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# Role of Urokinase-Type Plasminogen Activator Receptor in the Regulation of Angiogenic Properties of Sca1+ Vasculogenic Progenitor Cells

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## Summary

Neoangiogenesis is the key process determining myocardial regeneration after infarction. The urokinase-type plasminogen activator receptor (uPAR) is known to play an important role in the regulation of endothelial cell function and postnatal angiogenesis. However, uPAR its involvement in the regulation of the properties of vascular progenitor cells remains poorly studied.

**Aim:** to evaluate uPAR expression on the surface of resident cardiac vascular progenitor cells (rcVPCs) and its impact on angiogenic cell properties in vitro as well as postinfarction cardiac vascularization.

**Materials and Methods.** We used immunofluorescent analysis of cryosections of a murine myocardial infarction model to characterize vessels and rcVPCs, and evaluated the angiogenic properties potential of vasculogenic progenitor cells using the «tube assay» and induction of inducing differentiation in a specialized medium.

**Results.** We have found that the majority of Sca-1+ rcVPCs express the urokinase receptor and endothelial cell markers on their surface and are capable of proliferation and integration into the newly formed vessels in the injured area, indicating their possible involvement in the contribution to vascularization process after infarction. After acute ischemic injury, the accumulation of vasculogenic progenitor cells (8+2 and 27+7 cells per visual field, respectively; *P*=0.032) and vascularization processes (85+11 and 166+25 capillaries per visual field, respectively; P=0.033) were observed in myocardium of uPAR-/- animals, compared with wild-type animals. Our studies demonstrated that Sca-1+ rcVCPs derived from uPAR-/- murine hearts demonstrated a reduced ability to form capillary-like structures and endothelial differentiation compared with Sca-1+ rcVCPs from hearts of wild-type mice.

**Conclusion.** Thus, uPAR deficiency may lead to impaired vasculogenic properties of Sca-1+ rcVCPs, which is likely due to the loss of regulatory influence of specific ligands and the ability to interact with signaling mediators such as integrins. From the viewpoint of regenerative medicine, the modulation of uPAR activity can be considered as a potential target promising approach for targeted regulation of vasculogenic progenitor cells properties and postnatal angiogenesis.

## HIGHLIGHT

The urokinase-type plasminogen activator receptor is involved in the regulation of the angiogenic properties of Sca1+ vasculogenic progenitor cells.

***Keywords: urokinase receptor; vasculogenic cells; vasculogenesis; angiogenesis***

**Conflict of interest.** The authors declare no conflict of interest.

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## Introduction

Myocardial infarction is characterized by massive myocardial cell death and associates with genetic, molecular and cellular alterations, leading to changes in cardiac structure and size and causing gradual loss of heart function [1–3]. Acute ischemic damage of cardiac muscle triggers reparative response with the formation of a new vascular network and restoration of tissue perfusion being an integral component of it. The classical mechanism of vasculogenesis, i.e., new vessel formation from circulating endothelial progenitor cells from bone marrow, has been shown to contribute minimally to the revascularization of the damaged myocardium [4, 5]. Formation of new vessels occurs exclusively due to the endogenous pool of endothelial cells and resident cardiac vasculogenic progenitor cells (rcVPCs) present in the myocardium [4]. Several research groups have isolated and characterized cardiac-derived rcVPCs based on the expression of the Sca-1 surface marker. This population of cardiac cells is characterized by lack of expression of endothelial (CD31) and hematopoietic markers (CD45), they exhibit a profile of mesenchymal cell surface markers (CD34-, CD29+, CD90+, CD105+ and CD44+) and have been reported to be capable to differentiate toward endothelial and smooth muscle cells [6–8]. Despite a long history of studying these cells, the precise mechanisms controlling the vasculogenic behavior of rcVPCs remain poorly understood.

This study suggested that the urokinase-type plasminogen activator receptor (uPAR) may be involved in the regulation of rcVPC status. uPAR is anchored in the membrane via the GPI anchor, which ensures its mobility within the membrane bilayer and allows local concentration of urokinase proteolytic activity toward the cell movement. The cascade of proteolytic reactions triggered by urokinase, including local formation of plasmin and activation of matrix metalloproteinases, promotes the destruction of the extracellular matrix in the path of the moving cell, the activation of growth factors, and the release of growth factors sequestered in the matrix [9–11]. However, in addition to the activation of extracellular proteolysis, most cellular responses modulated by the urokinase system require transmembrane signaling, which is mediated by the interaction of uPARs with intermediary proteins that provide signal transmission via intracellular pathways regulating cell status.

The aim of the study was to evaluate the expression of uPAR on the surface of rcVPCs and uPAR impact to the in vitro angiogenesis and postinfarction cardiac vascularization.

## Material and Methods

**Animals.** Male C57BL/129 mice (wild-type) and uPAR gene knockout mice (uPAR-/- mice) [12], provided on a free-of-charge basis by the Faculty of Fundamental Medicine of the Lomonosov Moscow State University, were used in this study. Animal genotyping was performed by PCR in accordance with the protocol of the developer company. The experiments were approved by the ethical committee of Cardiology Research Medical Center.

**Myocardial infarction modeling.** Experimental myocardial infarction was induced according to the protocol described earlier [13]. There were 15 mice in each group (C57BL/129 (wild-type) and uPAR gene knockout (uPAR-/- mice)).

**Vessel detection in the murine myocardium.** Cardiac cryosections were fixed in 4% paraformaldehyde solution, washed in PBS buffer solution, stained with antibodies to von Willebrand factor (vW) (BD, USA) for 1 h, then washed and stained with antibodies conjugated with Alexa Fluor 594 (Invitrogen, USA). For the detection of smooth muscle alpha-actin, additional staining with antibodies conjugated with FITC dye (Sigma) was performed. Characterization of Sca-1+ rcVPCs was performed by staining the samples with antibodies to Sca1 (Biolegend, USA), uPAR (Santa Cruz, USA), CD34 (Abcam, USA), CD34 (Abcam, USA) markers for 1 h, then washing and staining with antibodies conjugated to Alexa Fluor 488, 594 (Invitrogen, USA). Cell nuclei were stained with DAPI (Sigma, USA). Vessels morphometric analysis was performed by counting the number of vW+ capillaries and vW+Sca1+ vessels per visual field using the Image J software (NIH, USA).

**Sca-1+ rcVPCs culture establishment.** To obtain Sca-1+ rcVPCs, murine hearts (Wt and uPAR-/- ) were removed from the thoracic cavity, washed in Krebs-Ringer solution with heparin, transferred into enzymatic solution (collagenase A (Roche), working concentration 1 mg/ml), and incubated in a Hybaid (Thermo Scientific, USA) shaker 2 times for 30 min at 37°C. After that, hearts were withdrawn, 5 ml of enzyme inactivation medium was added to the obtained cell suspension, and centrifuged at 300 g for 10 min. The precipitate was resuspended in the IMDM medium containing 2% fetal calf serum, B27 supplement, 20 ng/ml EGF and 40 ng/ml bFGF and transferred to gelatin-coated cups. The next day, the resultant cell culture was used to perform immunomagnetic selection using commercial selection kits and MS columns from Miltenyi biotec. At the first stage, hematopoietic cell depletion was performed using «Lineage Cell Depletion Kit» and then positive selection of Sca-1+ rcVPCs using the Cardiac Progenitor Cell Isolation Kit (Sca-1) was completed. Immunomag-

netic selection was performed according to the reagent kit manufacturer's recommendations.

**Formation of capillary-like structures of Sca-1+ rcVPCs on Matrigel surface.** The ability of Sca-1+ rcVPCs to endothelium-like behavior was evaluated using the model of formation of capillary-like structures on Matrigel surface. Cooled Matrigel (BD Bioscience, USA) in 350 μL volumes was applied to a 24-well culture plate and incubated for 1 h at 37°C until complete polymerization to gel texture. Cells were removed from the culture plates using Accutase solution and resuspended in EGM-2 (Endothelial Cell Growth Medium-2) supplemented with VEGF (10 ng/ml). Endothelial cells cultured in EGM-2 supplemented with VEGF (10 ng/ml) were used as a positive control. The cells were plated in wells with Marigel in the number of 80,000 per well. After 5 hours, the cells were fixed with 1% formalin solution. Microphotographs of 5 randomly selected fields in each well were obtained using an Axiovert 200M inverted microscope (Zeiss, Germany). Image J software (NIH, USA) was used for calculations.

**Differentiation of Sca-1+ rcVPCs into endothelial cells.** To induce cell differentiation in the vascular direction, the differentiation medium described earlier [14] was used (DMEM/F12 supplemented with 10% fetal calf serum, insulin-transferrin-selenite, and 10 ng/ml VEGF). The differentiation medium was replaced every 24 hours. The cells were cultured for 14 days. Endothelial differentiation was tested by immunofluorescent staining of cells with von Willebrand factor antibodies (DAKO, USA) and secondary antibodies conjugated with fluorescent labeling. Quantification of cells differentiated in the endothelial direction was performed using Image J software (NIH, USA).

**Microscopy and image analysis.** Myocardial cells and cryosections were analyzed using an Axiovert 200 M fluorescence microscope (Carl Zeiss, Germany) and AxioVision 3.1 software (Carl Zeiss, Germany).

**Statistical analysis.** The normality of the data distribution was assessed using the Kolmogorov–Smirnov test. Significance of differences between the samples was assessed using Mann–Whitney *U*-criterion. Statistical analysis of the results was performed using Statistica 8.0 software (StatSoft, Inc.). Data were presented as mean±standard deviation (*M*±*SD*).

## Results

On day 5 post myocardial infarction, there was a 2-fold decrease in the total number of capillaries in uPAR-/- animals in the necrotic area (Fig. 1, *c*), compared with wild-type animals (85+11 and 166+25 capillaries per visual field, respectively; *P*=0.033), indicating impaired myocardial vascularization after acute ischemic injury.

Taking into account the identified differences in the formation of vessels, the content of vasculogenic progenitor cells which may participate in this formation, was analyzed. Compared with wild-type mice, a 3-fold decrease in the number of Sca-1+ progenitor cells (Fig. 1, *a, d*) (27+7 and 8+2 cells per visual field, respectively; *P*=0.032) that can differentiate in the vascular direction and release proangiogenic growth factors was observed in uPAR-/- animals [6]. Most Sca-1+ rcVPCs expressed the urokinase receptor on their surface (Fig. 1, *b*) and were characterized by a lack of hematopoietic cell markers (CD34 and CD45), which precludes their bone marrow origin. We found that some Sca-1+ rcVPCs co-localized with endothelial cell markers (CD31, vW) and were part of the newly formed vessels in both the necrosis zone and the peri-infarct area.

In view of the identified signs of reduced vascularization of the injured zone in uPAR-/- animals, we conducted experiments to evaluate the angiogenic properties of these cells in vitro (Fig. 2).

Our studies showed that Sca-1+ rcVPCs obtained from the hearts of uPAR-/- mice showed a reduced ability to form capillary-like structures compared with Sca-1+ rcVPCs from the hearts of wild-type mice (Fig. 2, *a, b*). Morphometric calculations showed (Fig. 2, *c, d*) that the total length of vascular structures (57969+6998 (Sca1+uPAR-) and 83302+6464 (Sca1+Wt) relative units; *P*=0.037) and their branching capacity (1900+397 (Sca1+uPAR-) and 3322+501 (Sca1+Wt); *P*=0.036) was reduced in Sca-1+ rcVPCs compared with control cells. The impaired endothelial-like behavior associated with impaired endothelial differentiation ability of Sca-1+ rcVPCs induced by the dedicated medium (Fig. 3). Culturing Sca-1+ rcVPCs (from uPAR-/- and wild-type murine hearts) promoted the formation of capillary-like structures. Meanwhile, the ability of Sca-1+ rcVPCs obtained from uPAR-/- hearts to form vWF+ vascular structures was 5 times lower compared to cells from wild-type hearts (7+4 (Sca1+uPAR-) and 33+17 (Sca1+Wt) von Willebrand+ cell structures per visual field; *P*=0.01) (Fig. 3).

## Discussion

Our research show that some Sca-1+ rcVPCs express endothelial cell markers on their surface and are capable of proliferation and integration into the newly formed vessels in the injured zone, indicating their possible participation in vascularization after infarction. Urokinase receptor was found on the surface of most Sca-1+ rcVPCs, which can participate in the regulation of rcVPC function through interaction with urokinase and/or vitronectin [15, 16]. When uPARs interact with specific

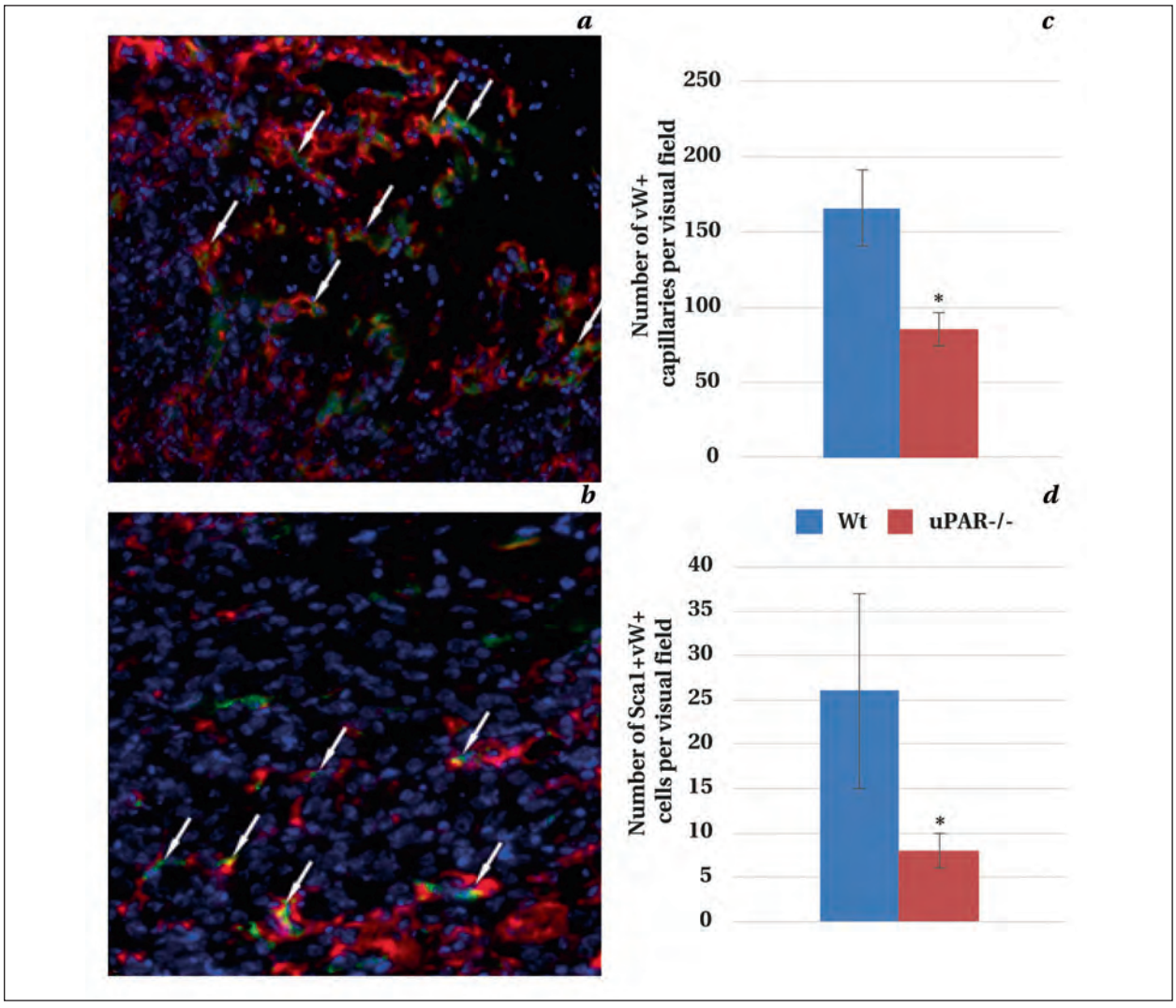


**Fig. 1. Sca1+ resident cardiac vasculogenic progenitor cells express von Willebrand factor (vW) and urokinase receptor (uPAR).** **Note.** *a* — vW (red), Sca1 (green). *b* — uPAR (red), Sca1 (green). Yellow staining indicates co-localization of signals. c — quantitative assessment of capillary content in the zone of postinfarction necrosis (day 5 after myocardial infarction) in wild-type and uPAR-/- mice. *d* — quantitative assessment of Sca1+vW+ capillary content in the zone of postinfarction necrosis (day 5 after myocardial infarction) in wild-type and uPAR-/- mice. For Fig. 1–3, data are presented as mean±standard deviation (*M*±*SD*). * — $P<0.05$.

ligands [17–19], intracellular signaling cascades are activated, promoting adhesion, cell proliferation, and vascular differentiation [20–22]. Consequently, this kind of interaction might serve as a potential stimulus for the regulation of rcVPC functions. Indeed, reduced accumulation of Sca-1+ vasculogenic progenitor cells and impaired postinfarction vascularization were observed in the heart of uPAR-/- animals after acute ischemic injury compared with wild-type animals. In addition, Sca-1+ rcVPCs derived from uPAR-/- mice hearts exhibited reduced endothelium-like behavior (formation of capillary-like structures) and angiogenic differentiation in vitro, compared with Sca-1+ rcVPCs from wild-type mouse hearts. Disrupted interactions between uPARs and integrins result in the suppression of the activity of Rac and Cdc42 minor Rho GTPases, thereby inhibiting their participation in the rearrangement of the cytoskeleton and cell motility, which leads to the loss of the cells' ability to integrate into the forming vessels and participate in vasculogenesis [23]. The revealed changes are similar to the ones observed in vasculopathy in patients with systemic scleroderma, where uPAR cleavage between the first and second domains by MMP12 is seen [24, 25]. Overproduction of MMP12 in endothelial cells and uPAR dysfunction led to suppression of uPA-induced migration, invasion, proliferation of vascular cells and formation of capillary-like structures on matrigel surface [26, 27]. Moreover, adding the anti-MMP-12 monoclonal antibodies promoted recovery of endothelial cell angiogenic activity including ability to migrate, invade and form vascular structures [24, 27]. Furthermore, the same researcher

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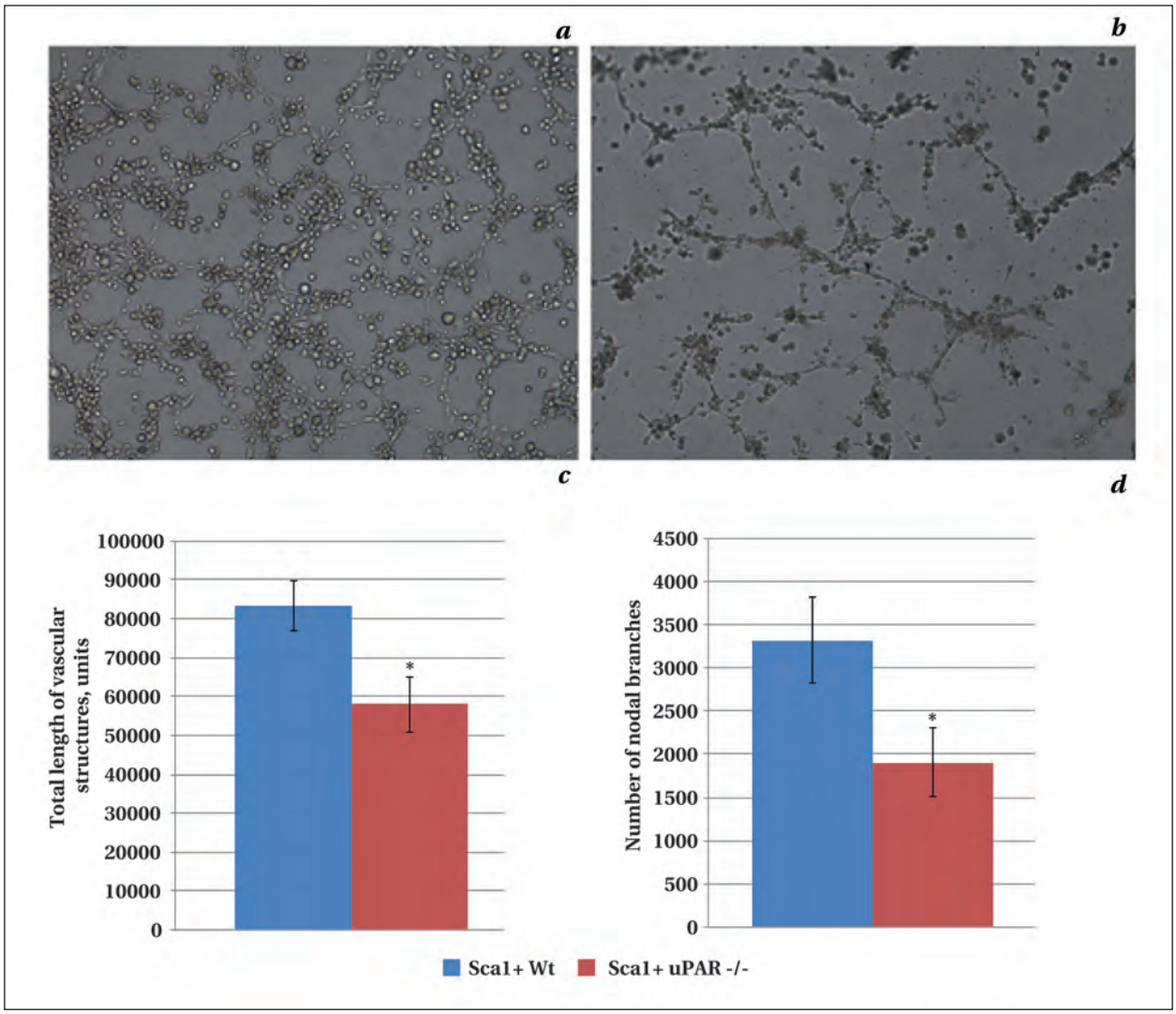


**Fig. 2. Representation of the ability of Sca-1+ rcVPCs isolated from wild-type murine hearts (*a*) and uPAR-/- (*b*) to form capillary-like structures in vitro.**
**Note.** *c, d* — quantitative assessment of the total length of vascular structures formed by Sca-1+ rcVPCs from the hearts of uPAR-/- and wild-type mice and their branching ability.* — $P<0.05$.

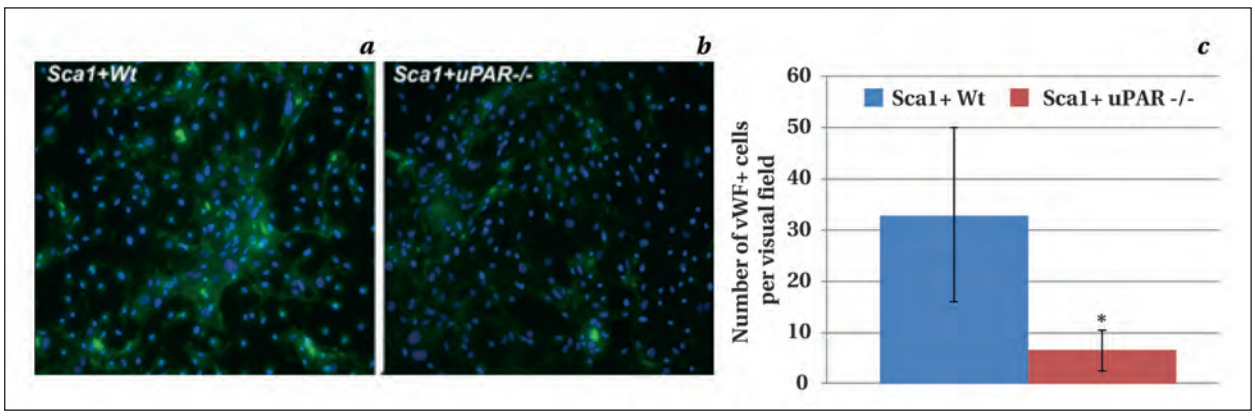


**Fig. 3. Representation of the differentiation ability of Sca-1+ rcVPCs isolated from the hearts of uPAR-/- and wild-type mice.**
**Note.** *a, b* — staining of Sca-1+ rcVPCs with antibodies to von Willebrand factor (vWF) (endothelial cell marker) (green) after cultivation in differentiation medium. *c* — quantification of the number of vWF+ structures after cultivation in differentiation medium. * — $P<0.05$.

team showed that uPAR cleavage in endothelial cells in systemic scleroderma leaded to loss of integrin-mediated uPAR binding to the actin cytoskeleton [28–30] thus abrogating a key step in vascular formation.

## Conclusion

The uPAR deficiency leads to impaired vasculogenic properties of Sca-1+ rcVPCs, which is probably associated with the loss of regulatory effect of specific ligands and the ability to interact with signaling mediators, such as integrins. From the viewpoint of regenerative medicine, modulation of uPAR activity can be considered as a potential target for specific regulation of the vasculogenic progenitor cells functions and postnatal angiogenesis.

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