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Expression and Functional Analysis of Tumor-Related Factor S100A4 in Antler Stem Cells

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Summary

Annual antler renewal is a stem cell–based epimorphic process driven by antler stem cells (ASCs) resident in antlerogenic periosteum (AP). Antlerogenic periosteal cells express a high level of S100A4, a metastasis-associated protein, which intrigued us to explore what role S100A4 could play in antler regeneration. The present study set out to investigate expression and effects of S100A4 in the ASCs and their progeny. The results showed that not only did cells from the AP express a high level of S100A4, but also the pedicle periosteum and the antler growth center. In the antler growth center, we found S100A4-positive cells were specifically located in blood vessel walls and in vascularized areas. In vitro, recombinant deer S100A4 protein stimulated the proliferation of the AP cells, promoted proliferation, migration and tube formation of human vascular endothelial cells, and enhanced migration of Hela cells, but not AP cells. These findings demonstrated that S100A4 in the ASCs may play a significant role in stimulating angiogenesis, proliferation, but not motility, of ASCs. Deer antlers offer a unique model to explore how rapid cell proliferation with a high level of S100A4 expression is elegantly regulated without becoming cancerous. **(J Histochem Cytochem XX:XXX–XXX, XXXX)**

Keywords

angiogenesis, cell migration, cell proliferation, deer antler, regeneration

Introduction

The S100 protein family, one of the largest subfamilies of calcium-binding proteins, plays crucial roles in a broad range of intracellular and extracellular functions, including regulation of cell cycle, cell growth, differentiation, motility, and invasion, thus shows a strong association with tumorogenesis.[1,2] S100A4, one of the members of the S100 protein family, is an 11-kD protein and was originally isolated from metastatic tumor cells by Ebralidze et al.[3] They found in spontaneous mouse mammary carcinoma cell lines that S100A4 was highly expressed in late stage cells (100% metastasis), intermediately expressed in intermediate stage cells (50% metastasis), and undetectable in early neoplastic stage cells (low or no metastasis). Based on the phenomenon of low-to-high transition during development of metastasis, the authors concluded that the level of S100A4 expression is closely associated with the progression of cancer metastasis, and thus termed S100A4 metastasin.

Loss- and gain-of-function analysis provided further evidence for the role of S100A4 in metastatic progression of tumors. The transfection of non-metastatic tumor

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cell lines with S100A4 endows them with metastatic properties whereas antisense- and ribozyme-mediated S100A4 inactivation in metastatic tumor cells abolishes their metastatic behavior.[4–6] Therefore, S100A4 has become a promising target for therapeutic applications by blocking tumor progression.[7] Despite this significance, the mechanism underlying the effects of S100A4 on metastasis is still not completely understood.

Unexpectedly, Li et al.[8] found that S100A4 protein was highly expressed in the antlerogenic periosteum (AP), a tissue from which deer pedicle and initial antler develop. However, S100A4 was almost undetectable in the pedicle periosteum (PP), a tissue that is directly differentiated from the AP and gives rise to subsequently regenerating antlers; undetectable in the reserve mesenchyme (RM), a tissue that is the derivative of the PP and is located in the center of antler growth; and undetectable in the facial periosteum (FP), a tissue that is nearby the AP but does not have capacity to regenerate. As cells of the AP, PP, and RM regions have attributes of stem cells, they are named antler stem cells (ASCs).[9,10] Based on the report by Li et al.,[8] the expression level of S100A4 in the ASCs during course of antler development has experienced a high-to-low transition, which is in sharp contrast to the situation of neoplastic transformation. Therefore, unveiling of the mechanism underlying this high-to-low transition in S100A4 expression during antler development may help to further dissect the role played by the molecule in cancer progression.

The aim of this study was 3-fold: (1) to take different approaches, including quantitative polymerase chain reaction (qPCR), western blot, immunofluorescent staining, and immunohistochemistry (IHC), to confirm the findings (high to low in S100A4 expression) made by Li et al.[8]; (2) to confirm whether recombinant deer S100A4 protein (D-S100A4) has similar effects reported previously with the ones from other species on cell proliferation, migration, and angiogenesis in vitro[11]; and (3) to explore the possible role played by S100A4 in the development of deer antlers, the only mammalian organs that once lost can fully regenerate.

Materials and Methods

Tissues Sampling and Cell Culture

The tissues for antlerogenic periosteal cells (APCs), pedicle periosteal cells (PPCs), antler tip reserve mesenchymal cells (RMCs), and facial periosteal cells (FPCs) were obtained from slaughtered male sika deer,[12] and the corresponding cell lines were created according to the previously reported methodology.[13] The deer tissues were sampled for primary culture or fixed for histology. Antlers of three sika deer were collected at their 30 days of growth and their tips (top 3 cm) were then removed, fixed, and embedded in paraffin wax. Tissue collection from sika deer was approved by CAAS animal ethics committee (CAAS201523). All of the above cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; Grand Island, USA), supplemented with 10% fetal bovine serum (FBS), 100 mg/ml of streptomycin, and 100 units/ml of penicillin, and grown in a humidified atmosphere with 5% CO_2 at 37C. Human vascular endothelial cells (HUVECs) were purchased from KeyGEN (KeyGEN; Nanking, China) and maintained in M199 (Sigma-Aldrich; Switzerland) supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin.

Lentiviral-Mediated RNA Interference

APCs with S100A4 gene knockdown [APC–RNA interference (APC-RNAi)] were established by our laboratory using lentiviral-mediated RNAi way.[14] The method for RNAi is described elsewhere.[15] Briefly, shRNA sequences targeting the deer S100A4 gene were designed: sense, 5′-cgcgtccccGCAACCTGGACTGCAACAAttcaagagaTTGTTGCAGTCCAGGTTGCttttttggaaat-3′, and antisense, 5′-cgatttccaaaaaGCAACCTGGACTGCAACAAtctcttgaaTTGTTGCAGTCCAGGTTGCgggga-3′.

The synthesized oligonucleotides were subsequently annealed into double-stranded small hairpin RNAs and inserted into the site between Cla1 and Mlu1 of the pLVTHM plasmid. Recombinant pLVTHM, pCMV, and pMD2G were co-transfected into 293T cells using lipofectamine 2000 reagent (Invitrogen; Gaithersburg, USA). Virus-containing supernatants were collected and concentrated to infect cultured APCs. The detection of GFP expression was initiated 48 hr after infection to determine the levels of siRNA expression. The GFP expressing cells were isolated via flow cytometry (BD FACSAria; USA), and effects of RNAi on S100A4 expression were analyzed using western blot analysis.

Flow Cytometry

CD9 is a cell surface marker and expresses in ASCs.[9,16] The lineage fidelity of the established ASCs lines was monitored using marker CD9 via flow cytometry, which is described elsewhere.[17] Briefly, the primary cultured APCs, PPCs, and RMCs were incubated, respectively, with 5% goat serum to block nonspecific staining for 30 min, then with anti-CD9 antibody (LS-C46004; LifeSpan, USA) for 1 hr at 4C after wash with cold phosphate-buffered saline (PBS). Then the cells were stained with Cy3-conjugated secondary antibody (ab150077)

for another 1 hr at room temperature. Isotype-matched mouse IgG was used as a negative control. Flow cytometry analysis was performed using FACSCalibur (BD Biosciences; USA), and the results were analyzed with Cellquest software (BD Biosciences).

Western Blot Analysis

Total cellular proteins were extracted from the cultured APCs, PPCs, RMCs, and FPCs (passage number ≤5) using a lysis buffer (Beyotime; Jiangsu, China). Supernatant proteins were separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (50 µg/lane) and transferred to polyvinylidene difluoride membranes (Sigma; St. Louis, USA). The membranes were blocked with 5% (w/v) bovine serum albumin (BSA) and immunoblotted with the anti-S100A4 antibody (ab27957, 1:200) or anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (ab181602, 1:2000) followed by secondary antibodies (goat anti-rabbit; ab6721, 1:2000) or anti-mouse IgG (ab6789, 1:2000) conjugated with horseradish peroxidase (HRP). Bands were visualized using chemiluminescence detection reagents (Thermo Scientific; Rockford, USA) applied to autoradiograph films. The quantification of S100A4 intensity of western blot bands was carried out using ImageJ software (version 2.1)

Real-Time qPCR

Total RNA was extracted from the APCs, PPCs, RMCs, and FPCs (passage number ≤5) using a Trizol total RNA isolation kit (Takara; Dalian, China) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 µg of total RNA (DNase-treated) using a Primescript reverse transcription–PCR kit (Takara). qPCR for S100A4 (primer sequences: 5'-TGGCATATCCCCTGGAGAAA-3'and 5'-TTCCGGG GTTGC TTATCAG-3') was performed on ABI 7500 (Applied Biosystem; Singapore) using QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's protocol, and GAPDH (primer sequences: 5'-GTCCGTTGTGGATCTGACCTG-3'and 5'-AGAGT GAGTGTCGCTGTTGAAGT-3') was used as a reference gene. The relative expression level of S100A4 was analyzed using the $2^{-\Delta\Delta Ct}$ method against GAPDH for normalization.

Immunofluorescent Staining

Immunofluorescence was carried out as described elsewhere.[18] Briefly, 10,000 cells were seeded to each well of 24-well plates a day before. The adhered cells were fixed with 4% formaldehyde for 30 min and blocked for 45 to 60 min with PBS Tween-20/BSA. Cells were incubated with diluted anti-S100A4 antibody (1:100) for 1 hr at room temperature. The fluorescein conjugated secondary antibody (ab150077, 1:500) was subsequently applied after proper wash. The nuclei of cells were counterstained with 4′,6-diamidino-2-phenylindole solution for 5 min at room temperature, and then examined under a fluorescent microscope.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked using a solution of 3% H_2O_2. Antigen retrieval was performed through boiling in a 10 mM sodium citrate buffer (pH 6.0) for 20 min. The slides were blocked in PBS plus 10% normal goat serum for 30 min and then incubated with anti-S100A4 antibody (ab27957, 1:500) for 2 hr at 37C. For isotype control, the primary antibody was replaced by rabbit IgG (ab171870). After rinsing in PBS followed by incubation with goat anti-rabbit IgG conjugated with HRP (ab6721) for 30 min. After rinsing in PBS, antigen in the sections were visualized with the DAB chromogen reaction solution (Maxim; Fuzhou, China). The sections were then counterstained with hematoxylin. The numbers of positive cells were counted using ImageJ software.

Production of Recombinant Sika D-S100A4

D-S100A4 was expressed and purified by a member of our library.[19] The protein was expressed by *Escherichia coli* BL21 (DE3), and the expression was inducted with 0.3 mM IPTG. The recombinant GST-S100A4 protein was purified from the cell extract using glutathione agarose (Sigma) and cleaved with PreScission Protease (GE Healthcare; USA).

MTT Cell Proliferation Assay

HUVECs were seeded at a density of 5×10^3/well in a 96-well plate. Various concentrations of recombinant sika D-S100A4 (10, 100, 1000 ng/ml and 10 µg/ml) were added to different wells and made the final volume up to 200 µl. Vascular endothelial growth factor (VEGF) at a concentration of 20 ng/ml was served as a positive control. Each sample was tested in triplicates and incubated for pre-determined time periods (24, 48, 72, and 96 hr) in a 37C incubator supplemented with 5% CO_2. After incubation, 20 µl 3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) reagent (5 mg/ml; Sigma) was added to each well and incubated for further 2 hr until a purple precipitate was visible. The medium was then

carefully removed and 150 µl dimethyl sulfoxide was added. Plates were shaken in the dark for 10 min, and the OD value was read at 490 nm using an enzyme-linked immunosorbent assay reader (TECAN; Grodig, Austria).

Migration Assay

The migration assay was performed using Ibidi cell migration plates (IBIDI; InVitro Technologies, Munich, Germany), consisting of silicon-based cell culture inserts with two reservoirs. Cultured cells were digested with 0.25% trypsin and collected by centrifugation. Cells were diluted to 2×10^5/ml, and 70 µl of cell suspension was added to each reservoir. Once the cells reached confluence, the inserts were removed and the wells were washed twice with PBS and filled with 400 µl/well of DMEM without FBS. S100A4 (100 ng/ml) or VEGF (20 ng/ml) was added to different wells. Reaction was stopped 24 hr after incubation by removing the culture medium; the cells were washed with PBS and immediately fixed for 30 min in 10% formalin and then stained for 5 min with 0.5% crystal violet dye. Each well was gently washed under tap water to remove any excess stain. The results of migrations were observed under a microscope and recorded with a digital camera (AMG/EVOS; USA). The numbers of migrated cells were counted using ImageJ software.

Tube Formation

The tube formation assay was performed as previously described.[20] Briefly, Matrigel matrix with reduced growth factor was prepared according to manufacturer's advice (BD Biosciences); 1 volume of Matrigel was mixed with 3 volumes of DMEM on ice. Fifty µl of mixture was add to each well of 96-well plates and allowed to gel for 30 min at 37C. HUVECs (1.5×10^4 cells) were suspended in 0.2 ml of the culture medium (containing 100 ng S100A4 or 20 ng/ml VEGF or 100 ng S100A4 plus 20 ng/ml VEGF in DMEM) and seeded into each well. Tube formation was observed under an inverted microscope 6 hr later. At least five representative fields were viewed.

Statistical Analysis

Data were presented as the means ± SEMs of three or more independent experiments. Statistical analysis was performed with the *t*-test using SAS (Statistical Analysis System) version 9.0, and values at $p<0.05$ were considered to be significant.

Results

Expression of S100A4 at Both mRNA and Protein Levels in the Cultured ASCs

Lineage fidelity of the established ASC lines was monitored via the expression of marker gene CD9 (Fig. S1). The expression level of S100A4 mRNA in the ASCs (the FPCs as a control) was measured using qPCR. All types of ASCs (APCs, PPCs, and RMCs) expressed high levels of S100A4 mRNA (Fig. 1A). Compared with the control FPCs, expression levels of S100A4 in APCs and RMCs were significantly higher ($p<0.05$), but not so in the PPCs ($p=0.062$). Surprisingly, the results of western blot analysis showed that expression of S100A4 protein in all three types of ASCs (APCs, PPCs, and RMCs) was significantly higher than that of the FPCs ($p<0.05$; Fig. 1B).

To confirm this new finding that all three types of the ASCs expressed high levels of S100A4 protein, we carried out immunofluorescent staining using a specific anti-S100A4 antibody. Again all three types of ASCs were intensively stained, whereas specific staining for the FPCs was barely seen (Fig. 1C).

S100A4-Positive Cells in the AP, PP, and Antler Growth Center

To pinpoint cell types that express S100A4 in the ASC tissues, we localized the S100A4-positive cells in the AP, PP, and antler growth center by IHC. The majority of the cells (87 ± 9%) in the AP were specifically stained (Fig. 2A), to a lesser extent in the PP (65 ± 12%; Fig. 2B), and the least in the FP (36 ± 11%; Fig. 2C). In the antler growth center, the type and number of S100A4-positive cells varied considerably among the different zones of the center (Fig. 3). S100A4-positive cells were detected in the majority of the RM zone (Fig. 3D), all cells of the discrete vascular walls in the pre-cartilage zone (Fig. 3E), all cells of the vascular walls and the majority of chondrocytes in the transitional zone (Fig. 3F), and all cells of the vascular walls in the cartilage zone (Fig. 3G). Little or no detection of S100A4 staining was shown in the dermis (Fig. 3C). These results demonstrated that S100A4 is expressed not only in different types of the ASCs, but also in some of their progeny.

Angiogenic Activity of D-S100A4 In Vitro

The role of D-S100A4 in stimulating proliferation, migration, and tube formation of endothelial cells was determined using cell proliferation, migration, and tube

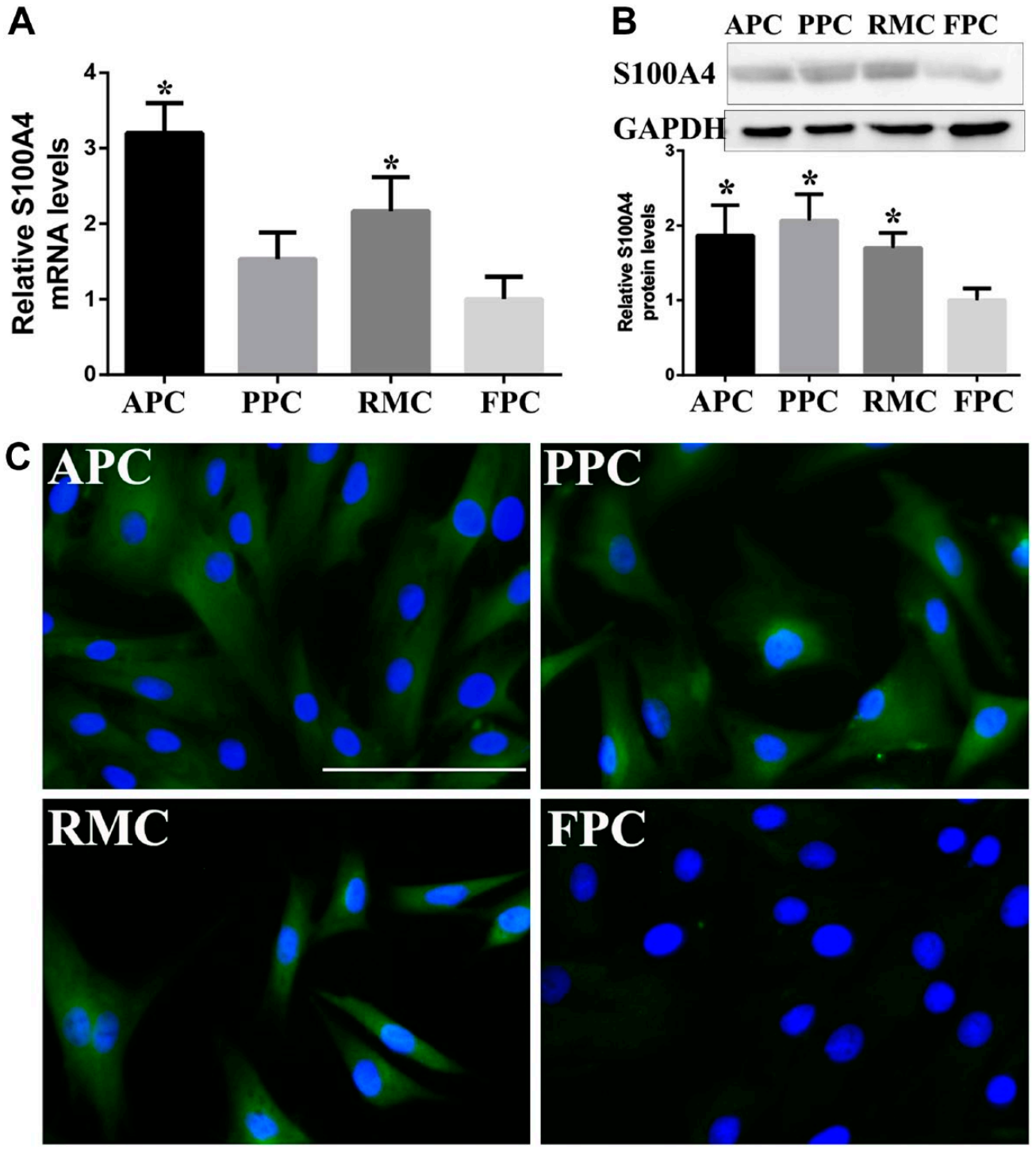

Figure 1. Expression of S100A4 at both mRNA and protein levels in the cultured ASCs. (A) mRNA expression, through qPCR, in the APCs and RMCs was significantly higher than the control FPCs. (B) Protein expression, through western blot analysis and normalized by GAPDH, was higher in all ASCs compared with the control FPCs. (C) Immunofluorescent staining of S100A4, showing a higher level of expression in the cultured ASCs than the control FPCs. Note that the FPCs were almost devoid of specific staining. The data, means ± SEMs; *$p<0.05$, significantly different from the FPCs. Bar = 100 µm. Abbreviations: ASC, antler stem cell; qPCR, quantitative PCR; APC, antlerogenic periosteal cell; RMC, reserve mesenchymal cell; FPC, facial periosteal cell; GAPDH, glyceraldehyde phosphate dehydrogenase; PPC, pedicle periosteal cell.

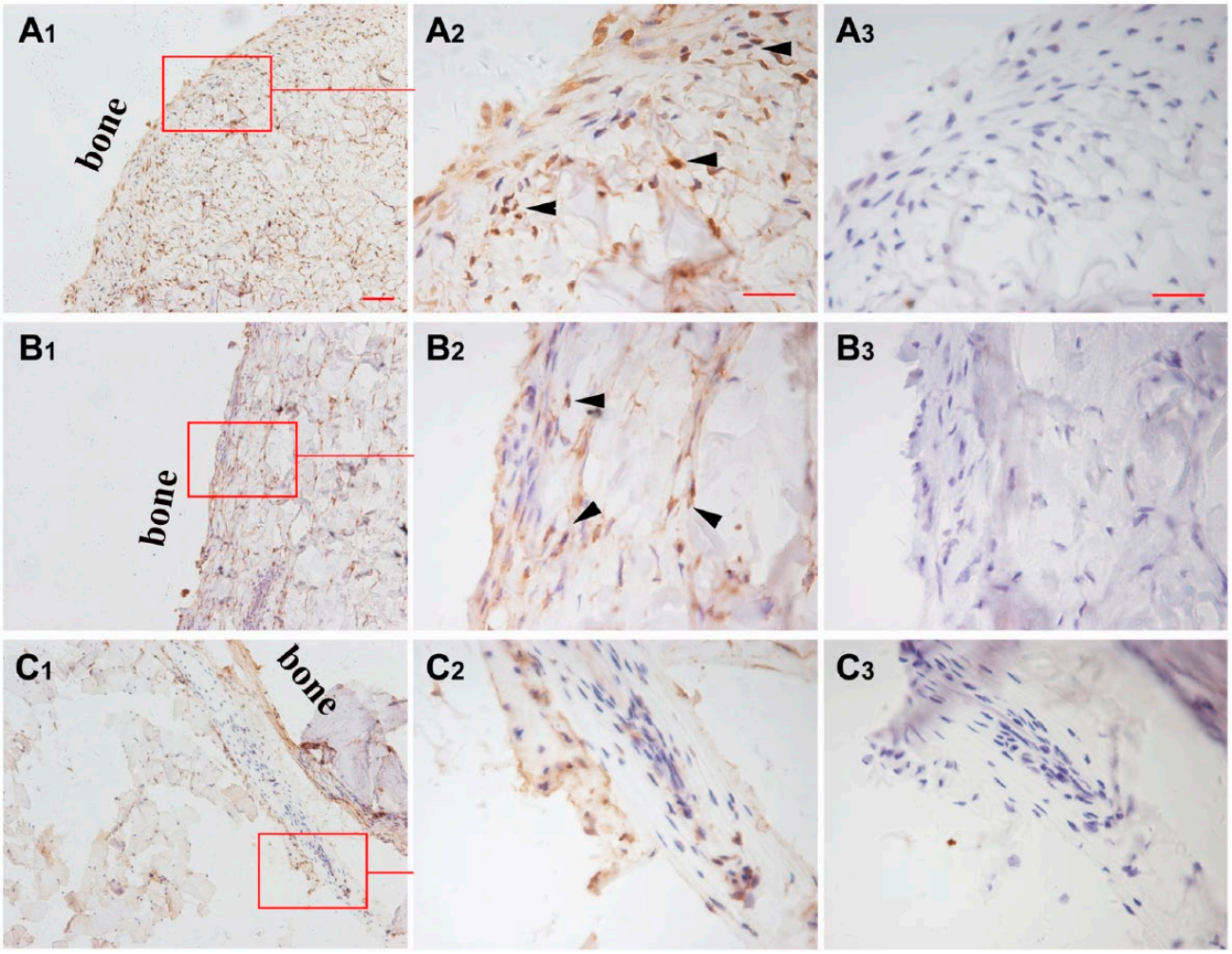

Figure 2. IHC of S100A4 in the antler stem cell tissues. (A1–A3) the AP tissue. Numerous S100A4-positive cells (brown cells; arrows) were mainly located in the bone side of the periosteum in A1 and A2, but not in the control A3. (B1–B3) the PP tissue. Relatively less number of cells that were S100A4-positive (brown cells; arrows) were localized compared with the AP tissue, but not in the control B3. (C1–C3) The FP tissue. S100A4-positive cells (brown cells) were hardly detectable in the FP tissue. Bar = 200 μm (A1), r100 μm (A2 and A3). Abbreviations: IHC, immunohistochemistry; AP, antlerogenic periosteum; PP, pedicle periosteum; FP, facial periosteum.

formation assays. Our results showed that both 100 and 1000 ng/ml D-S100A4 significantly increased the proliferation rate of HUVECs (Fig. 4A; $p<0.05$), demonstrating a similar effect as shown by S100A4 protein from other species.

The effect of D-S100A4 on the migration of HUVECs showed that 100 ng/ml D-S100A4 significantly stimulated the migration of HUVECs compared with the control (Fig. 4B and C; $p<0.05$). Interestingly, D-S100A4 at 100 ng/ml combined with VEGF at 20 ng/ml had an additive effect on the migration of HUVECs (Fig. 4C; $p<0.01$). Therefore, D-S100A4 may stimulate migration through a pathway alternative to VEGF.

In the presence of D-S100A4 (100 ng/ml), the tube-forming activity of HUVECs was highly significantly enhanced compared with the control (Fig. 4D and E; $p<0.01$). Likewise, treatment with VEGF also significantly increased the number of formed tubes by HUVECs. Consistent with the migration results, an additive effect on tube formation of HUVECs was also detected with the combined treatment of D-S100A4 (100 ng/ml) and VEGF (20 ng/ml; Fig. 4D and E). Overall, D-S100A4 has a strong angiogenic activity.

Effects of D-S100A4 on the Proliferation and Migration of the APCs

Effects of RNAi on S100A4 expression in the APC-RNAi cell line (established by our laboratory[14]) were analyzed using western blot analysis (Fig. S2). The results showed that D-S100A4 significantly increased

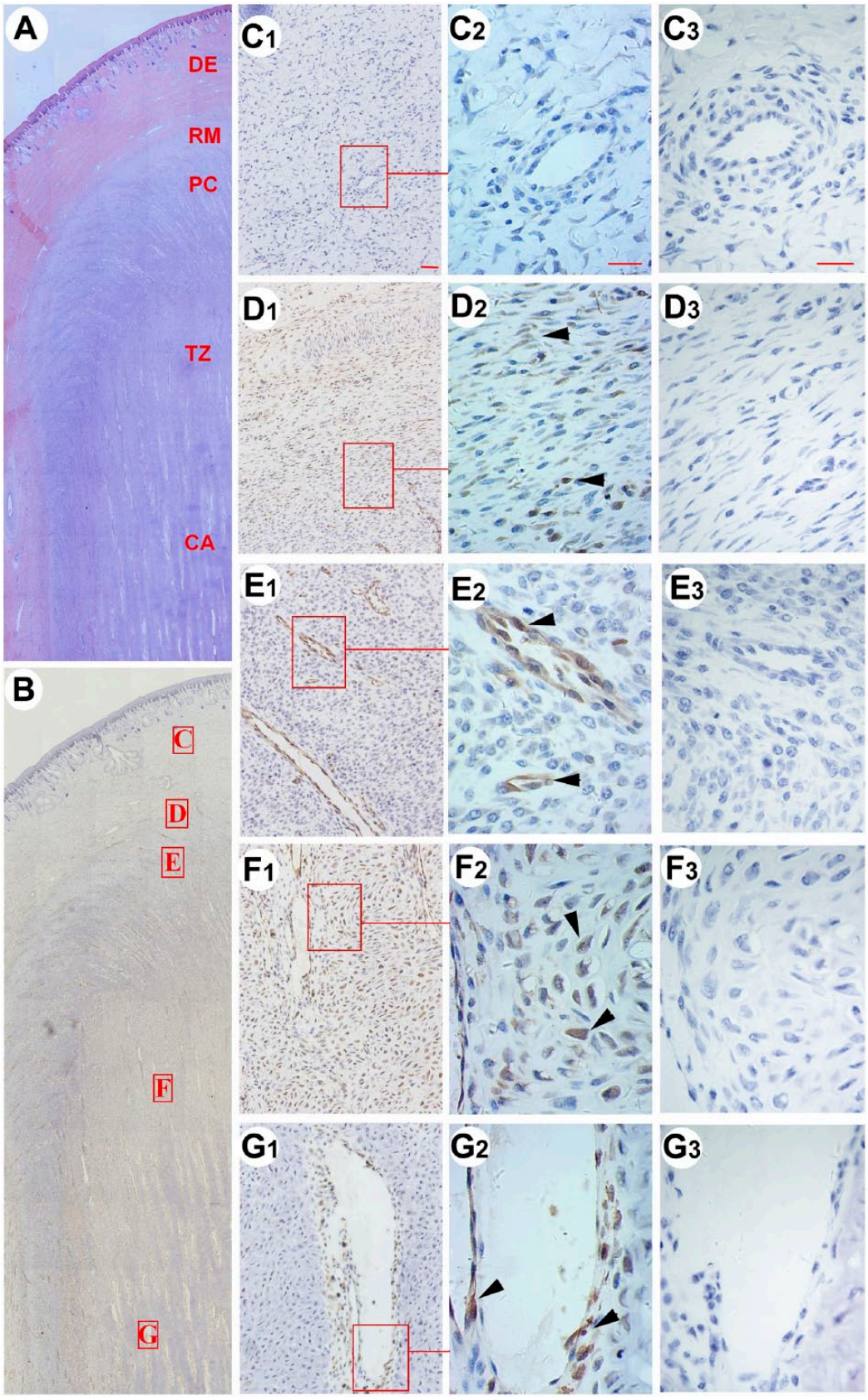

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Figure 3. IHC localization of S100A4 in the growth center tissue of antlers. (A) Antler tip section, H&E staining (montage), to indicate different zones of an antler growth center. (B) Antler tip section stained with anti-S100A4 antibody (montage). C to G in (B) are corresponding to the boxes in the C1 to G1, respectively. C2 to G2 are higher magnifications in the insets of C1 to G1, respectively. C3 to G3 are the negative controls in the similar areas of C2 to G2. (C1–C3) Dermal zone. DE tissue was almost devoid of S100A4-positive cells. (D1–D3) RM zone. The majority of the cells in the RM were stained positive with anti-S100A4 antibody (arrowheads). (E1–E3) PC zone. S100A4-positive cells were solely located in the walls of blood vessels (arrowheads). (F1–F3) TZ zone. Cartilage columns were densely studded with S100A4-positive cells (arrowheads). (G1–G3) CA zone. S100A4-positive cells were mainly lined around blood vessels and vein sinuous (arrowheads). Bar = 200 μm (C1), and 100 μm (C2 and C3). Abbreviations: IHC, immunohistochemistry; H&E, hematoxylin and eosin; DE, dermis; RM, reserve mesenchyme; PC, pre-cartilage; TZ, transition zone; CA, cartilage.

proliferation ($p<0.05$) of APCs at 24 hr, APC-RNAi at 24 and 48 hr, and FPCs at 24 and 48 hr (Fig. 5). However, the addition of D-S100A4 did not significantly stimulate proliferation of Hela cells compared with the controls (Fig. 5).

To test the effects of D-S100A4 on cell migration, APCs, APC-RNAi, FPCs, and Hela cells were treated with D-S100A4 (100 ng/ml). The results showed that D-S100A4 could significantly increase the migration of Hela cells ($p<0.05$), but not APCs (Fig. 6).

Discussion

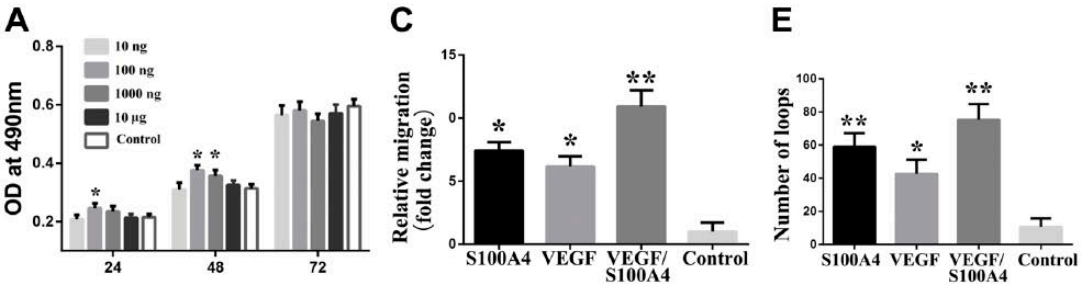
This is the first study of the expression and function of S100A4 in ASCs. We showed for the first time that S100A4 is significantly expressed in all three types of ASCs. S100A4-positive cells are localized in the AP, PP, and antler growth center tissues, and S100A4 may play a critical role in regulating ASCs. Specifically, D-S100A4 (1) promotes cell proliferation, (2) stimulates angiogenesis, and (3) enhances the migration of cancer cells (Hela cells), but not APCs.

In adult animals, S100A4 expression is restricted to very few types of normal tissue/cells, whereas it is frequently overexpressed in cancerous tissues.[21,22] Unexpectedly, Li et al.[8] reported that S100A4 protein highly expressed in APCs (normal antler cells) but not in their progeny, the PPCs. However, the present study demonstrated S100A4 expression in not only PPCs, but also RMCs, which are the PPCs' progeny. The RMCs all highly expressed S100A4 at both the transcriptional and translational levels (Figs. 1–3). This discrepancy between the two studies may not be attributed to different deer species (red deer vs sika deer) or different methods (2-DE vs IHC) used. S100A4 is known to be a secreted protein,[23] and our IHC detected the expression of S100A4 in ASCs and some of other antler cells (such as those in the lining of the blood vessel's wall), which means that these S100A4-positive antler cells either secreted S100A4 to or uptook from the extracellular environment. We believe that the ASCs express S100A4, as this gene expressed at both mRNA and protein levels in ASCs (Fig. 1). More importantly, the fact that antler cells do not become cancerous under the pressure of high expression of S100A4 is already impressive and worth further exploration.

A large body of evidence has demonstrated that S100A4 promotes cell proliferation and tumor growth.[7,24,25] The down-regulation of S100A4 expression suppresses cell proliferation in many cancer cells.[6,26] The annual full regeneration of deer antlers is unique among mammalian organs in that it displays an unprecedented growth rate during the growth phase.[9] The rapid growth of antlers is mainly dependent on the proliferation of the cells residing in the antler tip, named the antler growth center.[27] The antler growth center comprises four zones: reserve mesenchyme, pre-cartilage, transition, and cartilage.[12] Clark et al.[28] performed the BrdU injection assay to detect mitotic cells in the antler growth center, and found numerous mitotic cells in the mesenchyme and pre-cartilage zones. Interestingly, there exists a large overlying area in the antler growth center between cells that expressed S100A4 (the present study) and those that showed BrdU incorporation,[28] suggesting that S100A4 may serve as a mitogen for ASCs.

To be commensurate with the growth rate of antlers, blood vessels in the antlers must elongate at the same pace. Therefore, strong angiogenic factors must be essential. VEGF is a significant regulator of angiogenesis,[29] and although expression of VEGF was found in the pre-cartilage, it was not directly associated with the vascular cells.[30] S100A4 is also known as a potent angiogenic factor in many tumors.[31–33] In the present study, we found S100A4 specifically located in blood vessel walls and there were numerous S100A4-positive cells in the vascularized tissues of the pre-cartilage zone (Fig. 3).

Angiogenesis is a complex process involving multiple endothelial cell activities, including migration, proliferation, and tubulogenesis.[34] In our study, D-S100A4 increased proliferation, migration, and tube formation of HUVECs (Fig. 4). Therefore, we believe that one of the roles of S100A4 in the antler growth center is to promote blood vessel formation. A previous study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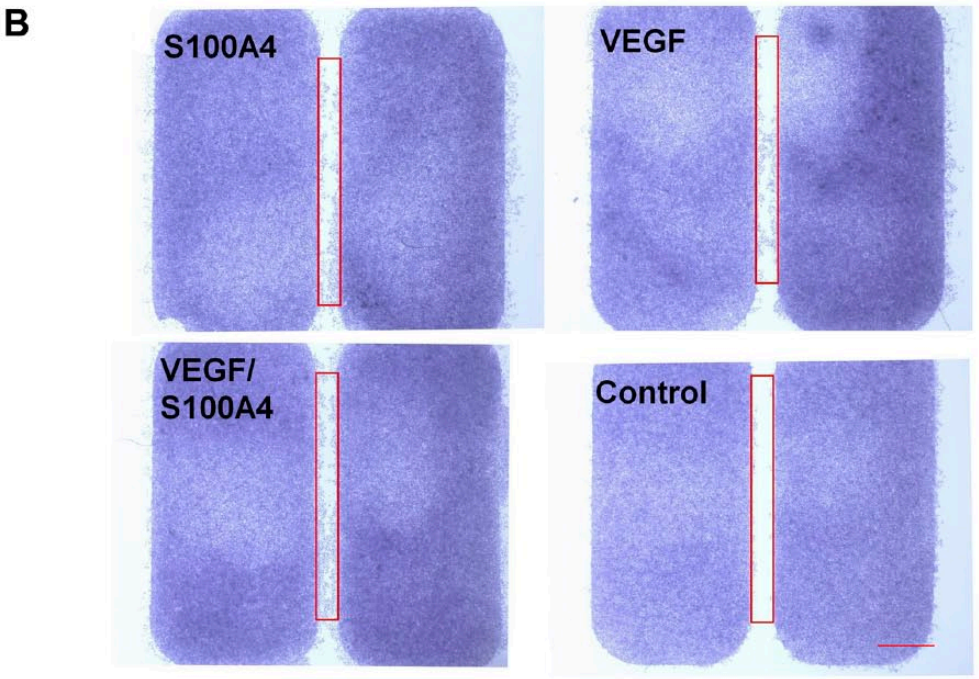
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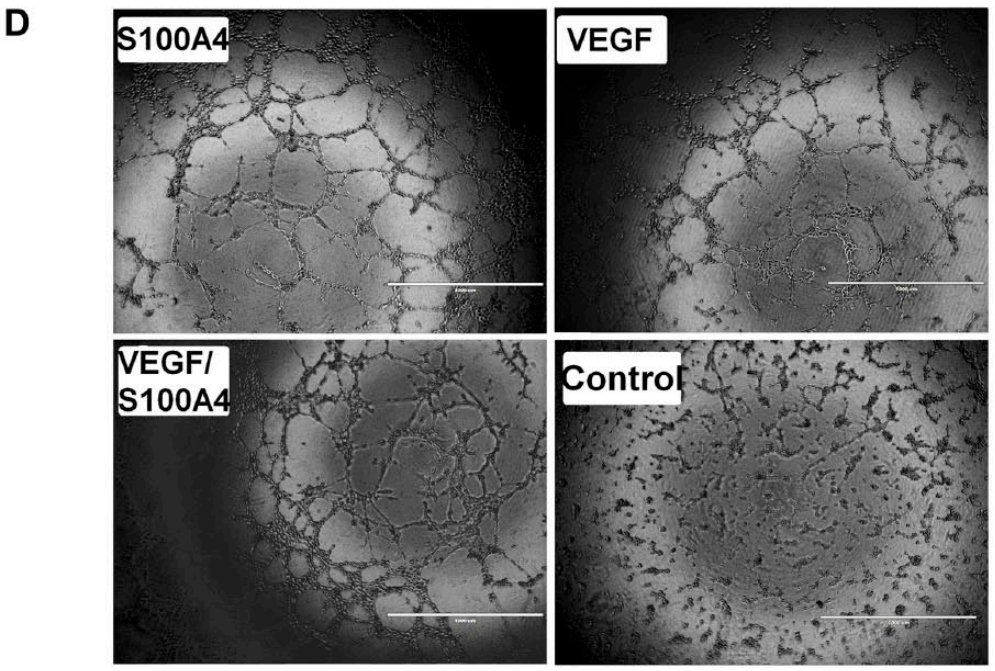
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Figure 4. Angiogenic activity of deer S100A4 protein (D-S100A4) on HUVECs in vitro. (A) MTT proliferation assay. Dose–response effects of D-S100A4 on the proliferation of HUVECs. The most effective dosage was found to be 100 ng/ml at 24 and 48 hr. (B) Migration assay. D-S100A4 (100 ng/ml) significantly enhanced migration of HUVECs, and had synergistic effects with VEGF on HUVEC migration. (C) Bar graph to quantify the results of cell migration assay. Numbers of the migrated cells were counted using ImageJ software, and the relative value is the fold change of treatment/control. (D) Tube formation assay. D-S100A4 (100 ng/ml) significantly increased number of loops from HUVECs, and had synergistic effects with VEGF (20 ng/ml) on HUVEC tube formation. (E) Bar graph to quantify the results of loop numbers. Numbers of loops were counted using ImageJ software. All controls in the figure were negative control (without addition of any growth factor). The data, means ± SEMs; *$p<0.05$, **$p<0.01$, significantly different from the control. Bar = 1000 μm, in both B and D. Abbreviation: HUVEC, human vascular endothelial cell; MTT, 3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; OD, optical density; VEGF, vascular endothelial growth factor.

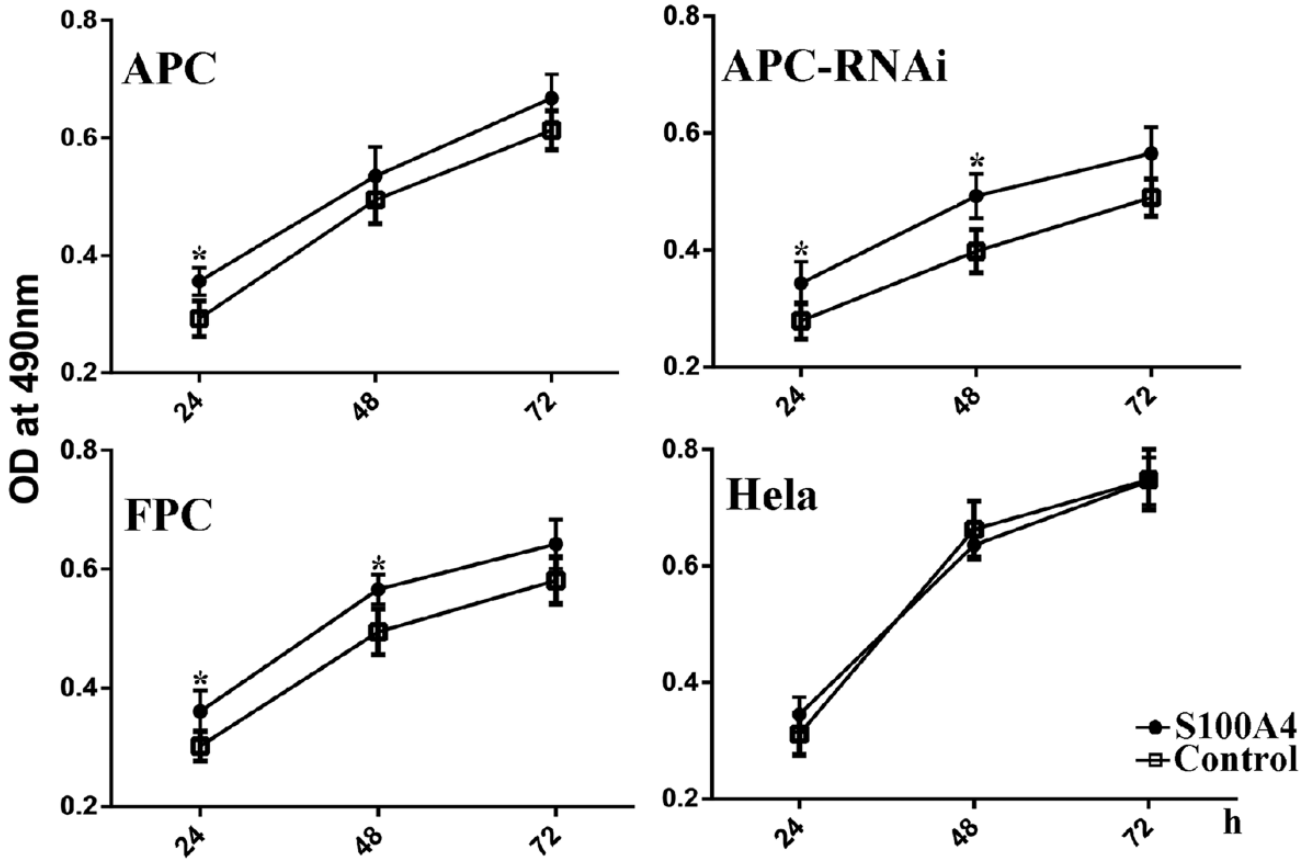

Figure 5. Effects of D-S100A4 on the proliferation of the APCs. D-S100A4 (100 ng/ml) significantly stimulated proliferation of the APCs at the time point of 24 hr, APC-RNAi at the points of 24 and 48 hr, and FPCs at the points of 24 and 48 hr, but no significant effect was detected for the cancer cell line Hela cells. Values are the average of triplicates in each experiment and are expressed as means ± SEMs. *$p<0.05$, significantly different from the control. Abbreviations: APC, antlerogenic periosteal cell; FPCs, facial periosteal cell; OD, optical density.

reported that S100A4 synergizes with VEGF in promoting endothelial cell migration[31] and our results in the ASC model are consistent with this report.

Metastasis is generally defined as the spread of malignant cells from the primary tumor to establish secondary growth in a distant organ[21] and accounts for most of deaths from cancer.[35] S100A4 is involved in the stimulation of cell motility in several tumor cell lines.[36–38] S100A4 enhances cell motility through several ways, such as (1) promoting directional motility via myosin-IIA,[39] (2) increasing the activity of matrix metalloproteinases causing higher cell dissociation,[40] and (3) down-regulating adhesion molecule E-cadherin and β-catenin.[41] In the present study, we found that D-S100A4 protein could promote motility of Hela cells, an epithelial cancer cell line, but not APCs, mesenchymal cell lines. As S100A4 is one of key factors involved in epithelial-to-mesenchymal transition,[42] we infer that S100A4 may have differential effects on different cell lines whether they are epithelial or mesenchymal.

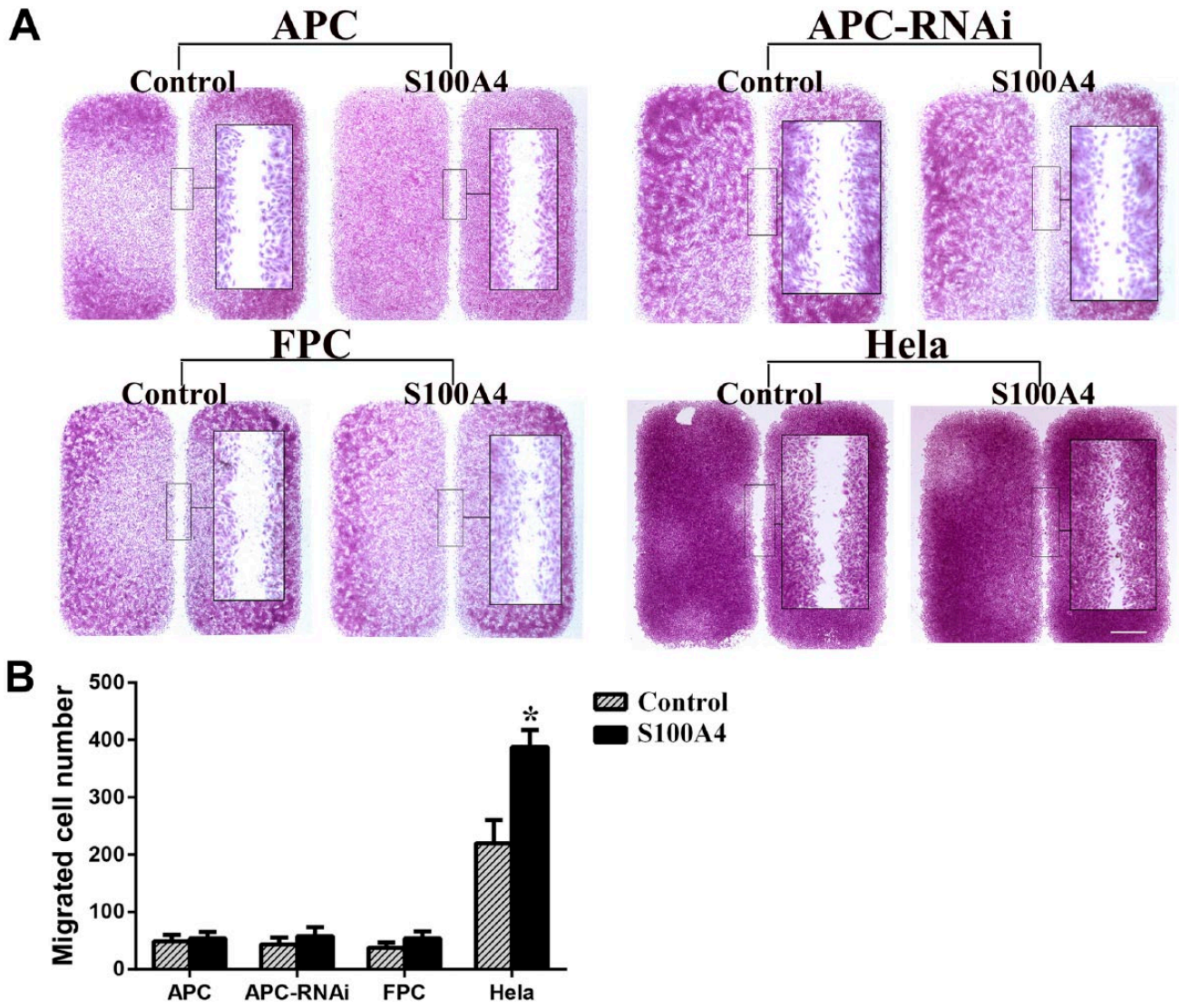

Figure 6. Effects of D-S100A4 on the migration of the APCs. (A) D-S100A4 (100 ng/ml) did not significantly stimulate migration of the APCs, but significantly stimulated migration of the Hela cells. Each inset is the higher magnification of corresponding zone in the black box. (B) Bar graphic to quantify the results of cell migration assay. Numbers of migrated cells were counted using ImageJ software. The data, means ± SEMs; *$p<0.05$, significantly different from the control. Bar = 1000 µm. Abbreviations: APC, antlerogenic periosteal cell; FPC, facial periosteal cell.

Genes in some of the S100A4-related transduction pathways that are involved in cell migration were also detected in the transcriptome of the ASCs (unpublished work). Whether S100A4 in ASCs has the capability to promote cell motility, and whether similar mechanisms would be involved if it were to occur, remains to be elucidated. Coincidently, CD9, cell surface marker of ASCs, is a suppressor of metastasis that modulates cell adhesion and migration. Currently, we do not know yet if CD9 could interact with S100A4 in this particular case. We infer that CD9 may play an important role in ASCs to counteract the high-level S100A4. Therefore, ASCs may offer a unique opportunity to explore how nature has effectively regulated the high expression level of S100A4 during rapid cell proliferation without going cancerous.

In conclusion, our work demonstrated that S100A4 is highly expressed in ASCs and some of their differentiated progenies. S100A4 may stimulate angiogenesis and cell proliferation during antler growth, but not migration of these antler cells. The study of the regulatory mechanism of S100A4 during antler growth may illuminate a unique way to control this metastasis-associated protein.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

D-TW and C-YL conceived and designed the study; D-TW, W-HC, and H-MS performed the experiments; D-TW, W-HC, and H-XB performed the data analyses; D-TW and C-YL wrote the paper; and W-HC, H-XB, and H-MS reviewed and edited the manuscript. All authors read and approved the manuscript.

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