[Frontiers In Bioscience, Landmark, 23, 1848-1863, June 1, 2018]

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

Zhen Liu[1,2], Hai-ping Zhao[1,2], Da-tao Wang[1,2], Chris McMahon[3], Chun-yi Li[1,2]

[1]Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, Changchun, Jilin, P. R. China, [2]State Key Laboratory for Molecular Biology of Special Economic Animals, Jilin, P. R. China, [3]AgResearch Ltd, Ruakura Research Centre, Private bag 3123, Hamilton 3240, New Zealand

TABLE OF CONTENTS

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 (PIGF) (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). PIGF, 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 PIGF, 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, and100 µg/ml streptomycin (Invitrogen, USA)); incubated at 37 °C in a 5% CO_2 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 forth 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/cm^2, 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 μM) 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±SEM.

3.3. Cytoskeleton staining

The AP, PP and FP cells were seeded at a density of 1×10^4 cells/cm^2 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 μM) 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 YuanyeInc., 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 40min. 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 μM). 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≤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 μM) or insulin (10^{-6} M) overnight. These cells were then trypsinized and re-seeded at a density of 3×10^4 cells/cm^2 in 96-well plates, which were pre-coated with Growth Factor Reduced (GFR) Matrigel (20 μl/well, 354263, BD, USA) and cultured for a further 4h. Cells were then transferred to DMEM containing MTT (20 μl/well) for 4h, and washed twice with PBS (to eliminate non-adhered cells). DMSO was added to each well (150 μl/well) to dissolve the formazan salts. The OD_{570} 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 μM LY294002), the treatment 2 (T2, the control medium containing 30 μM 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 μl/well) and the plates were then incubated at 37 °C in a 5% CO_2 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 μM LY294002, or the control medium containing 30 μM 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).

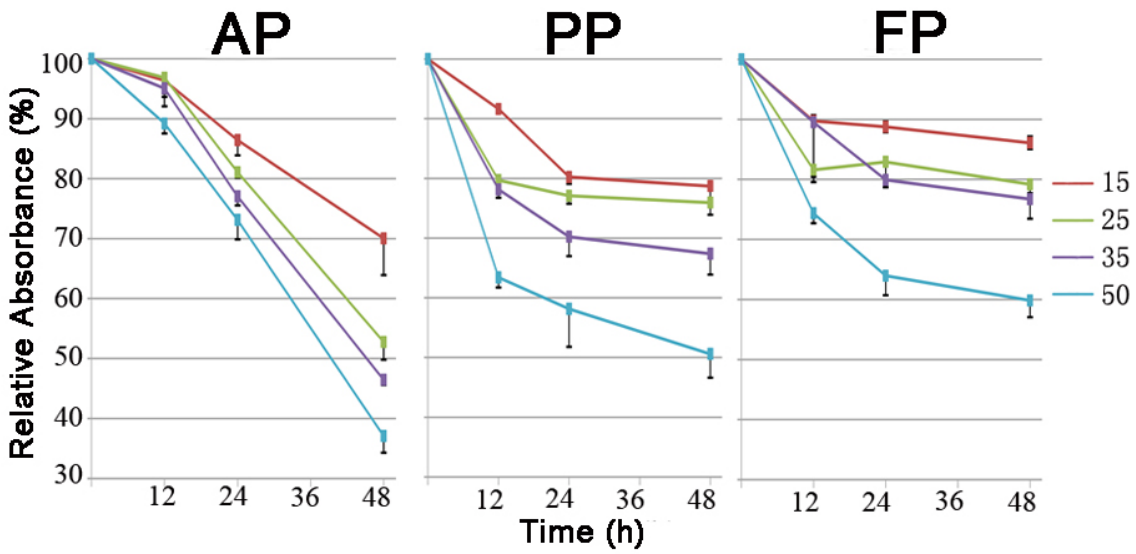

Figure 1. Effects of LY294002 on proliferation rates of the AP, PP and FP cells. Time points of the treatment: 12, 24 or 48h, and doses of the treatment: 0, 15, 25, 35, or 50 μM. Relative absorbance (%) = ($A_{treated\ group}$ / $A_{control\ group}$) x 100%. Mean ± S.D (n=3). AP, antlerogenic periosteum; 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 μM) or KU-0063794 (30 μM) for 3h. After harvesting, total RNA was isolated from each cell line using PureLink™ RNA Mini Kit (Life Technologies, USA) according to the manufacturer's recommendation, and reverse-transcribed into cDNA using the PrimeScript™ 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 μM) 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 μM: 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

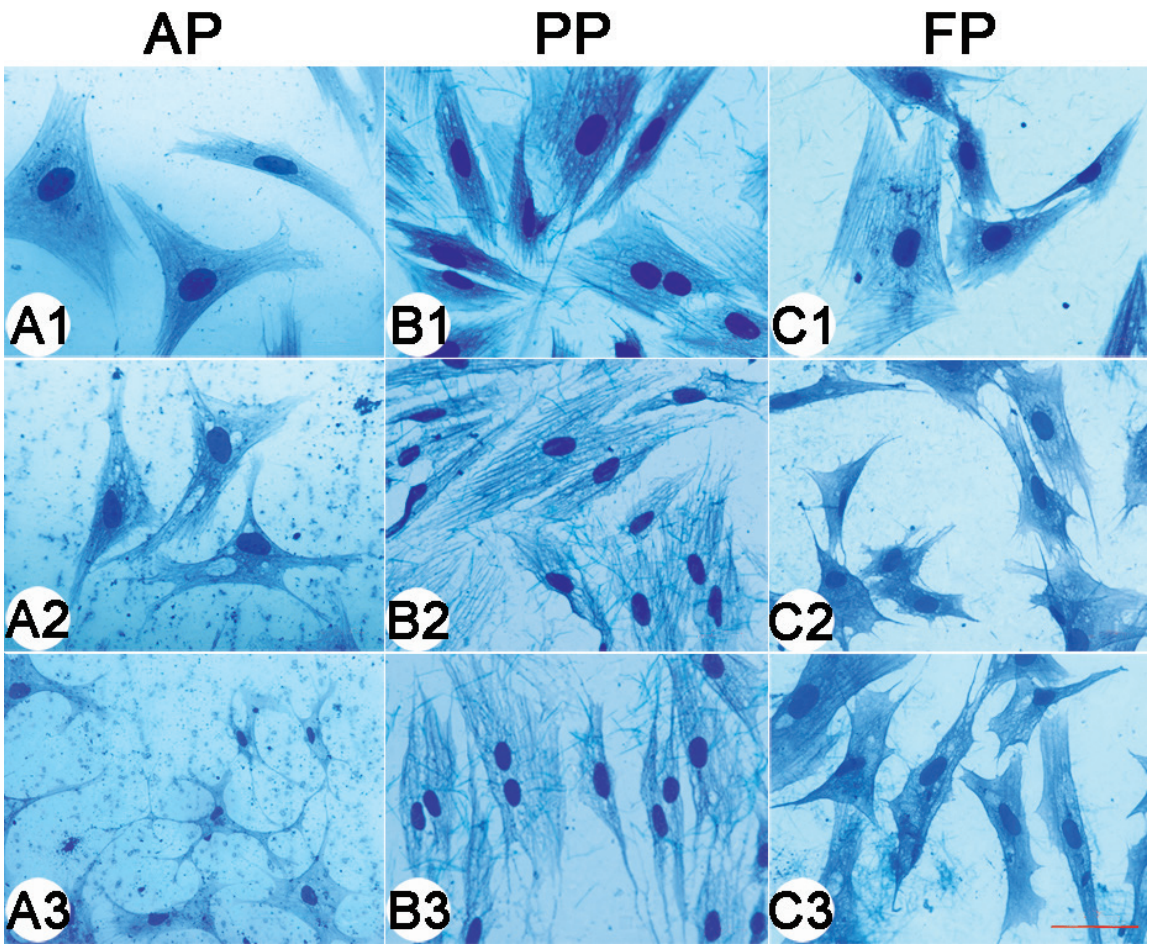

Figure 2. Effects of LY294002 on cytoskeleton of the AP, PP and FP cells. Coomassie brilliant blue staining. A1-A3 Cytoskeleton of the AP cells cultured in the control medium or the control medium containing LY294002 (35 μM or 50 μM). B1-B3 Cytoskeleton of the PP cells cultured in the control medium or the control medium containing LY294002 (35 μM or 50 μM). C1-C3 Cytoskeleton of the FP cells cultured in the control medium or the control medium containing LY294002 (35 μM or 50 μM). Bar= 400 μm. 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 ± 2.36	1.69 ± 0.35	3.36 ± 0.41
	LY294002 (50 μM)	96.8 ± 3.17	2.54 ± 0.48	0.66 ± 0.15
PP cells	Untreated	96.82 ± 1.83	1.2 ± 0.31	1.98 ± 0.42
	LY294002 (50 μM)	95.97 ± 3.01	1.49 ± 0.27	2.54 ± 0.58
SP2/0 cells	Untreated	27.63 ± 4.75	61.63 ± 5.09	10.74 ± 2.59
	LY294002 (50 μM)	72.44 ± 3.86	27.50 ± 3.23	0.06 ± 0.04

Data are presented as mean ± 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

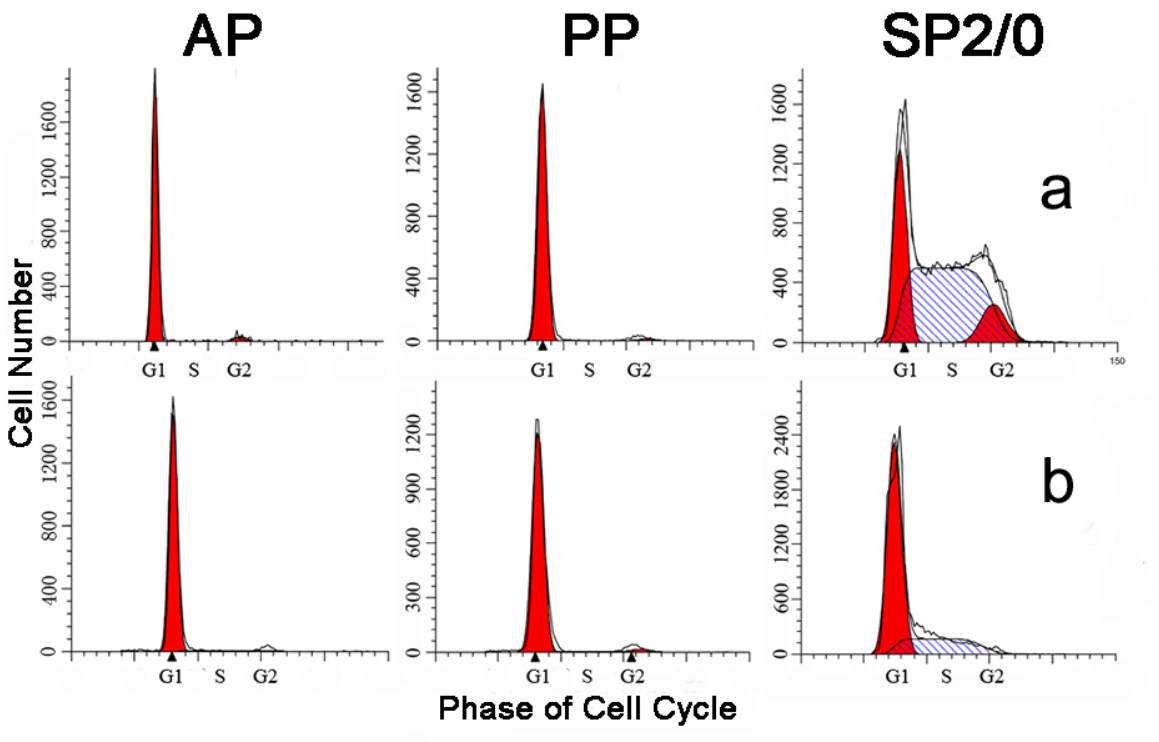

Figure 3. Effects of LY294002on cell-cycle distribution of the AP, PP and SP2/0 cells. Data were analyzed using ModFit LT Software to estimate the percentages of cells at each phase of G0/G1, S and G2/M. **a** The AP, PP and SP2/0 cells were cultured in the control medium. **b** The AP, PP and SP2/0 cells were cultured in the control medium containing LY294002 (50 μM).

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±0.0135	0.2747± 0.0292	0.6035± 0.0181	0.2508± 0.0096
LY (5×10^{-5} M)	0.3638 ± 0.0078**	0.3689± 0.0135**	0.6186± 0.0029	0.1214± 0.0106**
Insulin (10^{-6} M)	0.4671 ± 0.0174**	0.4730 ± 0.0125**	0.9392 ± 0.0364**	0.3560 ± 0.0045**

Data are presented as mean ± 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 μM LY294002 (Figure 4C1), or the control medium containing 30 μM 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

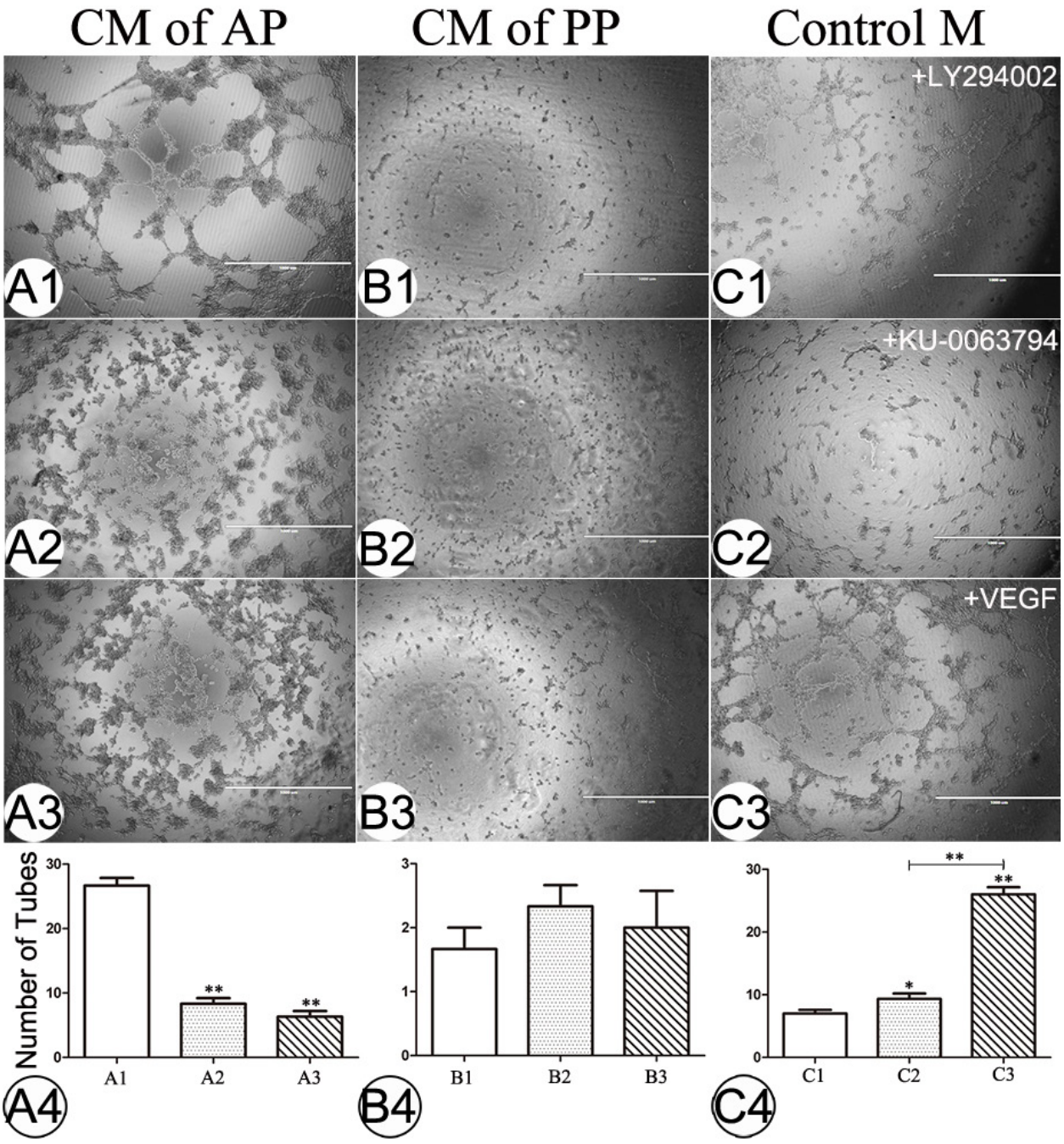

Figure 4. Effects of the AP or PP conditioned medium on tube formation with or without LY294002 or KU-0063794. A1-A3 HUVECs were cultured in the CM of the AP cells cultured in NC, T1 or T2. B1-B3 HUVECs were cultured in the CM of the PP cells cultured in NC, T1 or T2. C1-C3 HUVECs were cultured in the 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 μm.

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%↓).

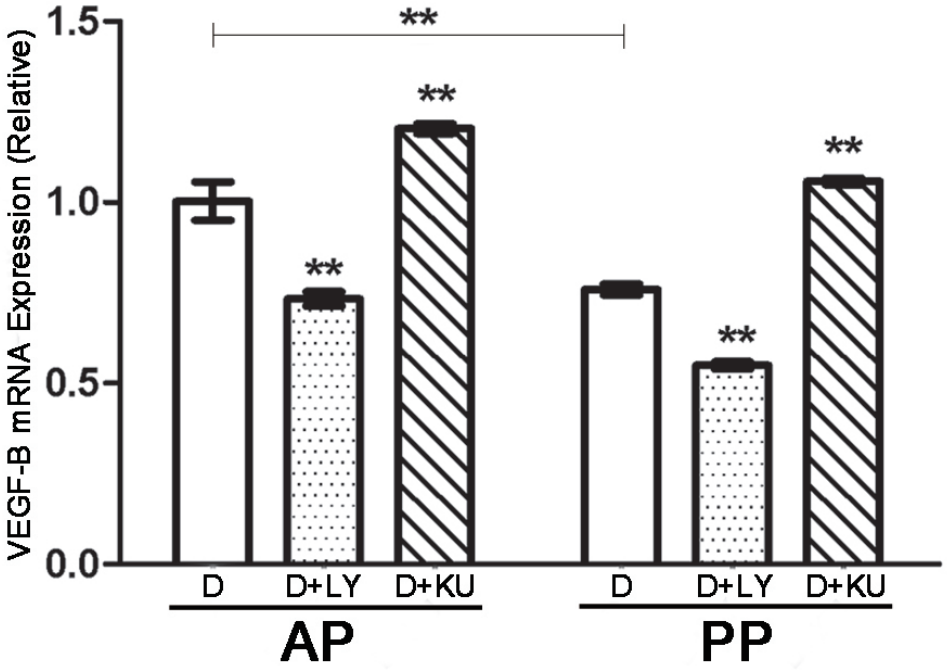

Figure 5. Effects of LY294002 on expression of VEGF-B mRNA in the AP or PP cells. LY294002 significantly decreased (p<0.01) and KU-0063974 significantly increased (p<0.01) the expression level of VEGF-B mRNA in both the AP and the PP cells, whereas in the control medium, the relative expression level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

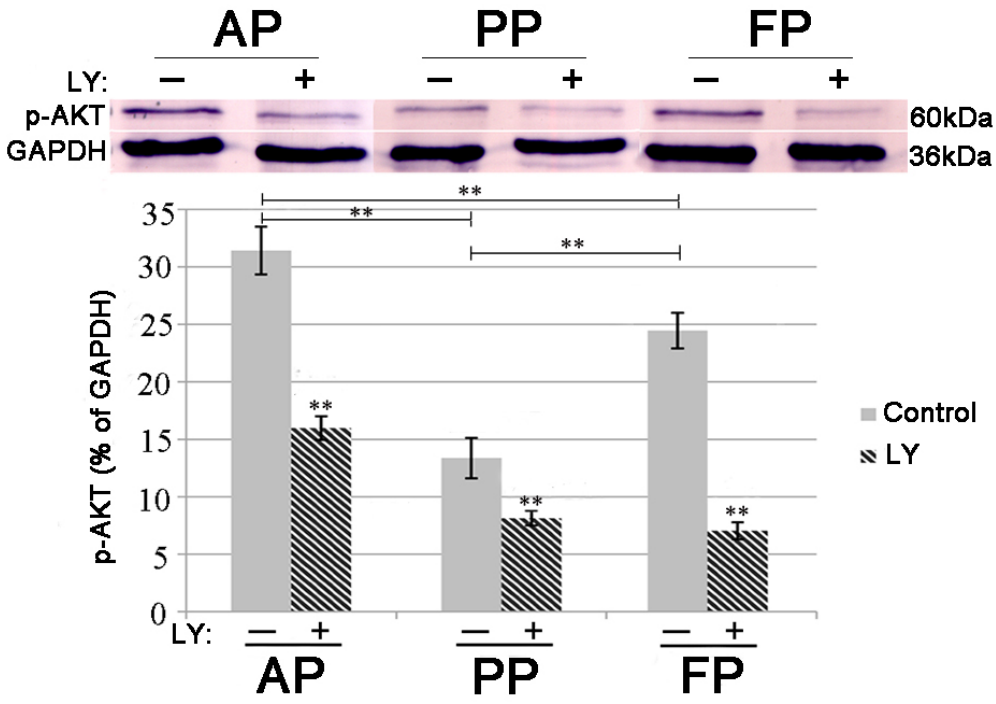

Figure 6. Effects of LY294002 on the expression of p-AKT protein in the AP, PP or FP cells. Addition of LY294002 in the control medium significantly decreased the expression level of p-AKT in the AP, PP or FP cells (p<0.01). In the control medium only, the expression level of p-AKT in the AP cells was 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 ± 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-1α, NOS, mTOR/p70S6K1, FOXO and GSK-3β (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 thePI3K/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 fromthe Deer Antler Research Group, Institute of Special Wild Economic Animals and Plants, ChineseAcademy 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).

7. REFERENCES

1. Stocum DL. Developmental mechanisms of regeneration. *Foundations of Regenerative Medicine: Clinical and Therapeutic Applications* 100 (2009). DOI: 10.1016/B978-012369410-2.50008-5

2. Li C, Yang F, and Sheppard, A. Adult stem cells and mammalian epimorphic regeneration-insights from studying annual renewal of deer antlers. *Curr Stem Cell Res Ther* 4, 237-251 (2009). DOI: 10.2174/157488809789057446

3. Li C, Suttie JM, and Clark DE. Histological examination of antler regeneration in red deer (Cervus elaphus). *Anat Rec A Discov Mol Cell Evol Biol* 282, 163-174 (2005). DOI: 10.1002/ar.a.20148

4. Li C, Littlejohn R, Corson I, and Suttie J. Effects of testosterone on pedicle formation and its transformation to antler in castrated male, freemartin and normal female red deer (Cervus elaphus). *Gen Comp Endocrinol* 131, 21-31 (2003). DOI: 10.1016/S0016-6480(02)00625-1

5. Kierdorf U, Stoffels E, Stoffels D, Kierdorf H, Szuwart T, and Clemen G. Histological studies of bone formation during pedicle restoration and early antler regeneration in roe deer and fallow deer. *Anat Rec A Discov Mol Cell Evol Biol* 273, 741-751 (2003). DOI: 10.1002/ar.a.10082

6. Li C, Mackintosh CG, Martin SK, and Clark DE. Identification of key tissue type for antler regeneration through pedicle periosteum deletion. *Cell Tissue Res* 328, 65-75 (2007). DOI: 10.1007/s00441-006-0333-y

7. Kierdorf H, Kierdorf U. State of determination of the antlerogenic tissues with special reference to double-head formation. *In: Brown RD (ed) The biology of deer*. Springer, New York, 525-531 (1992). DOI: 10.1007/978-1-4612-2782-3_123

8. Li C, and Suttie JM. Deer antlerogenic periosteum: a piece of postnatally retained embryonic tissue. *Anat Embryol* 204, 375-388 (2001). DOI: 10.1007/s004290100204

9. Li C, and Suttie JM. Light microscopic studies of pedicle and early first antler development in red deer (Cervus elaphus). *ANAT REC* 239, 198-215 (1994). DOI: 10.1002/ar.1092390211

10. Rolf HJ, Kierdorf U, Kierdorf H, Schulz J, Seymour N, Schliephake H, Napp J, Niebert S, Wölfel H, and Wiese KG. Localization and characterization of STRO-1 cells in the deer pedicle and regenerating antler. *PLoS One* 3, e2064 (2008). DOI: 10.1371/journal.pone.0002064

11. Mount J, Muzylak M, Allen S, Okushima S, Althnaian T, McGonnell I, and Price J. Antlers may regenerate from persistent neural crestlike stem cells, Advances in deer biology. *Proc 6th Int Deer Biol Congr, Prague: Research Institute of Animal Production* 161 (2006).

12. Li C, and Suttie JM. Electron microscopic studies of antlerogenic cells from five developmental stages during pedicle and early antler formation in red deer (Cervus elaphus). *ANAT REC* 252, 587-599 (1998). DOI: 10.1002/(SICI)1097-0185(199812)252:4%3C587::AID-AR9%3E3.0.CO;2-I

13. Hartwig H, Schrudde J. Experimentelle Untersuchungen zur Bildung der primären Stirnauswüchse beim Reh (Capreolus capreolus L.). *Z Jagdwiss* 20, 1-13 (1974).

14. Goss RJ, and Powel RS. Induction of deer antlers by transplanted periosteum I. Graft size and shape. *J Exp Zool A Ecol Integr Physiol* 235, 359-373 (1985). DOI: 10.1002/jez.1402350307

15. Li C. Exploration of the mechanism underlying neogenesis and regeneration of postnatal mammalian skin—deer antler velvet. *Int J Med Biol Front* 16, 1-19 (2010).

16. Li C, Clark DE, Lord EA, Stanton JAL, and Suttie JM. Sampling technique to discriminate the different tissue layers of growing antler tips for gene discovery. *Anat Rec (Hoboken)* 125-130 (2002). DOI: 10.1002/ar.10120

17. Zhao Y, Yao B, Zhang M, Wang S, Zhang H, and Xiao W. Comparative analysis of differentially expressed genes in Sika deer antler at different stages. *Mol Biol Rep* 40, 1665-1676 (2013). DOI: 10.1007/s11033-012-2216-5

18. Yao B, Zhao Y, Wang Q, Zhang M, Liu M, Liu H, and Li J. De novo characterization of the antler tip of Chinese Sika deer transcriptome and analysis of gene expression related to rapid growth. *Mol Cell Biochem* 364, 93-100 (2012). DOI: 10.1007/s11010-011-1209-3

19. Li C, Harper A, Puddick J, Wang W, and McMahon, C. Proteomes and signalling pathways of antler stem cells. *PLoS One* 7, e30026 (2012). DOI: 10.1371/journal.pone.0030026

20. Bader AG, Kang S, Zhao L, and Vogt PK. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 5, 921-929 (2005). DOI: 10.1038/nrc1753

21. Chen J. The Src/PI3K/Akt signal pathway may play a key role in decreased drug efficacy in obesity - associated cancer. *J Cell Biochem* 110, 279-280 (2010).

22. Johnson SM, Gulhati P, Rampy BA, Han Y, Rychahou PG, Doan HQ, Weiss HL, and Evers BM. Novel expression patterns of PI3K/Akt/mTOR signaling pathway components in colorectal cancer. *J Am Coll Surg* 210, 767-776 (2010). DOI: 10.1016/j.jamcollsurg.2009.12.008

23. Wickenden JA, and Watson CJ. Key signalling nodes in mammary gland development and cancer. Signalling downstream of PI3 kinase in mammary epithelium: a play in 3 Akts. *Breast Cancer Res* 12, 202 (2010). DOI: 10.1186/bcr2558

24. Welham M, Storm M, Kingham E, and Bone H. Phosphoinositide 3-kinases and regulation of embryonic stem cell fate. *Biochem Soc Trans* 35, 225-228 (2007). DOI: 10.1042/BST0350225

25. Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 296, 1655-1657 (2002). DOI: 10.1126/science.296.5573.1655

26. Chen J, Crawford R, Chen C, and Xiao Y. The key regulatory roles of the PI3K/Akt signaling pathway in the functionalities of mesenchymal stem cells and applications in tissue regeneration. *Tissue Eng Part B Rev* 19, 516-528 (2013). DOI: 10.1089/ten.teb.2012.0672

27. Karar J, and Maity A. PI3K/AKT/mTOR pathway in angiogenesis. *Front Mol Neurosci* 4 (2011). DOI: 10.3389/fnmol.2011.00051

28. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, and Semenza GL. Modulation of hypoxia-inducible factor 1α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* 60, 1541-1545 (2000).

29. Yamazaki Y, and Morita T. Molecular and functional diversity of vascular endothelial growth factors. *Mol Divers* 10, 515-527 (2006). DOI: 10.1007/s11030-006-9027-3

30. Ferrara N, and Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161, 851-858 (1989). DOI: 10.1016/0006-291X(89)92678-8

31. Lohela M, Bry M, Tammela T, and Alitalo K. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Curr Opin Cell Biol* 21, 154-165 (2009). DOI: 10.1016/j.ceb.2008.12.012

32. Alitalo K, Tammela T, and Petrova TV. Lymphangiogenesis in development and human disease. *Nature* 438, 946-953 (2005). DOI: 10.1038/nature04480

33. Iyer S, and Acharya KR. Role of placenta growth factor in cardiovascular health. *Trends Cardiovasc Med* 12, 128-134 (2002). DOI: 10.1016/S1050-1738(01)00164-5

34. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, and De Klerck B. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 8, 831-840 (2002). DOI: 10.1038/nm731

35. Mould AW, Greco SA, Cahill MM, Tonks ID, Bellomo D, Patterson C, Zournazi A, Nash A, Scotney P, and Hayward NK. Transgenic overexpression of vascular endothelial growth factor-B isoforms by endothelial cells potentiates postnatal vessel growth in vivo and in vitro. *Circ Res* 97, e60-e70 (2005). DOI: 10.1161/01.RES.0000182631.33638.77

36. Wright CE. Effects of vascular endothelial growth factor (VEGF) A and VEGFB gene transfer on vascular reserve in a conscious rabbit hindlimb ischaemia model. *Clin Exp Pharmacol Physiol* 29, 1035-1039 (2002). DOI: 10.1046/j.1440-1681.2002.03773.x

37. Silvestre JS, Tamarat R, Ebrahimian TG, Le-Roux A, Clergue M, Emmanuel F, Duriez M, Schwartz B, Branellec D, and Lévy BI. Vascular endothelial growth factor-B promotes in vivo angiogenesis. *Circ Res* 93, 114-123 (2003). DOI: 10.1161/01.RES.0000081594.21764.44

38. Bhardwaj S, Roy H, Gruchala M, Viita H, Kholova I, Kokina I, Achen MG, Stacker SA, Hedman M, and Alitalo K. Angiogenic responses of vascular endothelial growth factors in periadventitial tissue. *Hum Gene Ther* 14, 1451-1462 (2003). DOI: 10.1089/104303403769211664

39. Reichelt M, Shi S, Hayes M, Kay G, Batch J, Gole GA, and Browning J. Vascular endothelial growth factor - B and retinal vascular development in the mouse. *Clin Exp Ophthalmol* 31, 61-65 (2003).

40. Aase K, von Euler G, Li X, Pontén A, Thorén P, Cao R, Cao Y, Olofsson B, Gebre-Medhin S, and Pekny M. Vascular endothelial growth factor-B–deficient mice display an atrial conduction defect. *Circulation* 104, 358-364 (2001).

41. Li C, and Suttie J. Tissue collection methods for antler research. *Eur J Morphol* 41, 23-30 (2003). DOI: 10.1076/ejom.41.1.23.28106

42. Sun H, Yang F, Chu W, Zhao H, McMahon C, and Li C. Lentiviral-mediated rnai knockdown of cbfa1 gene inhibits endochondral ossification of antler stem cells in micromass culture. *PLoS One* 7, e47367 (2012). DOI: 10.1371/journal.pone.0047367

43. Zhang M, Wang J, and Chen Y. Effects of mechanical pressure on intracellular calcium release channel and cytoskeletal structure in rabbit mandibular condylar chondrocytes. *Life sciences* 78, 2480-2487 (2006). DOI: 10.1016/j.lfs.2005.10.043

44. Malinda KM, Nomizu M, Chung M, Delgado M, Kuratomi Y, Yamada Y, Kleinman HK, and Ponce ML. Identification of laminin α1 and β1 chain peptides active for endothelial cell adhesion, tube formation, and aortic sprouting. *FASEB J* 13, 53-62 (1999).

45. Shimizu Y, and Hont SW. Regulating integrin-mediated adhesion: one more function for PI 3-kinase?. *Immunol Today* 17, 565-573 (1996). DOI: 10.1016/S0167-5699(96)10061-X

46. Guilherme A, and Czech MP. Stimulation of IRS-1-associated phosphatidylinositol 3-kinase and Akt/protein kinase B but not glucose transport by β1-integrin signaling in rat adipocytes. *J Biol Chem* 273, 33119-33122 (1998). DOI: 10.1074/jbc.273.50.33119

47. Li C, Yang F, Haines S, Zhao H, Wang W, Xing X, Sun H, Chu W, Lu X, and Liu L. Stem cells responsible for deer antler regeneration are unable to recapitulate the process of first antler development—revealed through intradermal and subcutaneous tissue transplantation. *J Exp Zool B Mol Dev Evol* 314, 552-570 (2010).

48. Zhao G, Cai C, Yang T, Qiu X, Liao B, Li W, Ji Z, Zhao J, Zhao H, and Guo M. MicroRNA-221 induces cell survival and cisplatin resistance through PI3K/Akt pathway in human osteosarcoma. *PLoS One* 8, e53906 (2013). DOI: 10.1371/journal.pone.0053906

49. Chen G, and Khalil N. TGF-β1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir Res* 7, 2 (2006). DOI: 10.1186/1465-9921-7-2

50. Fujihara M, Comizzoli P, Keefer CL, Wildt, D. E., and Songsasen, N. Epidermal growth factor (EGF) sustains in vitro primordial follicle viability by enhancing stromal cell proliferation via MAPK and PI3K pathways in the prepubertal, but not adult, cat ovary. *Biol Reprod* 90, 86 (2014). DOI: 10.1095/biolreprod.113.115089

51. Fletcher DA, and Mullins RD. Cell mechanics and the cytoskeleton. *Nature* 463, 485-492 (2010). DOI: 10.1038/nature08908

52. Pollard TD, and Cooper JA. Actin, a central player in cell shape and movement. *Science* 326, 1208-1212 (2009). DOI: 10.1126/science.1175862

53. Janmey PA. The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiol Rev* 78, 763-781 (1998).

54. Theisen CS, Wahl JK, Johnson KR, and Wheelock MJ. NHERF links the N-cadherin/catenin complex to the platelet-derived growth factor receptor to modulate the actin cytoskeleton and regulate cell motility. *Mol Biol Cell* 18, 1220-1232 (2007). DOI: 10.1091/mbc.E06-10-0960

55. Wheeler D, Sneddon WB, Wang B, Friedman PA, and Romero G. NHERF-1 and the cytoskeleton regulate the traffic and membrane dynamics of G protein-coupled receptors. *J Biol Chem* 282, 25076-25087 (2007).
DOI: 10.1074/jbc.M701544200

56. Heuser JE, and Kirschner M. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J Cell Biol* 86, 212-234 (1980).
DOI: 10.1083/jcb.86.1.212

57. Metzner B, Barbisch M, Bachmann F, Czech W, and Norgauer J. Evidence of the Involvement of Phosphatidylinositol 3-Kinase in the Migration, Actin Stress Fiber Formation, and &agr; vβ3-Integrin-Mediated Adherence of Human Melanoma Cells. *J Invest Dermatol* 107, 597-602 (1996).

58. Jiménez C, Portela RA, Mellado M, Rodríguez-Frade JM, Collard J, Serrano A, Martínez-a C, Avila J, and Carrera AC. Role of the PI3K regulatory subunit in the control of actin organization and cell migration. *J Cell Biol* 151, 249-262 (2000).

59. Li C. Development of deer antler model for biomedical research. *Recent Adv Res Updat* 4, 255-274 (2003).

60. Hartwell LH, and Kastan MB. Cell cycle control and cancer. *Science* 266, 1821-1828 (1994).
DOI: 10.1126/science.7997877

61. Guo Q, Wang D, Liu Z, and Li C. Effects of p21 Gene Down-Regulation through RNAi on Antler Stem Cells In vitro. *PLoS One* 10, e0134268 (2015).
DOI: 10.1371/journal.pone.0134268

62. Goss R. Control of deer antler cycles by the photoperiod, Antler Development in Cervidae. *Antler Dev Cervidae Proc Int Symp Caesar Kleberg Wildl Res Inst 1st* 1-14 (1983).

63. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84, 345-357 (1996).
DOI: 10.1016/S0092-8674(00)81279-9

64. Li L, Xia Y, Wang Z, Cao X, Da Z, Guo G, Qian J, Liu X, Fan Y, and Sun L. Suppression of the PI3K—Akt pathway is involved in the decreased adhesion and migration of bone marrow - derived mesenchymal stem cells from non - obese diabetic mice. *Cell Biol Int* 35, 961-966 (2011).

65. Murillo CA, Rychahou PG, and Evers BM. Inhibition of α5 integrin decreases PI3K activation and cell adhesion of human colon cancers. *Surgery* 136, 143-149 (2004).
DOI: 10.1016/j.surg.2004.04.006

66. Goss RJ. SECTION III BASIC SCIENCES AND PATHOLOGY 24 Problems of Antlerogenesis. *Clin Orthop Relat Res* 69, 227-238 (1970).
DOI: 10.1097/00003086-197003000-00025

67. Kume T. Specification of arterial, venous, and lymphatic endothelial cells during embryonic development. *Histol Histopathol* 25, 637 (2010).

68. Hicklin DJ, and Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *JCO* 23, 1011-1027 (2005).
DOI: 10.1200/JCO.2005.06.081

69. Byrne AM, Bouchier-Hayes D, and Harmey J. Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). *J Cell Mol Med* 9, 777 (2005).
DOI: 10.1111/j.1582-4934.2005.tb00379.x

70. Clark DE, Li C, Wang W, Martin SK, and Suttie JM. Vascular localization and proliferation in the growing tip of the deer antler. *Anat Rec A Discov Mol Cell Evol Biol* 288, 973-981 (2006).
DOI: 10.1002/ar.a.20364

71. Lord E, Clark D, Martin S, Pedersen G, Gray J, Li C, and Suttie J. Profiling genes expressed in the regenerating tip of red deer (Cervus elaphus) antler. *In Antler Science and Product Technology* (2004).

72. Okumura N, Yoshida H, Kitagishi Y, Murakami M, Nishimura Y, and Matsuda S. PI3K/AKT/PTEN signaling as a molecular target in leukemia angiogenesis. *Adv Hematol* 2012 (2012).
DOI: 10.1155/2012/843085

73. Gómez-Raposo C, Mendiola M, Barriuso J, Casado E, Hardisson D, and Redondo A. Angiogenesis and ovarian cancer. *Clin Transl Oncol* 11, 564-571 (2009).

74. Wang H, Duan L, Zou Z, Li H, Yuan S, Chen X, Zhang Y, Li X, Sun H, and Zha H. Activation

of the PI3K/Akt/mTOR/p70S6K pathway is involved in S100A4-induced viability and migration in colorectal cancer cells. *Int J Med Sci* 11, 841 (2014).
DOI: 10.7150/ijms.8128

75. Mount J, Muzylak M, Allen S, Althnaian T, McGonnell I, and Price J. Evidence that the canonical Wnt signalling pathway regulates deer antler regeneration. *Dev Dyn* 235, 1390-1399 (2006).
DOI: 10.1002/dvdy.20742

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

Send correspondence to: Chunyi Li, State Key Lab for Molecular Biology of Special Economic Animals, 4899 Juye Street, Changchun City, Jilin, China, Tel: 0431-81919500, Fax: 0431-81919800, E-mail: Chunyi.li@agresearch.co.nz