Title: Equine Mesenchymal Stromal Cells retain a pericyte-like phenotype

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Running title: MSCs retain a pericyte-like phenotype

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Abstract

Mesenchymal Stem/Stromal Cells (MSCs) have been used in human and equine regenerative medicine, and interest in exploiting their potential has increased dramatically over the years. Despite significant effort to characterize equine MSCs, the actual origin of these cells and how much of their native phenotype is maintained in culture have not been determined. In this study, we investigated the relationship between MSCs, derived from adipose tissue (AT) and bone marrow (BM), and pericytes in the horse. Both pericyte (CD146, NG2 and αSMA) and MSC (CD29, CD90 and CD73) markers were detected in equine adipose tissue and co-localized around blood vessels. Importantly, as assessed by flow cytometry, both pericyte (CD146, NG2 and αSMA) and MSC (CD29, CD44, CD90 and CD105) markers were present in a majority (≥90%) of cells in cultures of AT- and BM-MSCs, however, levels of pericyte markers were variable within each of those populations. Moreover, the expression of pericyte markers was maintained for at least 8 passages in both AT- and BM-MSCs. Hematopoietic (CD45) and endothelial (CD144) markers were also detected at low levels in MSCs by qPCR. Finally, in co-culture experiments, AT-MSCs closely associated with networks produced by endothelial cells, resembling the natural perivascular location of pericytes in vivo. Our results indicate that equine MSCs originate from perivascular cells and moreover maintain a pericyte-like phenotype in culture. Therefore, we suggest that, in addition to classical MSC markers, pericyte markers such as CD146 could be used when assessing and characterising equine MSCs.
Introduction

Horses and humans are the two species in which interest in the therapeutic use of Mesenchymal Stem/Stromal cells (MSCs) is highest. In the horse, the last decade saw a dramatic increase in the use of MSC preparations [1,2], in particular for repair of musculoskeletal injuries [3-6], where conventional treatments have limited efficacy. The main clinical application of equine MSCs has been tendon and ligament repair [7-10], but other conditions such as joint disease and laminitis are also being considered [11]. Importantly, because musculoskeletal pathophysiology in horses resembles that in humans, the US Food and Drug Administration has approved the use of horses as a large animal preclinical model, which broadens the relevance of the findings obtained in equine.

Equine MSCs used clinically are mainly derived from bone marrow (BM) and adipose tissue (AT) [2,12,13]. BM samples are processed using a density gradient to isolate the mononucleated cell fraction while AT extracts are obtained by collagenase digestion [14]. Both BM and AT extracts contain heterogeneous cell populations that can be cultured and characterized for their MSC properties. Contrary to human MSCs, there are no standard guidelines to define equine MSCs and, therefore, the minimum criteria established in 2006 by the International Society for Cellular Therapy (ISCT) are commonly applied [15]. Specifically, ISCT guidelines state that MSCs must grow adherent to plastic and have the ability to differentiate into adipocytes, osteocytes and chondrocytes. Guidelines for adipose-derived MSC
surface markers have been more recently updated [16] to include CD44 in addition to CD105, CD73 and CD90, while CD45 and CD31 must be absent. In relation to equine MSCs, although tri-lineage differentiation is usually demonstrated, a variable immunophenotype is often reported for cells of different tissue origins, and additional makers such as CD29 are being considered to characterize MSCs [1,17-21].

Despite significant work to characterize their properties, the precise in vivo origin of equine MSCs and whether they maintain their original phenotype in culture have not been determined. Pericytes are perivascular cells, also known as Rouget or mural cells, and are embedded in the basement membrane of small blood vessels, capillaries and microvessels [22-24]. In humans, isolated pericytes expressing CD146, NG2, PDGFRβ and αSMA have been described as a native source of MSCs [25], but it is still not clear how the pericyte phenotype is maintained in cultured MSCs obtained from heterogeneous cell extracts of AT and BM.

To further understand the nature of equine MSCs, in this study we investigated the relationship between equine MSCs and pericytes both in native tissues and in culture, including a comparison between AT-MSCs and BM-MSCs.

Methods

Samples

Samples were obtained immediately post-mortem from 8 adult horses. All animal procedures were carried out according to the UK Home Office Animals (Scientific Procedures) Act 1986 with approval by the Ethical Review Committee, University of
Edinburgh. Tissues (adipose, bone marrow and large blood vessels) were kept on ice until cell extraction or were frozen in cold isopentane mounted in optimum cutting temperature compound (OCT; VWR Chemicals, Leicestershire, UK) for immunohistochemistry (IHC). Subcutaneous adipose tissue was obtained by dissection from the abdominal region close to the linea alba.

**Immunohistochemistry**

Samples were processed in a Leica CM1900 cryostat, allowed to air dry and processed right away or kept frozen at -80°C. For staining, tissues were fixed in ice cold acetone:methanol (50:50) and antibodies were prepared in diluent (003118; Invitrogen-Thermo Fisher Scientific, Paisley, UK) and, after staining, slides were mounted in fluoroshield with DAPI (Sigma-Aldrich, St Louis, USA). Primary antibodies used in the study are listed in table 1 and isotype controls were mouse IgG1κ 400101 (BioLegend, San Diego, USA), mouse IgG1 MCA928F (AbD Serotec-BioRad, Kidlington, UK), mouse IgG1 MAB002 (R&D Systems, Minneapolis, USA), mouse IgG1κ 557273 (BD, Oxford, UK), rabbit PRABP01 (AbD Serotec-BioRad). Secondary antibodies were AF488-conjugated (A11008 and A11029) and AF568-conjugated (A110037 and A10042). Isotypes and secondary antibodies alone were used as controls. Micrographs were produced using a Zeiss LSM710 confocal or Leica DMLB fluorescent microscope.

**Cell extraction and culture**
Adipose tissue samples were minced and digested for 45 min with collagenase II (1 mg/ml; Gibco-Thermo Fisher Scientific)/BSA (3.5%) at 37°C under agitation (100 rpm). Collagenase activity was stopped by addition of DMEM with 20% FBS (Thermo Fisher Scientific) and the lipid layer was removed after separation by gravity. BM-MSCs were obtained by aspiration of sternum marrow followed by centrifugation on a density gradient, and the mononucleated cell layer was harvested. Endothelial cells were harvested by tying up both ends of a small piece of blood vessel which had been washed with PBS and filled with collagenase II (1mg/ml). After a 45 min digestion, endothelial cells were collected and cultured.

To determine colony-forming unit-fibroblasts (CFU-F), cells were seeded at a density of 1, 5 and 25 cells/cm² and allowed to grow for 11 days, in tissue culture dishes of 55cm² growth surface area. Colonies were visualized by staining for phosphatase alkaline activity using a commercial kit (86R-1KT, Sigma-Aldrich). Briefly, cells were washed twice with PBS, then fixed with PFA-citrate acetate buffer for 30s and washed again with PBS, to which the alkaline-dye mixture was added.

**RNA extraction and gene expression analyses**

Cultured cells were harvested in Trizol (Thermo Fisher Scientific) and RNA was reverse transcribed to cDNA using SuperScript III (18080-044; Invitrogen-Thermo Fisher Scientific). PCR was performed using SensiFAST SYBR Lo-ROX kit (Bioline, London UK) and equine primers designed by us, specifically to recognize PDGFRβ (5’-GTGCCTACAAAGGCTCCCAT-3’ and 5’-CACAGTGGGATCTGGCACAA-3’), CD144 (5’-
TCTGCAGGACATCAATGA-3’ and 5’-CTTCAGGCACGGCAATACG-3’), NG2 (5’-GCGCATCATTGGGCCCTACTT-3’ and 5’-GCTGTCCACCTCTCTCCAG-3’), and 18S (5’-GCTGCCACCAGACTTG-3’ and 5’-GGGAATCAGGGTTCG-3’) or obtained from the literature [20]. Reactions proceeded in a MX3005P qPCR system (Stratagene, CA, USA). Results were analysed with Stratagene MxPro software and normalized to 18S levels quantified in the same samples.

Flow cytometry

Flow cytometry was performed in samples of AT- and BM-MSCs stained at 4°C with primary antibody for 1h and, when necessary, with secondary antibody for 30min. Primary antibodies are listed in Table 1. Secondary antibodies were AF488-conjugated (A11029; Invitrogen), AF405-conjugated (Ab175654; AbCam, Cambridge, UK) or APC-Cy7 conjugated (sc-3847; Santa Cruz, Paso Robles, CA, USA).

Isotype controls used were mouse IgG1κ 400101 (BioLegend), mouse IgG1 MCA928A647 (AbD Serotec-BioRad), mouse IgG1 MAB002 and IgG1 IC002A (R&D Systems), or rabbit PRABP01 (AbD Serotec-BioRad). Sytox blue (Thermo Fisher Scientific) was used as live cell stain. Isotypes and secondary antibodies alone were used as controls. Samples were run on a BD LSFortessa or BD FACSaria Fusion (BD Biosciences, San Jose, USA) and data analysed on FlowJo_V10 (LLC, Ashland, USA).

Cell differentiation
AT- and BM-MSC were differentiated into adipocytes, osteocytes and chondrocytes. Adipogenesis was induced by DMEM/F-12 (Life technologies) containing 7% rabbit serum (Gibco-Thermo Fisher Scientific), 3% FBS (Invitrogen), 1% Penicillin/Streptomycin (Gibco-Thermo Fisher Scientific), 1μM Dexamethasone (Sigma Aldrich), 0.5mM IBMX (Sigma Aldrich), 10μg/ml Insulin (Sigma Aldrich) and 100μM indomethacin (Sigma Aldrich). Adipocytes were stained with Oil red O (Sigma-Aldrich) as described before [26]. Chondrogenesis and osteogenesis were induced using StemPro differentiation kits following manufacturer’s instructions for 18 days (A10071-01 and A10072-01, respectively, Life Technologies). Chondrocyte pellets were embedded in Histogel (HG-4000; Invitrogen) and paraffin, and cut at 8 μm thickness. Slides were dewaxed and rehydrated and Alcian blue solution (1%) was added to the slide and incubated overnight. Then, the stain was removed and the slides were washed. Neutral red (1%) solution was added and incubated for 1 min and rinsed in water. Absolute ethanol and xylene were used to dehydrate the slides that were mounted with pertex. Osteogenic cultures were washed with PBS and fixed with PFA (4%) for 15 min and stored in PBS at 4°C. After washing with water, Alizarin Red S (A5533; Sigma-Aldrich) prepared in water with pH adjusted to 4.2 was added to cover the cellular monolayer. The plate was incubated at room temperature in the dark for 10min and washed with water. Negative controls were produced with non-differentiated cells and micrographs were taken in an Axiovert 25 Inverted Microscope (Zeiss, Oberkochen, Germany).
Angiogenesis

The fluorescent dyes PKH26 (20 µM) and PKH67 (20 µM) (both from Sigma-Aldrich) were used to label AT-MSC (red) and equine endothelial cells (green). Once labelled, cells were re-suspended in EGM-2 medium and seeded on ibiTreat µ-Slides (IB-81506; Thistle Scientific) coated with matrigel. Pictures were taken using a Zeiss Live Cell Observer/Deconvolution system.

Statistical Analysis

Results are shown as mean ± standard error of the mean, and were analysed by Students’ t-Test or Two-Way ANOVA followed by Tukey test using GraphPad Prim 6.0 software (GraphPad Software, La Jolla, CA, USA). Significance was set at p<0.05.

Results

Localization of pericytes in equine adipose tissue

The relative location of pericyte (CD146, NG2 and αSMA) and MSC (CD29, CD73 and CD90) markers was examined by immunohistochemistry in equine adipose or testis (Fig. 1 and Fig. S1). CD146 and NG2 were detected around small blood vessels as evidenced by the presence of adjacent endothelial cells stained with CD144 (Fig. 1A, B) in agreement with previous results from human samples [27]. Other cell types in the tissue (Fig. S1A, B) were negative for these pericyte markers, as well as the isotypes and secondary antibody controls (Fig. S1D-F), showing cross-reactivity by
CD146 and NG2 antibodies. αSMA was also perivascular (Fig. 1C), while none of the five different PDGFRβ antibodies tested gave a specific signal (data not shown). The classical MSC, which have been tested before in equine tissues [19], were also located around small blood vessels, as shown by the results of CD29 and CD90 (Fig. 1D, E). In addition, dual staining with antibodies against NG2 and CD29, or CD146 and CD73 (Fig. 1F, G and S1B) confirmed the co-localization of pericytes and MSC markers in equine adipose tissue, consistent with the notion that pericytes may give rise to MSCs in culture.

Characterization of AT- and BM-MSCs

Extracts from equine AT and BM were obtained by collagenase digestion and density gradient, respectively, and the resulting cells grew in uncoated tissue culture vessels showing a characteristic spindled-like morphology (Fig. 2A). In order to test the ability of AT- and BM-MSCs to form CFU-Fs, cells were seeded at 1, 5 and 25 cells/cm². After 11 days both AT- and BM-MSCs formed colonies (49.5±13.9 and 43.5±10.5 CFU-F, respectively, for cells seeded at a density of 5 cells/cm²) which were positive for alkaline phosphatase (Fig. 2B), as reported before for human MSCs [28] and pericytes [27,29].

Both AT- and BM-MSCs were multipotent. Adipogenic capacity was evidenced by Oil Red O staining showing accumulation of lipids in the cytoplasm (Fig. 2C), osteogenesis was evidenced by the dark red staining of calcium deposits produced by
Alizarin red S (Fig. 2D) and chondrogenesis was shown by Alcian blue staining of cartilage matrix in cell micromasses (Fig. 2E).

The expression of typical MSC markers was confirmed by flow cytometry (Fig. 3A) by using antibodies that have previously been validated or used in other equine studies [19,20]. The results showed that ≥99% of the cells were positive for CD29, CD44, CD90 and CD105 in both AT- and BM-MSCs. qPCR analyses confirmed comparable expression of CD44, CD73, CD90 and CD105 by the two cell types (Fig. 3B). Moreover, the haematopoietic maker, CD45, was not detected in AT-MSCs but was present at low levels in all BM-MSC cultures. Low levels of expression of the endothelial cell marker, CD144, were observed in both AT- and BM-MSCs, while CD31 was not detected. Overall, these results showed that our cells fulfilled the criteria for MSCs and confirmed that AT- and BM-MSC preparations are heterogeneous in nature.

**AT- and BM-MSCs express pericyte markers in culture**

Following from our finding that MSC and pericyte markers co-localize in equine tissues (Fig. 1) we wanted to determine whether the expression of pericyte markers would be maintained by MSCs in culture by using complementary data from flow cytometry and qPCR, and in order to do this we validated further the pericyte antibodies (Fig. S2). Flow cytometry showed the pericyte markers, CD146 and NG2, to be highly expressed (≥94.6%) in AT- and BM-MSCs, while αSMA was present at slightly lower levels (≥89.9%). Histograms showed a broad distribution in the
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**Discussion**

The perivascular location of pericytes in vivo (Fig. 1).

Stages AT-MSCs retained closely associated to the endothelial network, resembling endothelial cells beginning even before a network formed and, importantly, at later which became more condensed, in co-cultures, AT-MSCs tended to co-locate with quickly forming dense cores of cells that were interlinked by thin bridging extensions strands (Fig. 5). AT-MSCs cultured in the absence of endothelial cells organized network was important after seeding and became increasingly organized with dense cultured AT-MSCs and endothelial cells on matrigel in endothelial-only cultures, a cell culture AT-MSCs at passages 2 and 8, BM-MSCs and while CD146 and PDGFRβ levels remained unchanged across passages in both MSC types, NG2 expression decreased expressed at similar levels in AT- and BM-MSCs, and while CD146 and PDGFRβ levels in AT- and BM-MSCs at passages 2 and 8 (Fig. 4B). All pericyte markers were extended culture, we quantified the expression of CD146, NG2 and PDGFRβ by qPCR determined whether pericyte markers were maintained by MSCs during time determi...
Clinical MSCs are commonly obtained through culture of crude (non-purified) extracts typically from bone marrow or adipose tissue. Although equine MSCs are widely used clinically, there is a significant lack of knowledge regarding their in vivo origin, identity and maintenance of original phenotype in culture. This has hindered efforts to characterize equine MSCs as well as exploit their full therapeutic potential. Studies performed in humans [25,27,30] have shown that pericytes, a cell type extracts is obtained from bone marrow or adipose tissue. Although equine MSCs are widely used clinically, there is a significant lack of knowledge regarding their in vivo origin, identity and maintenance of original phenotype in culture. This has hindered efforts to characterize equine MSCs as well as exploit their full therapeutic potential. Studies performed in humans [25,27,30] have shown that pericytes, a cell type...
MSCs has been previously reported [31]. Our results showed a broad distribution in fluorescence intensity of both CD146 and NG2 in equine MSCs, with a small fraction of cells having no or very low expression, indicating cell heterogeneity and/or potential loss of pericyte immuno-phenotype in culture. Nonetheless, mRNA levels for these markers were sustained up to P8, except for NG2 in BM-MSCs populations. Around 10% of MSCs were negative for αSMA, a finding which may be explained by the observation that a subset of pericytes do not naturally express this marker [25,32]. Consistent with their pericyte immunophenotype was their tendency of equine AT-MSCs to closely associate with endothelial cell networks in co-cultures, a property that has been reported before for MSCs [33,34] and pericytes [35,36] in human but not in horse. Based on our results showing maintenance of a pericyte phenotype by MSCs over extended culture, it may be desirable to include pericyte markers, for example CD146, in the panel of markers currently used to immunophenotype equine MSCs. Since perivascular cells expressing CD146 are present in most body tissues [25,30,37], CD146 may serve as a universal MSC marker. In addition, CD146 has been identified as a marker of multipotency in human MSCs [38], which could provide obvious advantages for tissue regeneration purposes.

We detected CD29, CD44, CD90 and CD105 in a majority of cells in AT- and BM-MSC populations, in agreement with many [18,19,31,39] but not all [40] previous studies. A limited number of studies have compared the expression of MSC markers between AT- and BM-MSCs. In accordance with the present data, no significant differences were found in transcript levels of CD73, CD90, CD105 [41] between these
two cell types, while higher expression of CD44 and CD90 was reported for BM-MSCs compared to AT-MSCs [20]. These discrepancies may be related to the use of different extraction methods, cell culture conditions, analytical techniques and cell immunophenotyping reagents. As already suggested [19,42], better standardization of techniques used to collect, culture and analyse equine MSCs is needed before results from different studies can be meaningfully compared.

According to ISCT guidelines, all MSCs should be negative for CD45 and AT-MSCs should additionally lack CD31. Several previous studies did not detect CD45 in equine MSCs using flow cytometry [41,43-45], however, we (present study) and Radcliffe et al. (2010) did detect this marker by qPCR. CD31 (or other endothelial markers) have not been commonly considered in the characterization of equine MSC preparations, in part because ISCT recommendations on CD31 were just introduced in 2013 [15,16]. Although we did not detect CD31 in our preparations, CD144 (another endothelial marker) was present at low levels in all MSCs. Overall, these results demonstrate the heterogeneity of equine MSCs from different sources and highlight the necessity to use different approaches to fully characterize these preparations, an important consideration since cell heterogeneity may significantly impact the outcome of treatments [38,46].

In conclusion, our results from the analysis of equine tissues and MSC preparations in culture indicate that equine MSCs originate from pericytes and that they maintain a pericyte immunophenotype in culture. Taken together with data available in other species, our results indicate that the isolation and selective culture
of pericytes from crude extracts could provide a means to increase the quality and clinical efficacy of equine MSC preparations. Although selective harvesting and in vitro expansion of equine pericytes may be challenging, the findings obtained in this study represent an initial step towards that goal.

Acknowledgements

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Author disclosure statement

BP is co-inventor of human Perivascular Stem Cell-related patents filed from University of California, Los Angeles. The authors declare no competing interests.

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Equine Mesenchymal Stromal Cells retain a pericyte-like phenotype (doi: 10.1089/scd.2017.0017)

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Fig. 1. IHC of equine adipose tissue sections showing staining for the pericyte markers, CD146 (A), NG2 (B), αSMA (C), and the MSC markers, CD29 (D) and CD90 (E). CD144 was used as endothelial marker (red; A, B, D, E). Co-localization of pericyte (NG2 and CD146) and MSC markers (CD146 and CD73; F and G, respectively) in yellow (left panel) from the overlap of green (middle panel) and red (right panel) individual antibody fluorescence. DAPI was used to stain nuclei. Scale, 10 µm, is indicated by white bars.
Fig. 2. Photomicrograph of cultured AT- and BM-MSCs (A) which generated AP-positive colonies when grown at low density (1, 5 and 25 cells/cm²) for 11 days (B) and differentiated into adipocytes (C), osteocytes (D) and chondrocytes (E) visualised by oil red O, alizarin red S and Alcian blue counterstained with Neutral red, respectively. Inset shows detail of the chondrocyte micromasses.
Fig. 3. Flow cytometry histograms of AT- and BM-MSC. (A) upper and lower panels, respectively, showing positive staining of most cells for the MSC markers, CD29, CD73, and CD90, and negative staining for CD44 and CD45.

B. mRNA levels (AU) of CD44, CD73, CD90, and CD105 for AT-MSC (solid black bars) and BM-MSC (striped black bars) in AT-MSC and BM-MSC.

C. mRNA levels (AU) of CD11, CD44, and CD45 for AT-MSC (open black bars) and BM-MSC (striped black bars) in AT-MSC and BM-MSC.

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CD44, CD90 and CD105 (dark line; ≥99% cells for all antibodies by detection of AF488- or FITC-conjugate). Signal from isotype controls is shown by the grey peak, unstained curves were omitted for simplicity. (B) Results of qPCR analysis of the MSC markers, CD44, CD73, CD90 and CD105, the endothelial markers, CD31 and CD144, and the haematopoietic marker, CD45 in AT- and BM-MSCs. All results are shown as Mean±SEM; n=3 animals. ND = not detected. AU = Arbitrary units. *, p<0.05 and **, p<0.005.
Fig. 4. A) Flow cytometry histograms showing the proportions of AT- and BM-MSCs (upper and lower panels, respectively) that are positive for staining with the
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Isotype controls are shown by grey peaks and unstained curves were omitted for simplicity. B) Results of qPCR analysis of CD146, NG2 and PDGFRβ in AT- and BM-MSCs at passages 2 (P2) and 8 (P8). AU = Arbitrary units. All results are shown as Mean±SEM; n=3 animals. *, P<0.05.
Fig. 5 Microphotographs of cultured equine endothelial cells (Endo, labelled with the green fluorescent PKH26), AT-MSCs (MSCs, labelled with the red fluorescent
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### Table 1. List of antibodies used in the present study.

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<td>OX7</td>
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Supplementary data

Supplementary Fig. S1. IHC of immune-precipitate sections showing specific pericyte localization of pericyte markers (CD146 and NG2, A) and pericyte/MSC markers (NG2 and CD29, B) while the other set in the tissue were negative for these antibodies. C-F show negative controls, contained tissue (C), isotype controls for CD146 (D) and NG2 antibodies (E) and secondary antibody alone (F) that was used for the staining of NG2 shown in A and B. DAPI was used to stain nucleus. Scale 60 μm (A-E) or 100 μm (F) is indicated by white bars.
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Supplementary Fig. S2. Fluorescence-activated cell sorting of adipose stromal vascular fraction (SVF) showing a discrete population of CD146+ cells. A) SVF cells visualised in a dot-dot graph of Side Scatter-Area (SSC-A) vs. Height (SSC-H) were successively selected for CD146+ and CD146+ cells. B) and CD146+ cells were positive for fluorescence signals to obtain a distinct cell fraction expressing CD146. C) in agreement to the profiles obtained in humans (25). D, E show control of unstained cells and CD146 antibody isolation, respectively. As determined by qPCR, CD146+ cells expressed CD146, N02 and sTMs corresponding to the arbitrary values of 56.6±11.36, 27.3±12.2 and 51.2±16.8, respectively.