The Wnt5a/Ror2 pathway is associated with determination of the differentiation fate of bone marrow mesenchymal stem cells in vascular calcification

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Abstract. Accumulating evidence have demonstrated that mesenchymal stem cells (MSCs) are involved in the initiation and progression of various vascular diseases. Canonical Wnt signaling controls the fate of MSCs, and plays an important role in vascular calcification. However, vascular calcification can be inhibited by the non-canonical Wnt signaling pathway Wnt5a/Ror2. This study aimed to investigate whether the Wnt5a/Ror2 pathway is associated with determination of the differentiation fate of MSCs in vascular calcification. Direct co-cultures were established by seeding smooth muscle cells (SMCs) or calcified SMCs and MSCs together at ratios of SMCs or calcified SMCs $15x10^4$; SMCs or calcified SMCs $5x10^4$: MSCs $10x10^4$, SMCs or calcified SMCs $10x10^4$: MSCs $5x10^4$. Osteosynthesis-inducing medium (OS) was added to the culture medium in the groups of MSCs with non-calcified SMCs. Cells were cultured for nine days. Osteoblastic differentiation was evaluated by cell morphology and the activity of alkaline phosphatase (ALP) in cell lysates and ALP staining. Furthermore, we investigated the inhibition of Wnt signaling, and observed that the members of the non-canonical signaling pathway Wnt5a/Ror2 were expressed in each group. Additionally, MSCs cultured in culture media with OS did not differentiate into an osteoblast phenotype when in direct contact with non-calcified SMCs, irrespective of the number of MSCs. However, an osteoblast phenotype was observed when MSCs were cultured in media without OS differentiation towards direct contact with calcified SMCs, and the levels of osteoblastic markers had a direct correlation with the number of MSCs. Of note, the Wnt5a protein was associated with the levels of calcification, thus, although rarely detected in non-calcification, Ror2 mRNA in the non-calcified groups was significantly higher compared to that in the calcified groups. Therefore, the Wnt5a/Ror2 pathway is associated with determination of the differentiation fate of bone marrow mesenchymal stem cells in vascular calcification.

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Introduction

Vascular calcification affects our aging and dysmetabolic population (1). Vascular calcification is known to be an actively regulated osteogenic processes (2,3). An important step of osteogenic processes is the osteochondrocytic differentiation of bone marrow (BM)-derived mesenchymal stem cells (MSCs) (4). MSCs can be isolated from bone marrow, harbor expansion potential, and differentiate into mesenhymal cells, such as osteoblasts and chondrocytes (5), as well as into non-mesenchymal cells, such as vascular cells (4,6,7). Moreover, MSCs can enter the circulation and migrate into vascular and other connective tissues (8), especially at sites of injury and in tissue transplant grafts (9-11).

MSCs are of increasing interest for future therapeutic exploitation in the treatment of cardiovascular diseases in the setting of allogeneic hematopoietic stem cell transplantations (12). Although it is attractive to consider that growth of MSC in injured vascular tissue may simply regenerate normal vascular tissue, it is also possible for these cells to produce ectopic tissues, such as those observed in advanced atherosclerotic calcification (13). MSC are involved in the initiation and progression of various vascular diseases (10). Notably, Wnt-signaling controls the fate of MSCs, and plays an important role in vascular calcification (4). MSCs also contribute to the regulation of osteogenic mineralization during development and disease and are therefore important in vascular calcification (14,15). However, vascular calcification can be inhibited by the non-canonical Wnt signaling pathway, Wnt5a/Ror2. Therefore, it is important to determine whether this pathway is associated with determination of the differentiation fate of MSCs in vascular calcification, since few studies are currently available regarding this novel method (17). In this study, we used an *in vitro* cell-cell co-culturing system to observe whether MSCs directly interact with vascular calcification and investigate the Wnt5a/Ror2 pathway during the process.

Materials and methods

Rat bone-marrow-derived MSCs. The isolation and culture of male MSCs was performed as described in a previous study (17). MSCs from the rats were allowed to attach to culture flasks. After two days, non-attached cells were washed away and the cells on flasks were cultured for 1-2 weeks until near-confluence. MSCs were cultured in DMEM, supplemented with 10% heat-inactivated FBS (both from Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 Ag/ml streptomycin at 37˚C in 5% CO_2 and 95% air using trypsin-EDTA solution (Invitrogen Life Technologies) to digest and detach MSCs. MSCs were used at passage three.

Rat aortic SMCs. Rat aortic SMCs (A-10, ATCC) were grown in low-glucose DMEM. Osteosynthesis-inducing medium (OS), used only in one osteoblastic differentiation assay, contained the above described medium with 0.1 μM dexamethasone, 10 mM sodium β-glycerol-phosphate and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA). The culture medium was removed and replaced with fresh medium three times a week. SMCs were cultured for 21 days after the formation of calcified nodules.

Co-culture conditions. Direct co-cultures were established by seeding smooth muscle cells (SMCs) or calcified SMCs and MSCs together at ratios of SMCs or calcified SMCs $15x10^4$; SMCs or calcified SMCs $5x10^4$: MSCs $10x10^4$, SMCs or calcified SMCs $10x10^4$: MSCs $5x10^4$/1.7 cm^2 into six-pore plates. OS was added in the culture medium in the groups of MSCs with non-calcified SMCs. Cells were cultured in low-glucose DMEM supplemented with 10% FBS at 37˚C in a 5% CO_2 incubator for 9 days. The medium was changed every 3 days. Each co-culture experiment was performed three times to validate the results. Direct co-cultures were established by seeding together two different cell types at three distinct cell density ratios and culturing the cells for 9 days. Osteoblastic differentiation was then evaluated by cell morphology, the activity of alkaline phosphatase (ALP) in cell lysates and ALP staining. Cell proliferation was evaluated by protein content. Additionally the inhibition of Wnt-signaling was investigated to determine whether the non-canonical signaling pathway, Wnt5a/Ror2, was expressed in each group.

Flow cytometry. Passage three MSCs were trypsinized, washed with PBS and incubated with fluorescein isothiocyanator phycoerythrin-conjugated monoclonal antibodies (BioLegend) specifically for CD29, CD90, CD45, CD11b or PBS in 4˚C for 30 min. Analysis was performed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA), using CellQuest software.

von Kossa staining. For von Kossa staining, cells were fixed with paraformaldehyde (Sigma-Aldrich) at 4˚C for 45 min. The fixed cells were incubated in 5% silver nitrate for 30 min in ultraviolet, and air-dried color development (black) was complete. Calcification was observed under light microscopy.

ALP staining. For alkaline phosphatase (ALP) activity analysis, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature, and then stained with the BCIP/NBT Phosphatase Substrate System (KLP, Gaithersburg, MD, USA) following the manufacturer's instructions and observed under light microscopy.

ALP activity and protein content. ALP activity was determined from all the samples after being cultured for 9 days. Samples were extracted into an assay buffer containing 50 mM Tris-HCl, 0.1% Triton X-100 and 0.9% NaCl (pH 7.6) and the lysate samples were frozen. Lysate samples were then thawed and enzyme activity was determined in duplicate using 0.1 M 4-p-nitrophenylphosphate as a substrate (Jiancheng, Nanjing, China). Absorbance was read at 492 nm in a plate reader (Bio-Rad, Hercules, CA, USA). The total protein contents were determined by Bio-Rad Protein Assay (Bio-Rad).

Ror2 mRNA expression. Total RNA was isolated from the samples using TRIzol reagent according to the manufacturer's instructions (Invitrogen) and reverse transcribed into cDNA using a Toyobo reverse transcription kit (Toyobo). The real-time quantitative PCR was carried out with the ABI PRISM 7900 sequence detector system (Applied Biosystems) according to the manufacturer's instructions. GAPDH was used as an endogenous control. PCR reaction mixture contained SYBR-Green I (Takara), cDNA, and the primers. Primer sequences used for real-time quantitative PCR were: Ror2, upstream: 5'-ATCCAAGACCTGGACACAACAGA-3' and downstream: 5'-GAACCCCAGTGGCAGTGATG-3'. Relative gene expression level (the amount of target, normalized to the endogenous, control gene) was calculated using the comparative Ct method formula $2^{-\Delta\Delta Ct}$.

Western blot analysis. Cells were incubated with NET buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, 20 mM NEM, 1% NP 40, pH 7.4). Total protein was quantified using a BCA protein assay kit (Pierce-Perbio Science, Tattenhall, UK). The primary antibody used was anti-Wnt5a [rabbit polyclonal anti-Wnt5a (ab72583); Abcam, Cambridge, MA, USA] at a dilution of 1:1,000. For the normalization of protein loading, anti-GAPDH (cat. no. KC-MM-1302) was used at a dilution of 1:10,000. Equivalent amounts of protein were resolved by SDS-PAGE electrophoresis. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked in 5% BSA and incubated with the relevant antibody. Western blotting was carried out using the chemiluminescence detection system (Bio-Rad).

Statistical analysis. Results were shown as the mean ± SEM. The significance of differences was estimated by ANOVA followed by Student-Newmann-Keuls multiple comparison tests. $P<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS software (version 11.0; SPSS Inc., Chicago, IL, USA).

Results

Cultured passage three MSC. Passage three rat MSCs were positive at CD29 and CD90, but negative at CD45 and CD11b and different from hemopoietic stem cells (18) (Fig. 1).

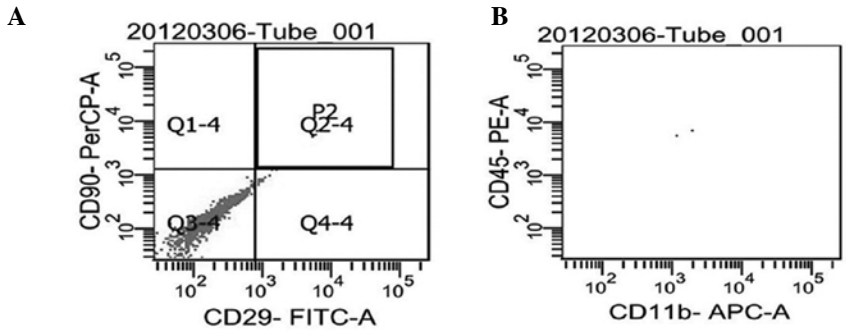

Figure 1. MSC cell-surface antigen analysis. (A) CD29 and CD90 expression was high at ~99.1 and ~96%, respectively, whereas the expression of (B) CD45 and CD11b was low at ~0.6%, respectively.

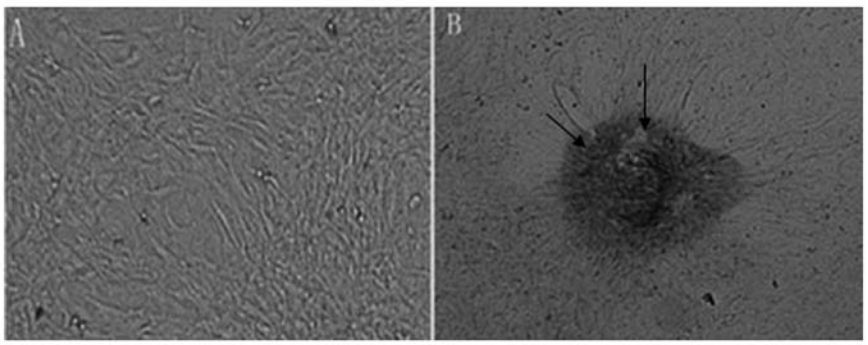

Figure 2. von Kossa staining showing SMCs cultured in medium with or without OS for 21 days. Representative photographs of von Kossa staining of SMCs cultured in media without OS. (A) No calcification is evident, (B, black arrowhead) black calcified nodules in SMCs cultured in media with OS. Original magnification, x100.

SMCs were cultured in medium with or without OS. SMCs were cultured in medium with or without OS for 21 days. Representative photographs of von Kossa staining are shown in Fig. 2. No calcification was detected in SMCs cultured without OS (Fig. 2A), whereas there were strongly positively stained black calcified nodules in SMCs cultured with OS (Fig. 2B, black arrowhead).

MSCs cultured in culture media with OS did not differentiate into an osteoblast phenotype when in direct contact with non-calcified SMCs, irrespective of the number of MSC. To determine whether non-calcified or calcified SMCs affected osteoblastic differentiation of co-cultured MSCs, ALP activity and ALP staining were quantified. A previous study demonstrated that OS significantly increased ALP activity when MSCs were cultured in the plastic culture flask (19). However, OS did not increase ALP activity when the cells were cultured in non-calcified SMCs. The results showed that the levels of all measured osteoblastic markers remained the same in the non-calcified SMCs directly co-cultured with MSCs at different cell ratios, ALP staining was negative, and there was no difference of ALP activity between the SMC15 (S15), SMC5:MSC10 (S5M10 + OS), and SMC10:MSC5 (S10M5 + OS) groups (Fig. 3).

MSCs cultured in media without OS differentiated into an osteoblast phenotype when in direct contact with calcified SMCs. A direct association was detected between the level of osteoblastic markers and the number of MSCs. By contrast, in the presence of MSCs, ALP activity ($P<0.05$) and ALP staining ($P<0.05$) were significantly higher in the calcified SMCs compared to the non-calcified SMCs in OS (Fig. 3). Moreover, the level of osteoblastic markers was dependent on the number of MSCs, with the level of osteoblastic differentiation of calcified SMC5: MSC10 (CS5M10) being higher compared to that of calcified SMC10: MSC5 (CS10M5) (Fig. 3B).

Wnt5a/Ror2 pathway may be associated with the differentiation fate of MSCs when in direct cell-cell contract with SMCs or calcified SMCs. Wnt5a protein is associated with the level of calcification, although it is rarely detected in non-calcification. Direct co-cultures were established by seeding together two different cell types at three distinct cell density ratios, culturing for 9 days, and examining the Wnt5a protein. Wnt5a protein was expressed in the groups CS15, CS5M10, CS10M5, with the highest expression evident in the CS5M10 group (Fig. 4). By contrast, no immunoreactivity for phosphotyrosine was detected in the groups S15, S10M5 + OS, while Wnt5a was slightly expressed in S5M10 + OS.

Ror2 mRNA in non-calcified cells was higher than that in calcified cells. Ror2 mRNA was quantitatively analyzed by real-time quantitative PCR. Ror2 mRNA in the groups with calcified SMCs was significantly reduced compared to that in groups S15, S5M10 + OS and S10M5 + OS ($P<0.001$) (Fig. 5). Compared to S10M5 + OS, Ror2 mRNA was expressed more

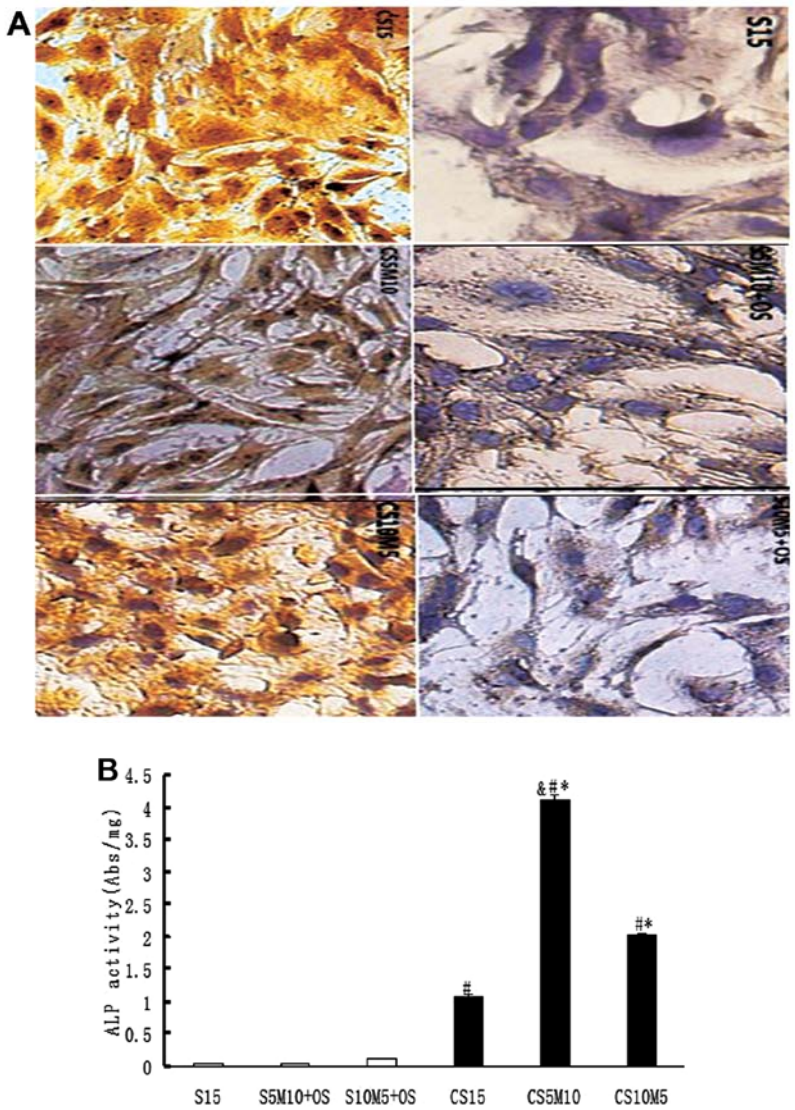

Figure 3. ALP staining (original magnification, x400) and ALP activity. Cells were seeded at a different cell density: SMCs or calcified SMCs $15x10^4$; SMCs or calcified SMCs $5x10^4$: MSCs $10x10^4$; SMCs or calcified SMCs $10x10^4$: MSC $5x10^4$; directly co-cultured for 9 days. ALP staining was positive when MSCs were directly co-cultured with calcified SMCs with a significant difference in the group of CS5M10, but negative when MSCs were directly co-cultured with non-calcified SMCs in culture medium with OS, with no significance detected when SMCs were cultured alone. ALP activity in the groups with calcified SMCs was higher compared to that in the groups with non-calcified SMCs ($^{\#}P<0.01$), ALP activity was higher in the group when MSCs were directly co-cultured with calcified SMCs compared to calcified SMCs cultured alone (CS15) ($^{*}P<0.05$). ALP activity in CS5M10 was higher than that in CS10M5 ($^{\&}P<0.01$).

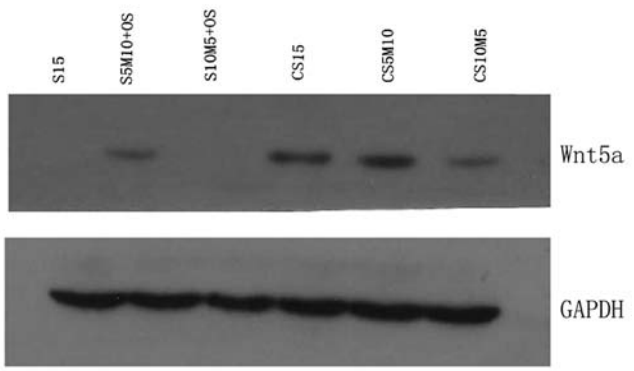

Figure 4. Western blotting shows expression of Wnt5a protein. Cells were seeded at a different cell density: SMCs or calcified SMCs $15x10^4$; SMCs or calcified SMCs $5x10^4$: MSCs $10x10^4$; SMCs or calcified SMCs $10x10^4$: MSCs $5x10^4$; directly co-cultured for 9 days. Wnt5a protein was expressed in the groups with calcified SMCs with the highest expression in CS5M10. However, Wnt5a was only slightly expressed in S5M10 in groups with no-calcified SMCs.

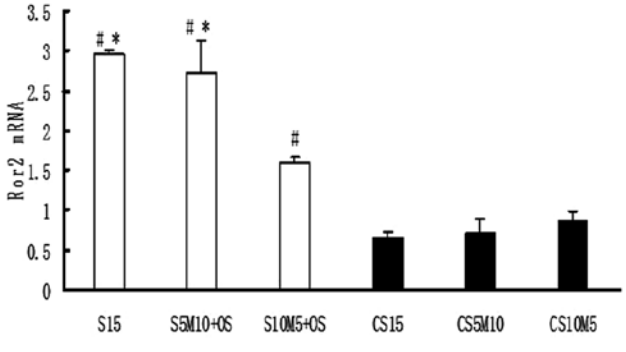

Figure 5. Ror2 mRNA expression. Cells were seeded at a different cell density: SMCs or calcified SMCs $15x10^4$; SMCs or calcified SMCs $5x10^4$: MSCs $10x10^4$; SMCs or calcified SMCs $10x10^4$: MSC $5x10^4$; directly co-cultured for 9 days. Ror2 mRNA in the groups with calcified SMCs was significantly reduced as compared to that in groups with non-calcified SMCs ($^{\#}P<0.001$). $^{*}P<0.05$ compared with S10M5 + OS. No difference was detected between CS15, CS5M10 and CS10M5.

in groups S15 and S10M5 + OS (P<0.05). Of note, there was no difference of Ror2 mRNA expression between CS15, CS5M10 and CS10M5.

Discussion

MSCs play a critical role in tissue regeneration and homeostasis (20). However, findings of previous study showed that circulating concentrations of stem-cell-mobilizing cytokines were associated with the levels of osteoprogenitor cells and aortic calcification severity (21). Major factors, such us local environment and resident cells, are likely to determine the fate of MSCs (8,22). A recent study has strongly suggested that cell-cell direct contact between resident cells and MSCs was critical in the differentiation of MSCs (12). Direct contact with distinct differentiated cells may be a critical determinant of mesenchymal stem cell fate in blood vessels and other connective tissues (8). To the best of our knowledge, this is the first study to examine whether MSCs should be co-cultured with non-calcified SMCs or calcified SMCs *in vitro* using a direct cell-cell co-culturing system. Direct cell contact affects the fate of MSCs (23). In this study, we found that MSCs did not differentiate into an osteoblast phenotype in the presence of non-calcified SMCs, whereas the spontaneous differentiation into bone-forming cells was observed when co-cultured with calcified SMCs, and dependent on the number of MSCs (Fig. 3).

However, the mechanism of action affecting this event remains to be determined. Many cell surface receptors, which have been used to create functional surfaces to enhance cell adhesion or alter cell morphology, have been identified, including cadherins (24) and cell adhesion molecules (CAM) (25). Wnt-signaling, which is mediated through cell-cell interaction and is involved in various developmental processes and cell functions (26), provides instructive cues for the recruitment, maintenance, and differentiation of MSCs (22). This signaling pathway on cell surface may be important in the differentiation of MSCs in direct cell-cell contact between MSCs and resident SMCs and calcified SMCs. It has been reported that canonical Wnt-signaling activates osteogenesis in mineralization under certain cellular contexts (27), and promotes the osteoblastogenesis of murine pluripotent mesenchymal and osteoprogenitor cells through the upregulation of RUNX2 (28) or osterix (29). However, canonical Wnt-signaling can be inhibited by the non-canonical Wnt-signaling pathway Wnt5a/Ror2 (16). Non-canonical signaling in general transduces through the c-Jun-N-terminal kinase (JNK)/planar cell polarity or the calcium-releasing pathways and regulates cell movement (30,31). Wnt5a, a member of the Wnt family is suggested to be involved in hydrophobic cell-cell interactions and is predominantly characterized as a non-canonical Wnt ligand that activates intracellular signaling via distinct receptors or co-receptors (31). Ror2, an orphan tyrosine kinase possessing an extracellular cysteine-rich Wnt-binding domain, has been shown to be a receptor for Wnt5a, inducing a non-canonical cascade involving the activation of JNK and inhibition of canonical signaling (30,32). The interaction between Ror2 and Wnt5a in order to mediate the non-canonical Wnt signaling pathway has received great attention in recent years (30,32). In addition, developmental phenotypes exhibiting an absence of Ror2 and Wnt5a lead to dwarfism, shortened limbs, facial abnormalities, ventricular septal defects in the heart, and abnormalities in lung development (33). In a previous study, it was found that Wnt5a may be involved in the pathogenesis of atherosclerosis as compared to protection from it (33), and that the expression of Wnt5a mRNA correlates with severity of atherosclerotic lesions (21). In this study, we investigated Wnt5a/Ror2 signaling in each group and observed that Wnt5a protein was expressed in the groups CS15, CS5M10 and SC10M5 with the highest expression being identified in CS5M10. By contrast, no immunoreactivity for phosphotyrosine occurred in the groups S15, S10M5 + OS, while Wnt5a was slightly expressed in S5M10 + OS (Fig. 4). Wnt5a protein was found to correlate with the severity of calcification, i.e., the more ALP activity, the more Wnt5a protein. Wnt5a was lightly expressed in S5M10 + OS (Fig. 4), likely due to the more MSCs in S5M10 + OS compared to S15 and S10M5 + OS.

The absence of Ror2 leads to enhanced Wnt/β-catenin signaling, specifically in cells that have lost Ror2 expression (34), indicating that the intracellular domain of Ror2 is required for functional Wnt5a/Ror2 signaling. Taken together, those studies have suggested that Wnt5a/Ror2 signaling may inhibit canonical Wnt signaling *in vivo,* suggesting that Ror2 is a potential therapeutic target for human disease (34). In this study, Ror2 mRNA in the groups with calcified SMCs was significantly reduced compared to that in groups S15, S5M10 + OS and S10M5 + OS (P<0.001). Compared to S10M5 + OS, a higher expression of Ror2 mRNA was observed in groups S15 and S10M5 + OS (P<0.05). However, no difference of Ror2 mRNA expression was detected between CS15, CS5M10 and CS10M5 (Fig. 5). Results of the present study show that the higher Ror2 mRNA expression, the less ALP activity of cells, and therefore less calcification since Ror2 mRNA in the groups with calcified SMCs was significantly reduced compared to that in groups S15, S5M10 + OS and S10M5 + OS (P<0.001). Compared to S10M5 + OS, a higher Ror2 expression of mRNA was detected in groups S15 and S10M5 + OS (P<0.05) (Fig. 5).

In summary, MSCs are able to differentiate into different cell phenotypes when in direct cell-cell contact with SMCs or calcified SMCs, depending on the number of MSCs when differentiated into calcified cell phenotype. Additionally Wnt5a/Ror2 signaling may be associated with determination of the differentiation fate of MSCs in this process. Wnt5a/Ror2 signaling and their regulators allow for the development of novel therapeutic strategies to prevent and treat valve and vascular calcification. Future studies should therefore focus on investigating the effect of Wnt5a/Ror2 signaling pathway in vascular calcification and stem cell therapy.

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