VEGF-A is not expressed in the ASCs (19), so we measured concentrations of VEGF-B mRNA. The results showed that when cultured in the control medium, the expression level of VEGF-B mRNA in the AP cells was significantly higher than that in the PP cells (p <0.01; Figure 5). Addition of LY294002 to DMEM significantly decreased, whereas addition of KU-0063794 significantly increased the

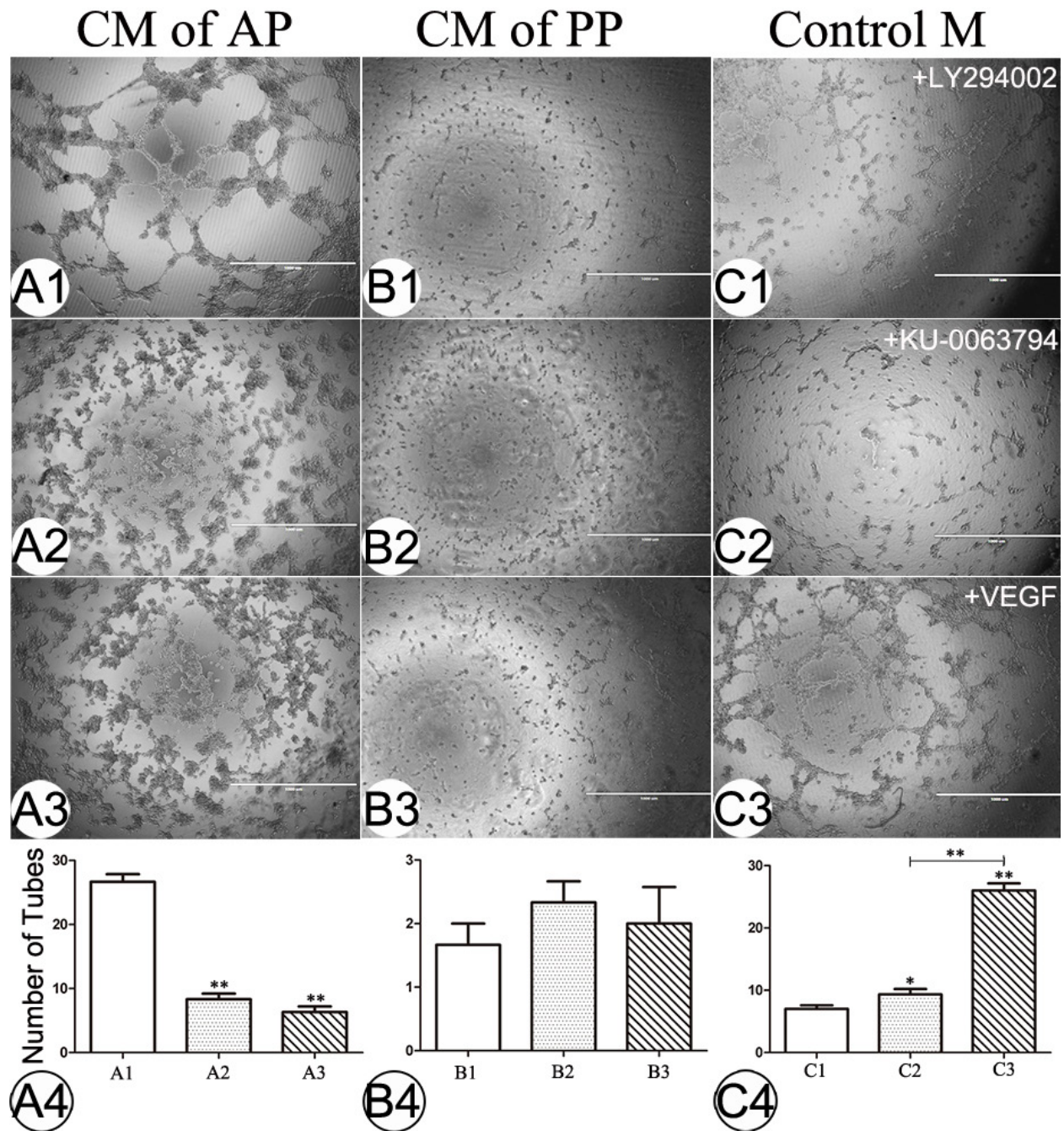


Figure 4. Effects of the AP or PP conditioned medium on tube formation with or without LY294002 or KU-0063794. A1-A3 HUVECs were cultured in the CM of the AP cells cultured in NC, T1 or T2. B1-B3 HUVECs were cultured in the CM of the PP cells cultured in NC, T1 or T2. C1-C3 HUVECs were cultured in the control medium containing LY294002, KU-0063794 or VEGF. A4-C4 The number of tubes was presented as bar graphs. CM, conditioned medium; M, medium. Bar=1000 μ m.

expression level of VEGF-B mRNA in both the AP and PP cells ($p<0.01$; Figure 5).

4.4. LY294002 induces a PI3K-dependent decrease of p-AKT

Activation of the PI3K/AKT pathway depends upon the degree of AKT phosphorylation (48).

Therefore, we measured the expression level of p-AKT in the AP and PP cells. The results (Figure 6) showed that the abundance of p-AKT in the AP cells was significantly higher than that in the PP cells ($p<0.01$). In addition, the expression level of p-AKT in the AP and PP cells was significantly reduced ($p<0.01$) after treatment with LY294002, even more so in the AP cells (around 50%↓) than in the PP cells (around 40%↓).

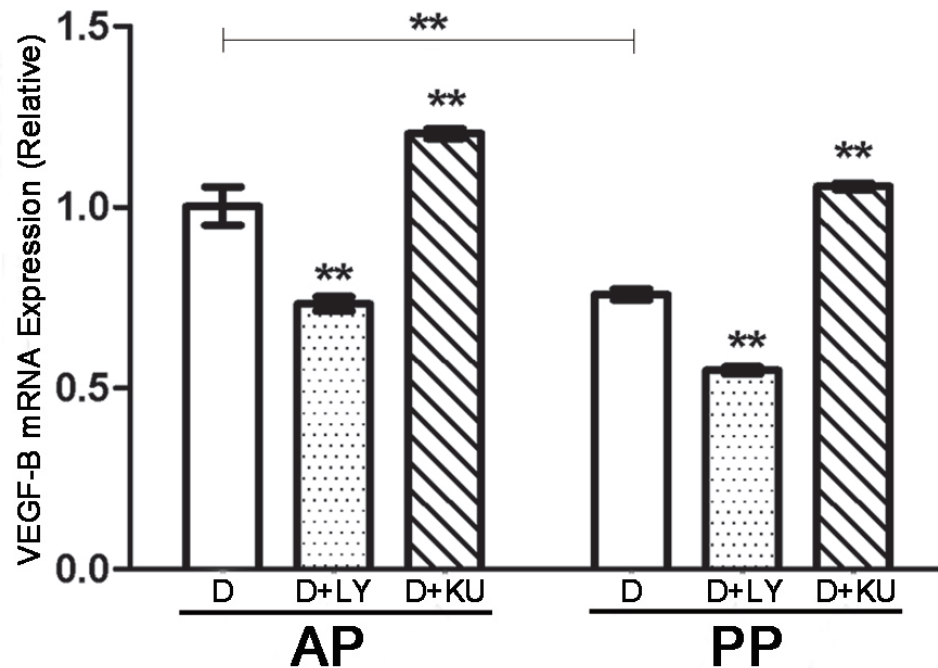


Figure 5. Effects of LY294002 on expression of VEGF-B mRNA in the AP or PP cells. LY294002 significantly decreased ($p < 0.01$) and KU-0063974 significantly increased ($p < 0.01$) the expression level of VEGF-B mRNA in both the AP and the PP cells, whereas in the control medium, the relative expression level of VEGF-B mRNA in the AP cells were significantly higher than in the PP cells ($p < 0.01$). All data represented as mean $\pm$ S.D. in triplicates.

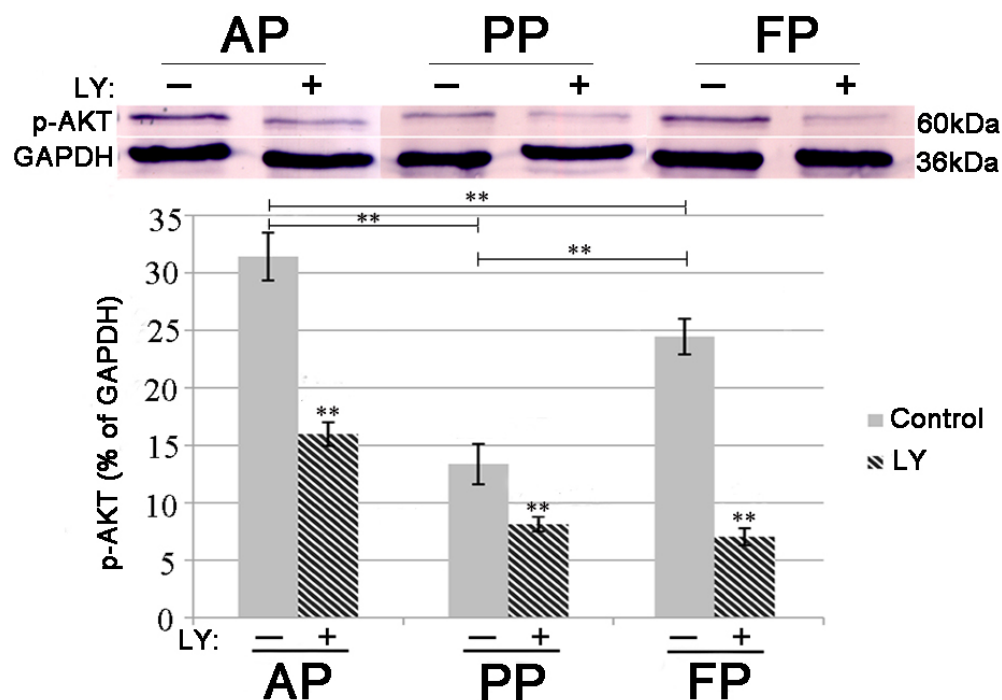


Figure 6. Effects of LY294002 on the expression of p-AKT protein in the AP, PP or FP cells. Addition of LY294002 in the control medium significantly decreased the expression level of p-AKT in the AP, PP or FP cells ($p < 0.01$). In the control medium only, the expression level of p-AKT in the AP cells was significantly higher than that in the PP cells ($p < 0.01$) or the FP cells ($p < 0.01$); and the expression level of p-AKT in the FP cells was significantly higher than in the PP cells ($p < 0.01$). GAPDH was used as a reference gene. All data represented as mean $\pm$ S.D. in triplicates. LY, LY294002.

Therefore, these results could have at least partially explained at molecular level why PI3K/AKT pathway has greater influences on the AP cells than on the PP cells.

5. DISCUSSION

This is the first comprehensive study of effects of the PI3K/AKT pathway *in vitro* on the AP and PP cells that are key cell types for antler generation and regeneration, respectively. Our results showed that the PI3K/AKT pathway plays a critical role in regulating the ASCs. Specifically, when this pathway being inhibited 1) cell proliferation was decreased and cell cytoskeleton collapsed, and the effect being more pronounced in the AP than in the PP cells. Therefore, this pathway may have a greater influence on antler generation than regeneration; 2) cell adhesion was enhanced; 3) cell cycle was not significantly affected; and 4) VEGF-B expression decreased, although VEGF-B is unlikely to be the key factor in regulating angiogenesis during antler generation or regeneration.

Cell proliferation is a complex process and is regulated by numerous factors and signal transduction pathways (49, 50). Our previous study showed that the most dominant signal transduction pathway involved in proliferation of AP cells (based on statistical significance) is the PI3K/AKT pathway, while in the PP cells it is the ERK/MAPK pathway (19). Consistent with these findings, in the present study we found that inhibition of the PI3K/AKT pathway decreased the rate of proliferation in the AP cells in a dose- and time-dependent manner, while being less responsive in the PP and the control FP cells. Therefore, the PI3K/AKT pathway is involved in proliferation of the ASCs, may play a more dominant role in regulating proliferation of the AP cells than the PP cells, and hence is more important for antler generation than for regeneration.

Cytoskeleton provides the structural basis for cell shape and is also involved in cell motility, transmembrane and intracellular transmission of chemical signals (51, 52). The main components of cytoskeleton are microtubules, actin filaments and intermediate filaments (53-55). In the present study, actin filaments in the ASCs were mainly observed because microtubules disappear when cells are treated with 1% Triton-X100 and the intermediate filaments are barely visible after staining with Coomassie Blue (56). Activation of the PI3K/AKT pathway alters cell shape and motility mainly through reorganization of the actin filaments (57, 58). Inhibition of the PI3K/AKT pathway, using LY294002 in the present study, caused more severe change in shape to the AP cells than to the PP cells (Figure 2A2). Again, this is likely due to cytoskeleton of the AP cells being predominantly maintained by the PI3K/AKT pathway, while that of

the PP cells is also regulated by additional pathways. Indeed, the most dominant pathway for the PP cells was found to be the actin cytoskeleton signaling pathway (19). Therefore, even if the PI3K/AKT pathway in the PP cells is significantly down-regulated, activation of actin cytoskeleton signaling would be able to partially compensate for the loss. Hence, the PP cells did not exhibit that dramatic change in morphology like the AP cells after inhibiting the PI3K/AKT pathway.

It is currently not known why there are differences between the AP and PP cells in the abundance, or expression level of regulating factors and in the activity of signal transduction pathways that control proliferation and that regulate the cytoskeleton. One plausible explanation is that antler development at different stages requires different regulatory mechanism. For example, antler regeneration is at least 10 times faster (10-30 mm/day) than initial pedicle/antler generation (around 1 mm/day) (59). Therefore, PP cells may require different stimuli conveyed by the ERK/MAPK and p38 MAPK pathways to induce the PP cells to proliferate at a faster rate than that of the AP cells, which mainly rely on the PI3K/AKT pathway. Furthermore, although both antler generation and regeneration require the progeny of the AP (9) and PP (3) cells, respectively, to form a growth centre, the distance that these cells migrate and rate of these progeny cells to form their respective growth centers may be different. Therefore, the AP and the PP cells may have a different organization of their cytoskeletons, which may require different signal transduction pathways to co-ordinate these changes.

Cell cycle is an integral part of cell proliferation and strict control of cell cycle progression for rapidly proliferating cells (G1 accumulation) is crucial to prevent them from undergoing neoplastic transformation (60). Our previous study showed that majority of the ASCs rest at G1 phase (61), although their multiplication rate is comparable to, or greater than that of cancer cells (2, 62). The accumulation of ASCs in the G1 phase was not influenced by significantly down-regulating expression of p21, a key regulatory factor that maintains a strong G1 checkpoint (61). Therefore, we considered that antlers may have a unique system where in rapid cell proliferation is initiated whilst the stability of the cell cycle distribution is effectively maintained. In the present study, we further confirmed the phenomenon of ASCs accumulation in the G1 phase and provided new evidence for the stability/rigidity of the cell cycle distribution of the ASCs because their accumulation at G1 was not altered by down-regulating the PI3K/AKT pathway. Therefore, the inherited cell cycle distributions of the ASCs could be vital to these cells and cannot be readily altered to ensure the unique organ, antler, to be developed, and at the same time will not become cancerous.

Cell adhesion is the binding of a cell to a surface or substrate, such as an extracellular matrix of another cell type (63). Previous studies have reported that the adhesion of mesenchymal stem cells or carcinoma cells was decreased when PI3K/AKT pathway was inhibited by LY294002 (64, 65). In contrast, in the present study adhesion of the ASCs was significantly increased ($p<0.01$) following inhibition of the PI3K/AKT pathway (Table 3). The reason and mechanism underlying this discrepancy are not known. We do know that both antler generation and regeneration require initiation of both proliferation and migration of the ASCs and their progeny to establish their respective growth centers (3, 9). Therefore, the co-ordinated use of additional pathways to those of PI3K/AKT and ERK/MAPK must be employed in a temporal manner to regulate the development of the growth centers and then stimulate proliferation of the ASCs and at the same time inhibit motility (increase adhesion) of these cells. The identity of these additional pathways is not known at present.

Deer antlers either in the process of generation or regeneration (even more so) are arguably the fastest growing mammalian organs and their rate of elongation can reach up to ~30 mm/day (66). Therefore, an ample supply of blood is needed to sustain this phenomenal rate of growth. Our previous study showed that when subcutaneously or intradermally transplanted elsewhere on the deer body, the AP cells could successfully initiate the development of an ectopic antler, while the PP cannot despite being able to survive in the ectopic site for a few years (47). Histological examination showed that some major blood vessels were either attracted to or newly generated by the ectopic pedicle/antler tissue, the derivative of the transplanted AP, whereas no obvious difference in blood vessels (number and size) was observed between the margin of the transplanted PP and the surroundings(47). Therefore, we concluded that the PP failed to initiate ectopic antler formation because it may be due to lacks of angiogenic activity. In the present study, conditioned medium of the AP cells stimulated tube formation by HUVECs, whereas the PP cells not only did not stimulate tube formation, but inhibited it. Therefore, the results from the present study provide further evidence for our previous conclusion.

VEGF is well known to be a key factor in activating angiogenesis (67-69). Amongst the five members of the VEGF family, VEGF-A and VEGF-B are thought to be the most relevant ones for antler development because the others either play a role in lymphangiogenesis (VEGF-C and -D) or are only expressed in the placenta (PIGF). Expression of VEGF-A mRNA was not detected in antler tissue (19, 70, 71), and VEGF-B has not been studied in antler development. Some studies showed that VEGF-B is angiogenic (35-37). In the present study, we found that although the expression level of VEGF-B mRNA

in the AP and PP cells were significantly increased after treatment with KU-0063794 ($p<0.01$). Therefore, VEGF-B may not be required for angiogenesis during antler development. The PI3K/AKT pathway could have also regulated angiogenesis in antler development through other downstream targets including HIF-1 α , NOS, mTOR/p70S6K1, FOXO and GSK-3 β (72, 73). For example, we showed in a recent study that S100A4, a member of S100 family, plays an indispensable role in blood vessel formation during antler development (Wang *et al*, in submission). In addition, Wang *et al*. (74) reported that over expression of S100A4 could increase the level of p-AKT and inhibition of PI3K/AKT pathway by LY294002 abrogates the effects of S100A4. Therefore, S100A4 may be implicated in regulating the PI3K/AKT pathway for angiogenesis in antler development. However, further studies are needed to test this hypothesis.

Collectively, we show here that the PI3K/AKT pathway regulates the ASCs *in vitro*, hence may be functionally involved in antler development. The more dominant role of the PI3K/AKT pathway in regulating the AP cells than PP cells is consistent with our previous findings, wherein we used 2-DE to show that the PI3K/AKT pathway predominates in the AP, while the ERK/MAPK predominates in PP cells (19). Interestingly, Mount *et al*. (75) found that the canonical Wnt pathway is predominantly expressed in the early antler regeneration bud. Therefore, antler development at different stages may require signaling via different transduction pathways, or there is a temporal co-ordination of multiple pathways to regulate antler development and regeneration.

6. ACKNOWLEDGEMENTS

Drs Zhen Liu, Hai-ping Zhao contributed equally to the paper. The authors would like to thank Dr Hongmei Sun for teaching tube formation assay; Dr Wenhui Chu for teaching techniques of cell culture and qPCR; Dr Dawn Coates for reading through the manuscript and giving critical comments; and staff from the Deer Antler Research Group, Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, for their help during the gestation of this paper. This work was supported by the Jilin province science and technology development plan item (20140204010YY); and the Project of Science and Technology Plans of Jilin province (20150204071NY).

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Abbreviations: AP: antlerogenic periosteum; PP: pedicle periosteum; ASCs: antler stem cells; FP: facial periosteum; VEGF: Vascular endothelial growth factor; HUVECs: human umbilical vein endothelial cells

Key Words: PI3K/AKT pathway; Antler Stem Cell; Antler generation; Angiogenesis; VEGF-B

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