FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 60, No. 3, 2022
pp. 271–279

ORIGINAL PAPER

VIA MEDICA

Wnt5a/Ror2 promotes vascular smooth muscle cells proliferation via activating PKC

Yaning Shi[1, 2, #], Hongfang Li[1, 3, #], Jia Gu[1, 3], Yongzhen Gong[1, 3], Xuejiao Xie[4], Duanfang Liao[1, 3], Li Qin[1, 3, *]

[1]Laboratory of Stem Cell Regulation and Application, Hunan University of Chinese Medicine, 410208, Changsha, Hunan, China

[2]Science and Technology Innovation Center, Hunan University of Chinese Medicine, 410208, Changsha, Hunan, China

[3]School of Pharmacy, Hunan University of Chinese Medicine, 410208, Changsha, Hunan, China

[4]School of Chinese Medicine, Hunan University of Chinese Medicine, 410208, Changsha, Hunan, China

[#]These authors contributed equally to this work.

Abstract
Introduction. Abnormal proliferation of vascular smooth muscle cells (VSMCs) can cause various vascular diseases, such as atherosclerosis, restenosis, and pulmonary hypertension. However, the effect and underlying mechanism of Wnt5a on the proliferation of VSMCs remain unclear. Our study aimed to investigate whether Wnt5a/Ror2 promotes vascular smooth muscle cell proliferation *via* activating protein kinase C (PKC), thereby effectively alleviating vascular proliferative diseases.
Material and methods. The proliferation of HA-VSMC cell line was evaluated by CCK-8, EdU, and Plate clone formation assays. The Wnt5a gene knockdown and overexpression were carried out by standard methods. The interaction between Wnt5a and Ror2 was explored by co-immunoprecipitation. Western blotting and immunofluorescence were used to determine the expression levels of key proteins in VSMCs.
Results. The present study found that the expression of Wnt5a protein increased significantly in the proliferation of VSMCs stimulated by 10% serum in a time-dependent manner. Furthermore, the proliferative rate of VSMCs overexpressing Wnt5a was dramatically accelerated, whereas Wnt5a knockdown using siWnt5a reversed thisproliferative effect. Wnt5a up-regulated the expression of receptor tyrosine kinase-like orphan receptor 2 (Ror2) by binding to it. Further studies indicated that Wnt5a induces the PKC expression in VSMCs and knockdown of Wnt5a or Ror2 could inhibit PKC phosphorylation.
Conclusions. Wnt5a could effectively promote the proliferation of VSMCs, which might be related to the binding of Wnt5a and Ror2 to activate PKC. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 3, 271–279)*

Keywords: vascular smooth muscle cells; Wnt5a; Ror2; PKC; cell proliferation

Introduction

Vascular smooth muscle cells (VSMCs) are the major cellular components in the media layer of arteries and possess prominent phenotypic plasticity. Generally, mature VSMCs remain in a quiescent state and exhibit the contractile phenotype. Once vessels are damaged, VSMCs change phenotype from a quiescent contractile state to a proliferative synthetic phenotype, which con-

***Correspondence address:**
Li Qin
School of Pharmacy
Hunan University of Chinese Medicine
Xueshi Road & 300
Changsha, Hunan, 410208, China
phone: +86-731-88458238
e-mail: lqin@hnucm.edu.cn

This article is available in open access under Creative Common Attribution-Non-Commercial-No Derivatives 4.0 International (CC BY-NC-ND 4.0) license, allowing to download articles and share them with others as long as they credit the authors and the publisher, but without permission to change them in any way or use them commercially.

©Polish Society for Histochemistry and Cytochemistry
Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0026
ISSN 0239-8508, e-ISSN 1897-5631

tributes to vascular remodeling and intimal hyperplasia [1]. It is well known that the abnormal proliferation caused by the phenotypic conversion of VSMCs can lead to a variety of vascular pathological changes, including atherosclerosis, pulmonary hypertension, restenosis, and other [2]. Therefore, exploration of the crucial molecular mechanisms of excessive proliferation of VSMCs will provide a useful strategy for the prevention and treatment of vascular diseases.

Wingless-type MMTV integration site family member 5a (Wnt5a), a member of the Wnt family which comprises of 19 highly conserved members that are secreted cysteine-rich glycoproteins [3, 4]. Wnt5a and its signaling pathway are involved in the regulation of diverse cellular physiological and pathological processes such as cell proliferation, migration, polarity, and survival [5]. A previous study has demonstrated that Wnt5a is highly expressed in the macrophage--rich regions of murine and human atherosclerotic lesions/plaques [6]. Moreover, Wnt5a can induce foam cell formation by increasing scavenger receptor CD36 expression, thereby promoting the development of atherosclerosis [7]. In contrast, the silencing of Wnt5a suppresses nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling, which in turn reduces the inflammatory response and prevents vascular damage in atherosclerosis [8]. However, the role and underlying mechanisms of Wnt5a in regulating the proliferation of VSMCs remain unclear.

Receptor tyrosine kinase-like orphan receptor 2 (Ror2) is a member of the Ror family that functions as a receptor or co-receptor for Wnt5a to trigger the non-canonical Wnt signaling pathway or block the canonical Wnt signaling pathway [9]. Accumulating evidence has demonstrated that the Wnt5a/Ror2 signaling pathway regulates cell proliferation during embryonic development [9, 10]. Moreover, the Wnt5a//Ror2 signaling pathway in mesenchymal stem cells facilitates the proliferation of gastric cancer cells by triggering the CXCL16–CXCR6 axis [11]. Wnt5a, as a non-canonical Wnt ligand, can activate the non-canonical Wnt/PKC pathway [12, 13]. Previous research has shown that the Wnt5a/PKC pathway promotes melanoma metastasis by inducing epithelial to mesenchymal transition and increasing lung cancer cell mobility and cisplatin resistance [12, 14]. The Protein kinase C (PKC) family of serine/threonine kinases is evolutionarily conserved and expressed in a wide variety of species [15]. Emerging evidence has shown that PKC is involved in the proliferation of VSMCs [16]. Interestingly, Ror2 activation by a noncanonical Wnt ligand can activate PKC and c-Jun N-terminal kinases (JNK) and promote N-methyl-d-aspartic acid (NMDA) receptor synaptic responses [17].

Herein, we explored the effect of Wnt5a on the proliferation of VSMCs and proposed that Wnt5a the induced proliferation of VSMCs by binding to Ror2 to activate PKC.

Materials and methods

Cell Culture. Human VSMCs (T/G HA-VSMC cell line) were obtained from professor Chaonan Qian (department of nasopharyngeal carcinoma, Sun Yat-Sen University, Guangzhou, China) Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin solution (Gibco) at 37°C in a humidified 5% CO_2 incubator.

Western blot assay. VSMCs lysates were prepared by using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor (Beyotime, Shanghai, China) and phosphatase inhibitors (Beyotime, Shanghai, China). The protein concentration was detected using a bicinchoninic acid (BCA) assay kit (Beyotime). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electro-transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: Wnt5a (ab72583, Abcam, USA; dilution, 1:500), Ror2 (sc--374174, Santa Cruz Biotechnology, Inc., USA; dilution, 1:500), p-PKC (ab75837, Abcam; dilution, 1:50,000), PKC (ab179521, Abcam; dilution, 1:2,000), α-Tubulin (AF0001, Beyotime; dilution, 1:1,000), GAPDH (AF5009; dilution, 1:2,000) and β-Actin (AF5001, Beyotime; dilution, 1:5,000). The membrane was washed and incubated with the peroxidase- conjugated anti- rabbit (SA00001-2, Proteintech, USA; dilution, 1:2,000) or anti-mouse (SA00001-1, Proteintech, USA; dilution, 1:2,000) secondary antibodies at room temperature (RT) for 2 h, and signals were detected using the enhanced chemiluminescence (ECL) plus kit (Millipore Corporation, USA) and visualizer (Tanon-5200, Shanghai, China).

Plasmids, lentivirus preparation, and small interference RNA (siRNA) transfection. A stable Wnt5a-overexpression VSMCs line (VSMC-Wnt5a) and VSMCs transfected with an empty vector (VSMC-NEG) were constructed by lentivirus transfection as described previously [18]. Lentiviral particles were packaged and transfected by Addgene and Invitrogen companies. Briefly, lentivirus was prepared by co-transfection of 293T cells with packaging plasmids psPAX2 (#12260, Addgene, USA), pMD2.G (#12259, Addgene, USA), pLenti6/V5-Wnt5a and pLenti6/V5 (V49610, Invitrogen, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Lentivirus-containing media were collected from plates at 24 h and 48 h after transfection. Then, VSMCs were incubated with lentivirus-containing media and 8 mg/mL polybrene for 24 h. All survived cells were selected

©Polish Society for Histochemistry and Cytochemistry
Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0026
ISSN 0239-8508, e-ISSN 1897-5631

by 5 μg/mL blasticidin. The cells were transiently transfected for 48 h with siRNA (Wnt5a-specific small interfering RNA, siWnt5a, forward: 5'-CGACUAUGGCUACCGCUUUdTdT-3', reverse: 3'-dTdT GCUGAUACCGAUGGCGAAA-5'); Ror-2-specific small interfering RNA, siRor2, forward: 5'-CAGUGAGCAAUGUGAGCAAdTdT-3', reverse: 3'-dTdTGUCACUCGUUACACUCGUU-5'; RiboBio, Guangzhou, China).

Cell viability. Cell viability was determined by using CCK8 assay kit (Beyotime). VSMCs, VSMC-Wnt5a and VSMC-NEG cells were seeded in 96-well microplates at a density of 3×10^3 cells/well. Next, the VSMC-Wnt5a cells were treated with siWnt5a (50 nM) for 24 h, 48 h and 72 h. The CCK8 reagent (10 μL) was added at a concentration of 10 μL/well, and the plates were incubated in a 5% CO_2 atmosphere at 37°C for 0.5 h. A microplate reader (BioTek, USA) was then used to determine the absorbance of each well at 450 nm.

EdU assay. VSMCs proliferation was evaluated using 5-ethynyl-2′-deoxyuridine (EdU) assay kit (C10310-1, RiboBio, Guangzhou, China) according to the manufacturer's instructions. Briefly, approximately 3×10^3/well VSMCs were cultured in 96-well plates for 24 h. At the culture endpoint, EdU labeling reagent (50 μM) was added to each well, followed by 2 h incubation at 37°C. The labeling medium was then removed, and the cells were fixed in 4% paraformaldehyde for 30 min at RT. Then, glycine was used to neutralize 4% paraformaldehyde and 0.5% TritonX-100 was utilized to permeabilize the cell membranes. Subsequently, the cells were incubated with premixed click additive solution for 30 min at RT. Finally, the cells were incubated with Hoechst 33342 for 15 min at RT to label the cell nuclei. Fluorescence was detected using an inverted fluorescence microscope (Olympus, Tokyo, Japan). EdU-positive cells ratio (%) = EdU-positive cells/total cells × 100%.

Plate clone formation assay. 500 cells were added into each well of a 6-well plate and cultured for 9 days. The cultured medium was changed every 3 days. Then, the cells were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet (Solarbio, Beijing, China) for 30 min. Cell clones with > 150 cells were counted and analyzed with an optical microscope.

Immunofluorescence assay. VSMC-Wnt5a was incubated on glass coverslips in a 24-well plate for 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde for 30 min. The cells were then permeabilized using PBS with 0.5% Triton X-100 for 20 min and blocked using PBS with 3% bovine serum albumin for 2 h at RT. Next, the cells were incubated using Wnt5a antibody (ab72583, Abcam, USA; dilution, 1:500) overnight at 4°C. After washing with PBS, the cells were labeled with a secondary antibody (goat anti-rabbit IgG H&L (Alexa Fluor® 488), ab150077, Abcam; dilution, 1:500) in TBST for 1.5 h at RT. The cells were then incubated using Ror2 antibody (sc-374174, Santa Cruz Biotechnology, Inc., USA; dilution, 1:300) overnight at 4°C. After washing with PBS, the cells were labeled with a secondary antibody (donkey anti-mouse IgG H&L (Alexa Fluor® 594), ab150108, Abcam; dilution, 1:500) in TBST for 1.5 h at RT. Finally, the cell nuclei were stained with DAPI for 10 min. An inverted fluorescence microscope was used to capture images.

Co-immunoprecipitation (Co-IP) assay. Co-IP assay was carried out using Co-IP Kit (26149, Thermo Fisher Scientific, USA), following the manufacturer's instructions. In brief, 10 μg of anti-Wnt5a antibody or another antibody (including Ror2 and IgG) was bound to AminoLink® Plus Coupling Resin. The cells were lysed with 500 μL of ice-cold IP lysis/wash buffer. The cell lysates were incubated with antibody-coupled resin overnight at 4°C. The resin was washed five times with IP lysis/wash buffer and then eluted with 60 μL of elution buffer. The eluted Wnt5a and Ror2 proteins were subjected to a Western blot assay.

Statistical analysis. The data were presented as means ± SD from three independent experiments. For comparisons between two groups, Student's *t*-test was used for independent samples. One-way analysis of variance (ANOVA) test was performed using Dunnett's method to compare multiple groups. $P < 0.05$ was considered a statistical significance. GraphPad Prism 8.0 software (GraphPad Software, Inc., USA) was used for all statistical analyses.

Results

Wnt5a accelerated the proliferation of VSMCs

To investigate whether Wnt5a was involved in the proliferation of VSMCs, we detected the expression of Wnt5a in proliferative VSMCs stimulated by 10% FBS. After starvation with a serum-free medium, VSMCs were treated with a medium containing 10% FBS for 0 h, 6 h, 12 h, and 24 h. The results showed that compared with the serum-free treatment group, 10% FBS significantly increased the expression of Wnt5a in a time-dependent manner in VSMCs (Fig. 1a). This finding indicates that the upregulation of Wnt5a was closely associated with the proliferation of VSMCs. Next, we constructed a stable Wnt5a-overexpression VSMC line (VSMC-Wnt5a) by lentivirus transfection to verify the effect of Wnt5a on VSMC proliferation. Compared with the VSMCs transfected with an empty vector (VSMC-NEG), the protein levels of Wnt5a were significantly increased in VSMC-Wnt5a, indicating that the stable Wnt5a-overexpression VSMC line was successfully established (Fig. 1b). As shown in Fig. 1c–e, overexpression of Wnt5a notably induced VSMCs proliferation in VSMC-Wnt5a cells as determined by CCK8 assay, EdU assays and plate clone formation assay compared to the VSMC-NEG group. These data suggest that Wnt5a could promote the proliferation of VSMCs.

Knockdown of Wnt5a inhibited the proliferation of VSMCs

Western blot results confirmed that siWnt5a successfully knocked down the expression of Wnt5a

©Polish Society for Histochemistry and Cytochemistry

10.5603/FHC.a2022.0026
ISSN 0239-8508, e-ISSN 1897-5631

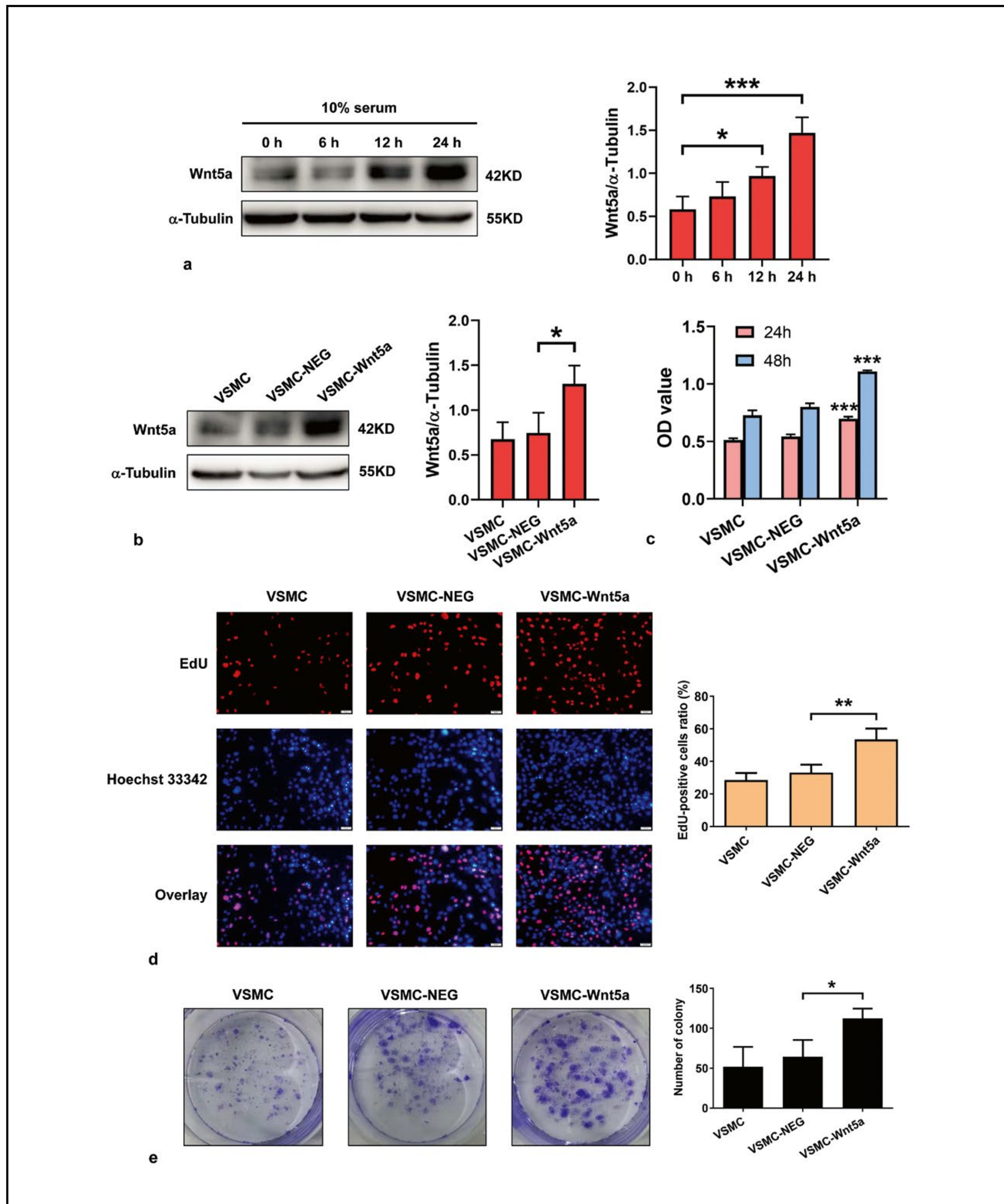

Figure 1. The effect of Wnt5a overexpression on the proliferation of vascular smooth muscle cells (VSMCs). **a.** The expression of Wnt5a protein in VSMCs treated with medium containing 10% FBS for 0 h, 6 h, 12 h and 24 h; **b.** Wnt5a expression in VSMC, VSMC-NEG and VSMC-Wnt5a was measured by western blot (WB) assay; **c.** Cell viability was examined by CCK-8 assays. $^{***}P < 0.001$ versus VSMC-NEG group; **d.** Cell viability was detected using EdU assays (magnification 100×, scale bar: 50 μm); **e.** The ability of cell clone formation was detected by plate clone formation assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The values represent the mean ± SD of three independent experiments.

©Polish Society for Histochemistry and Cytochemistry
Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0026
ISSN 0239-8508, e-ISSN 1897-5631

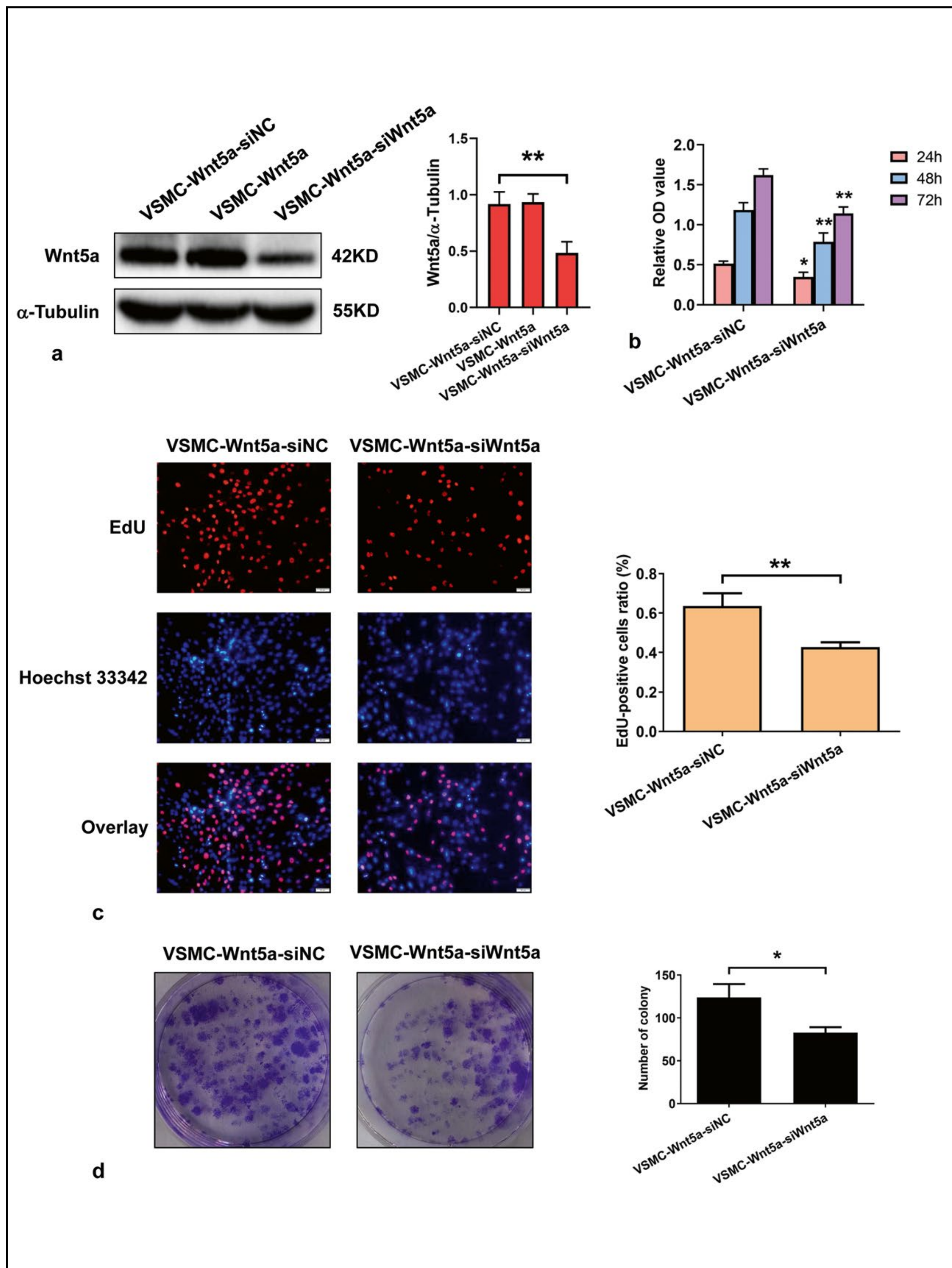

Figure 2. The effect of Wnt5a knockdown on the proliferation of VSMCs. **a.** The expression of Wnt5a in siWnt5a-interfered VSMC-Wnt5a was measured by WB assay; **b.** Cell proliferation was measured by CCK8 assay. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* VSMC-Wnt5a-siNC group; **c.** Cell proliferation was detected using EdU assays (magnification 100×, scale bar: 50 μm). **d.** Cell proliferation was examined by plate clone formation assay; $^{*}P < 0.05$, $^{**}P < 0.01$. The results represent the mean ± SD of three independent experiments.

©Polish Society for Histochemistry and Cytochemistry
Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0026
ISSN 0239-8508, e-ISSN 1897-5631

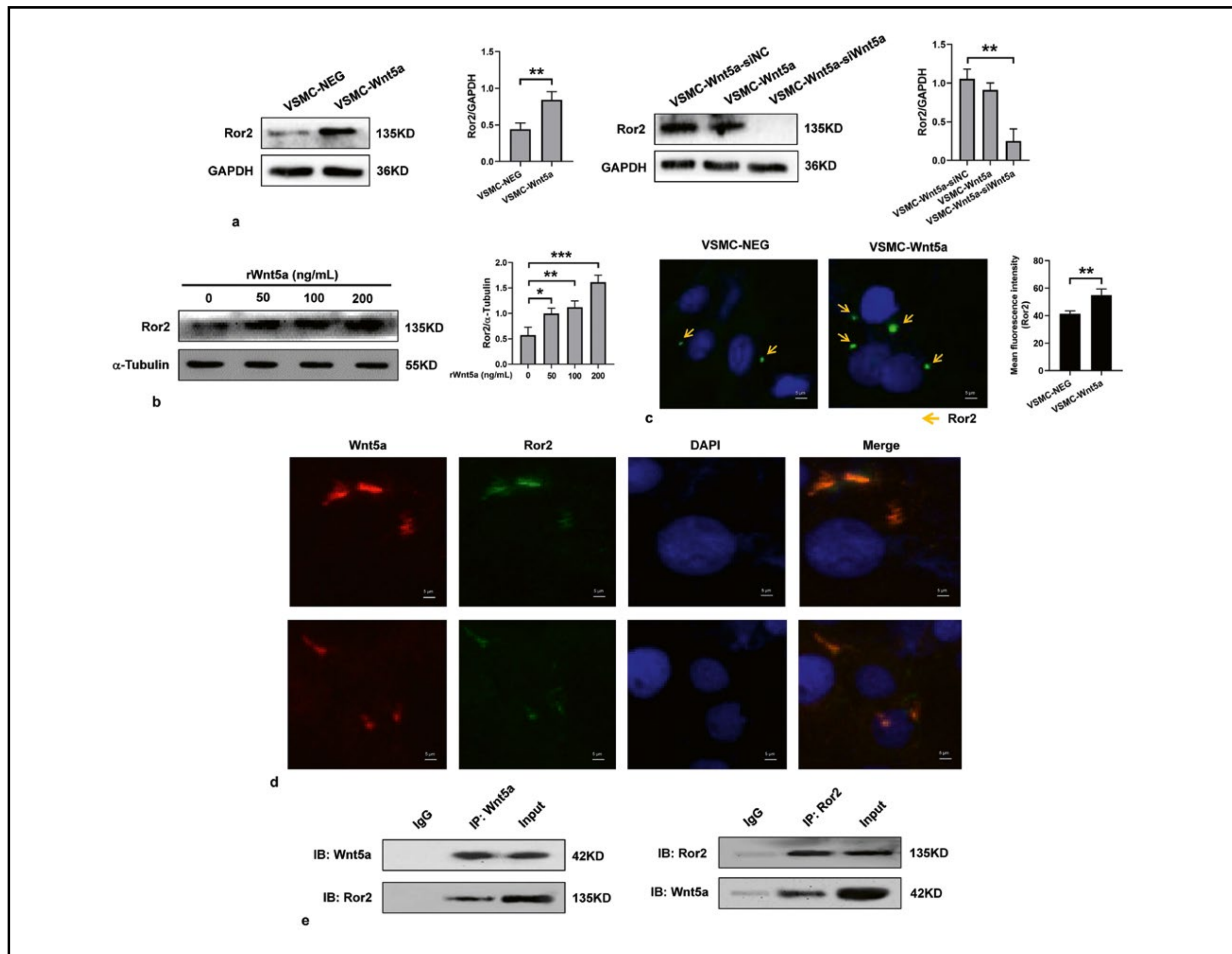

Figure 3. The interaction between Wnt5a and Ror2 in VSMCs. **a.** The expression of Ror2 in VSMC-NEG, VSMC-Wnt5a and siWnt5a-interfered VSMC-Wnt5a was detected by WB assay; **b.** The expression of Ror2 protein in VSMCs treated with different concentrations of rWnt5a (0 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL) for 72 h was determined by WB assay; **c.** The expression of Ror2 in VSMCs (scale bar: 5 μm) was detected by immunofluorescence assays; **d.** Double-immunofluorescence assay detected the co-localization of Wnt5a and Ror2 in VSMCs (scale bar: 5 μm); **e.** Cell lysates were immunoprecipitated with anti-Wnt5a antibody or anti-Ror2 antibody and blotted with anti-Ror2 antibody or anti-Wnt5a antibody. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent the mean ± SD of three independent experiments.

in VSMC-Wnt5a cells (Fig. 2a). The data from the CCK8 assay, EdU assays and plate clone formation assay showed that knockdown of Wnt5a could reverse the effect of Wnt5a on the proliferation of VSMCs (Fig. 2b–d). The results indicated that down-regulation of Wnt5a expression could reverse Wnt5a-induced VSMC proliferation.

Ror2 acted as a binding receptor for Wnt5a and was up-regulated by Wnt5a in VSMCs

We further explored whether Wnt5a/Ror2 signaling pathway was involved in the proliferation of VSMCs. As shown in Fig. 3a, the expression of Ror2 was increased in VSMC-Wnt5a cells, suggesting that Wnt5a enhanced Ror2 expression in VSMCs. Conversely, Wnt5a knockdown could reverse Wnt5a-induced elevation of Ror2 expression in VSMC-Wnt5a cells (Fig. 3a). Similarly, recombinant Wnt5a (rWnt5a) promoted the expression of Ror2 in VSMCs (Fig. 3b). The results of an immunofluorescence assay showed that Ror2 expression was positively correlated with Wnt5a in VSMC-Wnt5a cells (Fig. 3c).

The interaction between Wnt5a and Ror2 was investigated through a double-immunofluorescence assay. The data revealed that Wnt5a and Ror2 were spatially co-localized in VSMCs (Fig. 3d). Concurrently, the specific binding of Wnt5a and Ror2 in VSMCs was verified by using a co-immunoprecipitation assay (Fig. 3e). These findings indicate that Wnt5a could directly bind to Ror2 and increase the expression of Ror2 in VSMCs.

©Polish Society for Histochemistry and Cytochemistry

10.5603/FHC.a2022.0026
ISSN 0239-8508, e-ISSN 1897-5631

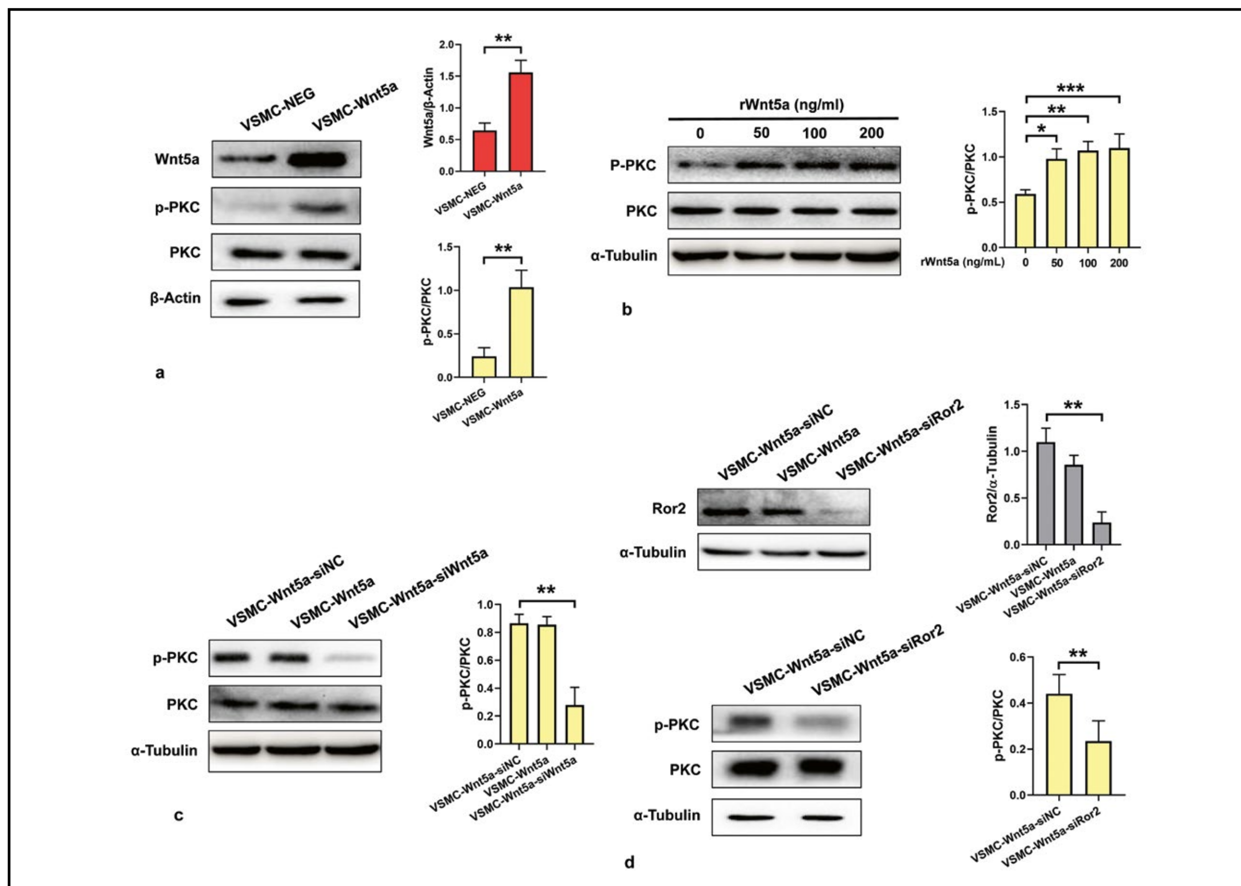

Figure 4. Wnt5a/Ror2 induced PKC in VSMCs. **a.** The expression of Wnt5a, p-PKC and PKC in VSMC-NEG and VSMC-Wnt5a; **b.** The protein levels of p-PKC and PKC expression in VSMCs treated with different concentrations of rWnt5a (0 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL) for 72 h; **c.** The expression of p-PKC and PKC in VSMC-Wnt5a-siNC and VSMC-Wnt5a-siWnt5a; **d.** The expression of Ror2, p-PKC and PKC in VSMC-Wnt5a-siNC and VSMC-Wnt5a-siRor2. The expression of all proteins was determined by WB assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All values represent the mean ± SD of three independent experiments.

Wnt5a/Ror2 activated PKC in VSMCs

Protein kinase C plays an important role in regulating the growth of VSMCs. Thus, we investigated whether PKC was involved in Wnt5a-induced VSMCs proliferation. The results disclosed that overexpression of Wnt5a or rWnt5a could activate PKC phosphorylation, while down-regulation of Wnt5a by using siWnt5a remarkably weakened the phosphorylation of PKC in VSMC-Wnt5a cells (Fig. 4a–c). By reducing Ror2 expression with siRor2, PKC phosphorylation in VSMC-Wnt5a was dramatically diminished (Fig. 4d). Overall, these findings suggest that Wnt5a/ /Ror2 might accelerate the proliferation of VSMCs by activating PKC.

Discussion

Mature VSMCs profile an extremely low proliferative rate and synthetic activity and express specific contractile proteins, whose biological functions are mainly involved in vasoconstriction and blood pressure regulation [19, 20]. The high plasticity of VSMCs is manifested during the process of vascular development and damage repair. VSMCs possess high proliferation, migration, and production of extracellular matrix components in vascular morphogenesis. Likewise, in response to vascular injury, VSMCs significantly improve their capabilities of proliferation, migration, and synthesis. Nevertheless, the plasticity of VSMCs is easily affected by the abnormal microenvironment, causing phenotypic transformations and vascular disease progression [21]. The excessive proliferation of VSMCs plays an important role in the pathomechanisms of vascular diseases, including atherosclerosis, pulmonary hypertension, restenosis after angioplasty, coronary artery bypass grafting failure [22, 23].

Wnt5a is involved in the development of various diseases such as atherosclerosis, cancer, and inflammation [24–26] and plays a critical role in numerous cellular processes from embryonic morphogenesis to postnatal development [5]. Our previous study has illustrated that the combination of Wnt5a and

©Polish Society for Histochemistry and Cytochemistry

Ror2 together suppresses the expression of adenosine triphosphate-binding cassette transporter A1 (ABCA1) promotes cholesterol accumulation and secretion of pro-inflammatory cytokines and nuclear translocation of NF-κB in VSMCs, and ultimately leads to atherosclerosis [16]. OxLDL increased Wnt5a expression, induced foam cell formation, and affected the migration and phenotype of VSMCs [27]. In the present study, our results demonstrated that Wnt5a could significantly promote VSMCs proliferation, while knockdown of Wnt5a reversed the VSMCs proliferation. Moreover, Wnt5a directly binds to Ror2 to increase its expression, while the down-regulation of Wnt5a reverses the increase of Ror2 expression in VSMC-Wnt5a.

PKC is particularly important in physiological processes for regulating gene expression, cell-cycle progression, cell migration, proliferation, differentiation, cell survival, and apoptosis [15]. Increasing evidence has shown that the activation of PKCδ stimulates the proliferation of VSMCs, while the inactivation of PKCεinhibits the growth of VSMCs [28, 29]. A previous study has revealed that interleukin-1 receptor-associated kinase-1 (IRAK1) induces VSMCs proliferation and neointimal hyperplasia by activating extracellular signal-regulated kinase 1/2 (ERK1/2) in a PKC-ε-dependent manner [30]. PKCα is increased in hypoxia-induced rat pulmonary artery smooth muscle cells (PASMCs). PKCα induces ERK1/2 phosphorylation, thereby leading to PASMC proliferation [31]. In contrast, inhibition of the PKC-ERK1/2 signaling pathway can suppress high glucose-induced VSMCs proliferation [32]. Furthermore, our previous study has verified that Wnt5a activates PKC signaling and subsequently induces epithelial-mesenchymal transition (EMT) and stemness characteristics, resulting in nasopharyngeal carcinoma (NPC) metastasis and tumorigenesis [33]. Herein, our results indicated that Wnt5a could enhance the phosphorylation of PKC in VSMCs. Contrarily, we found that inhibition of Wnt5a or Ror2 expression could reduce PKC phosphorylation in VSMC-Wnt5a. Thus, Wnt5a/Ror2 might accelerate VSMCs proliferation by inducing PKC expression.

In summary, our *in vitro* study demonstrated that Wnt5a could effectively promote the proliferation of VSMCs, which might be related to the interaction of Wnt5a and Ror2, thereby activating the downstream PKC. Therefore, these findings indicate that the Wnt5a/Ror2/PKC signaling pathway may be a potential and promising therapeutic target for the prevention and treatment of vascular proliferative diseases.

Statement of ethics

This manuscript does not contain any studies with human participants or animals performed by any of the authors. Therefore, this study did not require any ethical approval.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

TThis work was supported by the National Natural Sciences Foundation of China (No. 82274159, 81973668 and 81774130), the National Science Fund of Hunan Province (No. 2022JJ80088), the Key Project of the Educational Department of Hunan Province (No. 20A375, 21A0226), Open Fund of the State Key Laboratory Cultivation Base Co-constructed by the Ministry of Traditional Chinese Medicine Powder and Innovative Drug Research in Hunan Province (No. 21PTKF1004), the Scientific Research Project of Changsha Science and Technology Bureau (No. kq2004060), Key Project of Hunan Provincial Health Commission (202213055529), and First-Class Discipline of Pharmaceutical Science of Hunan.

References

1. Chistiakov DA, Orekhov AN, Bobryshev YV. Vascular smooth muscle cell in atherosclerosis. Acta Physiol (Oxf). 2015; 214(1): 33–50, doi: 10.1111/apha.12466, indexed in Pubmed: 25677529.
2. Wang D, Uhrin P, Mocan A, et al. Vascular smooth muscle cell proliferation as a therapeutic target. Part 1: molecular targets and pathways. Biotechnol Adv. 2018; 36(6): 1586–1607, doi: 10.1016/j.biotechadv.2018.04.006, indexed in Pubmed: 29684502.
3. Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol. 1998; 14: 59–88, doi: 10.1146/annurev.cellbio.14.1.59, indexed in Pubmed: 9891778.
4. Shi YN, Zhu N, Liu C, et al. Wnt5a and its signaling pathway in angiogenesis. Clin Chim Acta. 2017; 471: 263–269, doi: 10.1016/j.cca.2017.06.017, indexed in Pubmed: 28641961.
5. Kumawat K, Gosens R. WNT-5A: signaling and functions in health and disease. Cell Mol Life Sci. 2016; 73(3): 567–587, doi: 10.1007/s00018-015-2076-y, indexed in Pubmed: 26514730.
6. Christman 2nd MA, Goetz DJ, Dickerson E, et al. Wnt5a is expressed in murine and human atherosclerotic lesions. Am J Physiol Heart Circ Physiol. 2008; 294(6): H2864–H2870, doi: 10.1152/ajpheart.00982.2007, indexed in Pubmed: 18456733.
7. Ackers I, Szymanski C, Duckett KJ, et al. Blocking Wnt5a signaling decreases CD36 expression and foam cell formation in atherosclerosis. Cardiovasc Pathol. 2018; 34: 1–8, doi: 10.1016/j.carpath.2018.01.008, indexed in Pubmed: 29474941.
8. Yang L, Chu Y, Wang Y, et al. siRNA-mediated silencing of Wnt5a regulates inflammatory responses in atherosclerosis

©Polish Society for Histochemistry and Cytochemistry

through the MAPK/NF- B pathways. Int J Mol Med. 2014; 34(4): 1147–1152, doi: 10.3892/ijmm.2014.1860, indexed in Pubmed: 25050997.
9. Nishita M, Qiao S, Miyamoto M, et al. Role of Wnt5a-Ror2 signaling in morphogenesis of the metanephric mesenchyme during ureteric budding. Mol Cell Biol. 2014; 34(16): 3096–3105, doi: 10.1128/MCB.00491-14, indexed in Pubmed: 24891614.
10. He F, Xiong W, Yu X, et al. Wnt5a regulates directional cell migration and cell proliferation via Ror2-mediated noncanonical pathway in mammalian palate development. Development. 2008; 135(23): 3871–3879, doi: 10.1242/dev.025767, indexed in Pubmed: 18948417.
11. Takiguchi G, Nishita M, Kurita K, et al. Wnt5a-Ror2 signaling in mesenchymal stem cells promotes proliferation of gastric cancer cells by activating CXCL16-CXCR6 axis. Cancer Sci. 2016; 107(3): 290–297, doi: 10.1111/cas.12871, indexed in Pubmed: 26708384.
12. Yang J, Zhang K, Wu J, et al. Wnt5a increases properties of lung cancer stem cells and resistance to cisplatin through activation of Wnt5a/PCK signaling pathway. Stem Cells Int. 2016; 2016: 1690896, doi: 10.1155/2016/1690896, indexed in Pubmed: 27895670.
13. Rieger ME, Zhou B, Solomon N, et al. p300/ -catenin interactions regulate adult progenitor cell differentiation downstream of wnt5a/protein kinase c (PKC). J Biol Chem. 2016; 291(12): 6569–6582, doi: 10.1074/jbc.M115.706416, indexed in Pubmed: 26833564.
14. Dissanayake SK, Wade M, Johnson CE, et al. The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition. J Biol Chem. 2007; 282(23): 17259–17271, doi: 10.1074/jbc.M700075200, indexed in Pubmed: 17426020.
15. Isakov N. Protein kinase C (PKC) isoforms in cancer, tumor promotion and tumor suppression. Semin Cancer Biol. 2018; 48: 36–52, doi: 10.1016/j.semcancer.2017.04.012, indexed in Pubmed: 28571764.
16. Lu Q, Davel AP, McGraw AP, et al. PKC mediates mineralocorticoid receptor activation by angiotensin II to modulate smooth muscle cell function. Endocrinology. 2019; 160(9): 2101–2114, doi: 10.1210/en.2019-00258, indexed in Pubmed: 31373631.
17. Cerpa W, Latorre-Esteves E, Barria A. RoR2 functions as a noncanonical Wnt receptor that regulates NMDAR-mediated synaptic transmission. Proc Natl Acad Sci U S A. 2015; 112(15): 4797–4802, doi: 10.1073/pnas.1417053112, indexed in Pubmed: 25825749.
18. Zhang CJ, Zhu N, Liu Z, et al. Wnt5a/Ror2 pathway contributes to the regulation of cholesterol homeostasis and inflammatory response in atherosclerosis. Biochim Biophys Acta Mol Cell Biol Lipids. 2020; 1865(2): 158547, doi: 10.1016/j.bbalip.2019.158547, indexed in Pubmed: 31678514.
19. Owens GK, et al. Regulation of differentiation of vascular smooth muscle cells . Physiol Rev. 1995; 75(3): 487–517, doi: 10.1152/physrev.1995.75.3.487, indexed in Pubmed: 7624392.
20. Gordon D, Schwartz SM. Replication of arterial smooth muscle cells in hypertension and atherosclerosis. Am J Cardiol. 1987; 59(2): 44A–48A, doi: 10.1016/0002-9149(87)90175-5, indexed in Pubmed: 3544785.
21. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev. 2004; 84(3): 767–801, doi: 10.1152/physrev.00041.2003, indexed in Pubmed: 15269336.
22. Smith SA, Newby AC, Bond M. Ending restenosis: inhibition of vascular smooth muscle cell proliferation by cAMP. Cells. 2019; 8(11): 1447, doi: 10.3390/cells8111447, indexed in Pubmed: 31744111.
23. Wadey K, Lopes J, Bendeck M, et al. Role of smooth muscle cells in coronary artery bypass grafting failure. Cardiovasc Res. 2018; 114(4): 601–610, doi: 10.1093/cvr/cvy021, indexed in Pubmed: 29373656.
24. Bhatt PM, Lewis CJ, House DL, et al. Increased Wnt5a mRNA expression in advanced atherosclerotic lesions, and oxidized LDL treated human monocyte-derived macrophages. Open Circ Vasc J. 2012; 5: 1–7, doi: 10.2174/1877382601205010001, indexed in Pubmed: 25530821.
25. Bhatt PM, Malgor R. Wnt5a: a player in the pathogenesis of atherosclerosis and other inflammatory disorders. Atherosclerosis. 2014; 237(1): 155–162, doi: 10.1016/j.atherosclerosis.2014.08.027, indexed in Pubmed: 25240110.
26. Asem MS, Buechler S, Wates RB, et al. Wnt5a signaling in cancer. Cancers (Basel). 2016; 8(9): 79, doi: 10.3390/cancers8090079, indexed in Pubmed: 27571105.
27. Ackers I, Szymanski C, Silver MJ, et al. Oxidized low-density lipoprotein induces WNT5A signaling activation in THP-1 derived macrophages and a human aortic vascular smooth muscle cell line. Front Cardiovasc Med. 2020; 7: 567837, doi: 10.3389/fcvm.2020.567837, indexed in Pubmed: 33330641.
28. Xing Ap, Du Yc, Hu Xy, et al. Cigarette smoke extract stimulates rat pulmonary artery smooth muscle cell proliferation via PKC-PDGFB signaling. J Biomed Biotechnol. 2012; 2012: 534384, doi: 10.1155/2012/534384, indexed in Pubmed: 22754279.
29. Zhou H, Wang Y, Zhou Q, et al. Down-regulation of protein kinase c- by prolonged incubation with PMA inhibits the proliferation of vascular smooth muscle cells. Cell Physiol Biochem. 2016; 40(1-2): 379–390, doi: 10.1159/000452553, indexed in Pubmed: 27866196.
30. Jain M, Singh A, Singh V, et al. Involvement of interleukin-1 receptor-associated kinase-1 in vascular smooth muscle cell proliferation and neointimal formation after rat carotid injury. Arterioscler Thromb Vasc Biol. 2015; 35(6): 1445–1455, doi: 10.1161/ATVBAHA.114.305028, indexed in Pubmed: 25908764.
31. Jiang R, Shi Y, Zeng C, et al. Protein kinase Cα stimulates hypoxiainduced pulmonary artery smooth muscle cell proliferation in rats through activating the extracellular signalregulated kinase 1/2 pathway. Mol Med Rep. 2017; 16(5): 6814–6820, doi: 10.3892/mmr.2017.7478, indexed in Pubmed: 28901444.
32. Yang J, Han Yu, Sun H, et al. (-)-Epigallocatechin gallate suppresses proliferation of vascular smooth muscle cells induced by high glucose by inhibition of PKC and ERK1/2 signalings. J Agric Food Chem. 2011; 59(21): 11483–11490, doi: 10.1021/jf2024819, indexed in Pubmed: 21973165.
33. Qin Li, Yin YT, Zheng FJ, et al. WNT5A promotes stemness characteristics in nasopharyngeal carcinoma cells leading to metastasis and tumorigenesis. Oncotarget. 2015; 6(12): 10239–10252, doi: 10.18632/oncotarget.3518, indexed in Pubmed: 25823923.

Submitted: 25 March, 2022
Accepted after reviews: 15 September, 2022
Available as AoP: 21 September, 2022

©Polish Society for Histochemistry and Cytochemistry