

Short communication

TRPC EXPRESSION IN MESENCHYMAL STEM CELLSFREDERIC TOROSSIAN^{1,2}, AURELIE BISSON¹, JEAN-PIERRE VANNIER²,
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Abstract: Transient receptor potential canonical (TRPC) channels are key players in calcium homeostasis and various regulatory processes in cell biology. Little is currently known about the TRPC subfamily members in mesenchymal stem cells (MSC), where they could play a role in cell proliferation. We report on the presence of TRPC1, 2, 4 and 6 mRNAs in MSC. Western blot and immunofluorescence staining indicate a membrane and intracellular distribution of TRPC1. Furthermore, the decrease in the level of TRPC1 protein caused by RNA interference is accompanied by the downregulation of cell proliferation. These results indicate that MSC express TRPC1, 2, 4 and 6 mRNA and that TRPC1 may play a role in stem cell proliferation.

Key words: Mesenchymal stem cells, TRPC1, Cell proliferation

INTRODUCTION

The identification of several types of stem cells over the past decade has opened interesting possibilities for the development of cellular therapy. Multiple studies have explored crucial cellular functions such as differentiation, proliferation, exocytosis and apoptosis in umbilical cord, embryonic and mesenchymal stem cells [1-4]. These functions are controlled by intracellular calcium-mediated processes. Augmenting the cytosolic calcium concentration occurs through the cooperation of several calcium transporters that mobilize extra- and/or intracellular calcium pools.

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Abbreviations used: MSC – Mesenchymal stem cells, TRP – Transient receptor potential, TRPC – Transient receptor potential canonical

The transient receptor potential (TRP) channels are a superfamily of ionic channels with at least 33 members subdivided into seven subfamilies according to their molecular weight and physiological and physiopathological properties. The canonical subfamily (TRPC) is divided into 4 groups according to sequence similarity [5, 6]. TRPC1 and 2 belong to the first and second groups, TRPC3, 6 and 7 form the third, and TRPC4 and 5 are in the fourth. These proteins are composed of 6 transmembrane segments with N- and C-terminal cytoplasmic regions. The different motifs of these cytoplasmic regions allow the assembly of TRPC subunits to form a heterotetrameric plasma membrane channel or an association with other proteins such as the Orai 1 or IP3 receptors, to which they confer sensitivity during intracellular calcium store depletion [7, 8]. When associated with TRPC4 or 5 [9], TRPC1 may form a functional membrane channel, while the homotetramer appears to be distributed to intracellular compartments.

With the exception of TRPC2, the TRPC proteins were shown to be expressed in a large variety of tissues [for review, see 10], but the different levels of their expression may suggest distinct functions [11]. A notably high level of TRPC1 was detected in the myocytes, neurons and adenohypophyseal cells [11]. High levels of TRPC3 and TRPC7 are found in the pituitary gland, and of TRPC4 in the bone. TRPC5 is preferentially present in the central nervous system, and TRPC6 appears to be more ubiquitously distributed [11]. Interestingly, the production of TRPC2 seems to be limited to the vomeronasal organ and testis [12, 13]. There is growing interest in TRPC channels in adult stem cells and other cells with high proliferative and differentiation capacities. For instance, the expression of TRPC1 was recently demonstrated in primary umbilical cord blood CD34⁺ cells [14] and in the C2C12 myoblast line, where it is involved in the initial phase of differentiation [15]. However, little is known about the expression of the TRPC subfamily members in mesenchymal stem cells (MSC) possessing high proliferative and differentiation potentials. Here, we provide the first proof that some TRPC family members are expressed in MSC and that they may further play a role in stem cell proliferation.

MATERIALS AND METHODS

Cell culture

Bone marrow was collected from New Zealand white rabbit (Charles River, Chatillon-sur-Chalaronne, France) according to the recommendations of the Consultative French Ethical Committee. Bone marrow mononuclear cells were isolated via Ficoll density gradient centrifugation (Eurobio, Les Ulis, France). MSCs were separated from the CD45⁺ fraction using a mouse anti-rabbit CD45 antibody (Serotec, Cergy Saint Christophe, France) magnetically labelled with rat anti-mouse IgG1 microbeads (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The separated cells were 99% CD45⁻, as previously found with other protocols [16]. 6x10⁶ CD45⁻ cells were seeded in four-well

microplates in a previously described expansion medium [16] containing 60% low glucose-DMEM (Eurobio), 40% MCDB-201 (Eurobio), insulin-transferrin-selenium supplement (1:100; Invitrogen, Cergy Pontoise, France), 1% bovine serum albumin (Sigma, Saint Quentin Fallavier, France), 10^{-9} M dexamethasone (Merck, Fontenay-sous-Bois, France), 10^{-4} M ascorbic acid-2-phosphate (Sigma), 10 ng/ml endothelial growth factor (Euromedex, Souffelweyersheim, France), 10 ng/ml leukemia inhibitory factor (Euromedex), 10 ng/ml platelet-derived growth factor (Peprotech, Neuilly-Sur-Seine, France), 2% fetal calf serum (Eurobio), 100 U/ml penicillin (Sarbach, Suresnes, France) and 100 µg/ml streptomycin (Diamant, Puteaux, France). The cells were cultured in a humidified atmosphere at 5% CO₂ and 37°C. The medium was changed twice a week until 90% confluence of adherent cells had been achieved. For the subcultivation, the MSCs were detached with 0.05% Trypsin-EDTA and placed in a 25-cm² tissue culture flask.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The total cellular RNA was extracted from subconfluent cultures using Trizol (Invitrogen). The RNA concentration and integrity were analyzed using an Agilent RNA 6000 Nano LabChip kit with an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). Reverse transcription (Invitrogen), and the polymerase chain reaction (Thermo Scientific, France) was performed according to the manufacturer's instructions.

The primers for TRPC1, 3 and 4 were described by Lee *et al.* [17], for TRPC2 by Chu *et al.* [18] and for TRPC6 by Yu *et al.* [19]. The others primers were defined using the Primer Selection Tool program (Biotools site, University of Massachusetts Medical School, USA) and were: 5'-CTA TGA GAC CAG AGC TAT CGA TG-3' and 5'-CCA CCA GGG AGA TGA CAT TGT ATG-3' for TRPC5; and 5'-GAG AAG GAG AAG CTG GAG CA-3' and 5'-AGA GAA CCC CCA GGG TGA G-3' for OCT-4. The primer-annealing step was realized at 55°C for TRPC1, 4 and 6, and at 54-64°C for TRPC2, 3 and 5 according to the touchdown protocol, or at 60°C for OCT-4.

Transfection of siRNA

TRPC1 short interfering RNA (siRNA; Eurogentec, Angers, France) was used: (1) GCC CAC CUG UAA GAA GAU ATT and (2) UAU CUU CUU ACA GGU GGG CTT. A sequence without homology with any known gene (scramble siRNA) was used as a control. siRNA was bound with INTERFERinTM (Ozyme, Saint Quentin Yvelines, France) into L-DMEM (Eurobio) for 10 min at room temperature, and applied to the MSC culture, where a fresh prewarmed expansion medium without antibiotics was added. The final concentration of the TRPC1 siRNA (Eurogentec) and scramble siRNA was 100 or 150 nM. After 24 h of incubation, the transfection medium was removed and the MSCs were incubated for another 24 h in the expansion medium.

Western blot

Control and post-transfection MSCs were cultured in the expansion medium with 100 or 150 nM TRPC1 siRNA (Eurogentec) or with 150 nM scramble siRNA. For the protein extraction, 5 million cells were placed in a lysis buffer composed of 100 mM TrisHCl at pH 8, to which a protease inhibitor cocktail containing antipain, calpain inhibitor I and II, chymostatin, TLCK and trypsin-inhibitor (cOmplete Mini, Roche Diagnostics, USA) was added. A buffer adjusted to pH 7.4 composed of 0.36 M sucrose and 14 mM Hepes was also added. The lysates were clarified by centrifugation for 15 min at 3,000 g and 4°C. The supernatant was centrifuged again for 30 min at 50,000 g and 4°C. The supernatant containing the proteins was stocked at -80°C. For the membrane protein extraction, the pellet was incubated in buffer composed of TrisHCl 50 mM, NaCl 150 mM, EDTA 5 mM, KI 1 M and TritonX100 1%, and the pH was adjusted to 7.5 and centrifuged at 145,000 g for 1 h at 4°C. The protein concentration was determined using the Bradford method.

Western blots were performed using the XCell II system (Invitrogen) according to the manufacturer's instructions. The goat anti-TRPC1 antibody (Cliniscience, Montrouge, France) was used at a 1:200 dilution and the universal immunoperoxidase polymer anti-goat antibody (Nichirei Biosciences, Tokyo, Japon) was used at a 1:2000 dilution. Antibody binding was revealed using the enhanced chemiluminescence system (Amersham, GE Healthcare, Orsay, France). The densities of the blot bands, expressed in arbitrary units, were obtained using Image Processing and Analysis in Java (ImageJ, NIH Public domain).

Test of MSC proliferation

Transfected cells were seeded at a density of 25,000 per well in a microplate in the expansion medium described above. Simultaneously, a standard range corresponding to 25,000, 50,000, 100,000, 150,000, 200,000 and 400,000 non-transfected cells per well was prepared. After 24 h of incubation in a humidified atmosphere at 5% CO₂ and 37°C, the standard range cells were fixed in acetic alcohol (95% absolute ethanol, 2% acetic acid, 3% H₂O) whereas the TRPC1 siRNA (Eurogentec) or scramble siRNA was transfected into the other cells. After 24 h of incubation, the transfection medium was removed, and the cells were incubated for another 24 h in the expansion medium and fixed in acetic alcohol. The standard range and transfected cells were stained with Crystal Violet for 30 min, rinsed with dH₂O, dried for 1 h, and then extracted with 1 ml of 10% acetic acid. The optical density of the extracts was evaluated using spectrophotometric measurement at 570 nm (Biotech's Power Wave X Microplate Spectrophotometer, Biotech Instruments, Fisher Scientific). The relative effect of the TRPC1 siRNA (Eurogentec) on cell proliferation was evaluated in comparison to cells transfected with scramble siRNA. The data was compared using Student's *t*-test.

Immunofluorescence assay

After 24 h of incubation in the expansion medium, the MSCs were fixed in a 4% paraformaldehyde solution, rinsed 3 times with PBS and incubated in a blocking buffer composed of PBS, 0.1% Tween and 5% milk for 45 min at room temperature. Then the cells were incubated with a goat anti-TRPC1 antibody (1:50, Cliniscience) overnight at 4°C. After the cells had been rinsed 3 times with PBS, a donkey anti-goat secondary antibody conjugated to Alexa Fluor 594 (1:100, Invitrogen) was added for 1.5 h at room temperature. The nuclei were stained with Hoechst solution (1:5000, Sigma) for 15 min at room temperature. After washing in PBS, the cells were observed with a Leica DMI6000B fluorescence microscope with a Leica CTR 6000 light source.

RESULTS AND DISCUSSION

There is a lack of knowledge about TRPC channel expression in bone marrow MSC, so RT-PCR was used to determine which types of channel are expressed in these cells. To be sure of having immature MSC to work with, we demonstrated their ability to express the OCT-4 gene, a recognized marker of stem cell immaturity [20]. It is represented in Fig. 1A by a 231-bp apparent band obtained via RT-PCR analysis. In a more selective way, the studied cells showed the capacity to differentiate into osteoblasts, chondrocytes and adipocytes, characterized by distinct features like calcium phosphate, glycosaminoglycans and lipids, demonstrating their mesenchymal origin (data not shown).

The analysis of the TRPC family expression evidenced that the MSC mobilize the TRPC1, 2, 4 and 6 genes (Fig. 1B, image 1). For TRPC4, we detected mRNA with a slightly greater size than expected, but it was absent from the rabbit brain extracts (Fig. 1B image 2) used as a classical positive control for the expression of all the regular TRPC family members [10]. This observation suggests that MSC may express an already described splice variant [21]. In the same way, for TRPC2, the weak band of higher weight may correspond to the cDNA of TRPC2A, identified as spliced form of TRPC2 [22, 23].

Since the TRPC1 protein plays a role in important functions such as proliferation, contraction, differentiation or secretion in a large variety of normal and pathological cells [for a review, see 10, see also 24], we chose to analyse its cellular localization in the MSC and sought a possible role for it in cell proliferation. Using Western blot, we were able to detect the presence of an approximately 49-kDa TRPC1 protein band in both the cytosolic and membrane fractions (Fig. 1C). While this molecular weight does not correspond to the generally reported values of around 100 kDa [25], it is close to the isoform of TRPC1 detected in rat neurons [26]. Indeed, a short isoform has already been

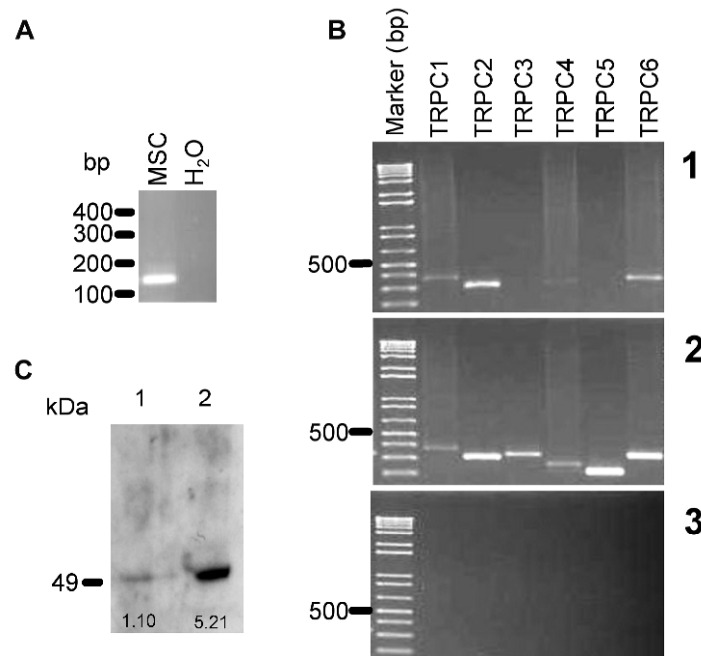


Fig. 1. The identification of OCT-4 and TRPC1, 2, 4 and 6 in rabbit bone marrow mesenchymal stem cells (MSC). A – The amplification of a 151-bp band corresponding to OCT-4 mRNA in the MSC. Distilled water was used as a control. B – TRPC1, 2, 3, 4, 5 and 6 (372, 310, 331, 265, 220 and 327 bp, respectively) were analyzed in the MSC (image 1) and in rabbit brain tissue (image 2). Distilled water was used as a control (image 3). C – A western blot analysis of the TRPC1 protein in a membrane (lane 1) and cytosol extract (lane 2) of MSC containing 10 μ g of proteins per lane. The apparent molecular weight of the TRPC1 protein is 49 kDa. The densities of blot bands, expressed in arbitrary units, are indicated below the bands.

detected via RT-PCR in salivary gland cells [27] or in immature CD34⁺ cells [14] and shown to disappear and be progressively replaced by the unspliced TRPC1 form during that differentiation into megacaryocytes [14]. Further immunofluorescence staining corroborated these results by showing that a large majority of MSC express TRPC1 (Fig. 2A). The most intense staining (Fig. 2B) appeared as a large juxtannuclear network suggesting an endoplasmic reticulum (ER) localization of TRPC1, as previously reported by Alfonso *et al.* [9]. Interestingly, rare cells, marked by a very luminous perinuclear halo (> 1.4 arbitrary units in comparison to < 0.22 for others), seemed to produce a higher quantity of TRPC1. Notably, a high expression of this protein in human myoblasts was observed during the initial stage of differentiation and its diminution coincided with an increase in the T-type voltage-dependent calcium channel expression essential for myotube formation [15]. In dividing cells, TRPC1 staining appeared as a densely spun web formation displaced to the border zone of division and as peripheral luminous spots suggesting a membrane

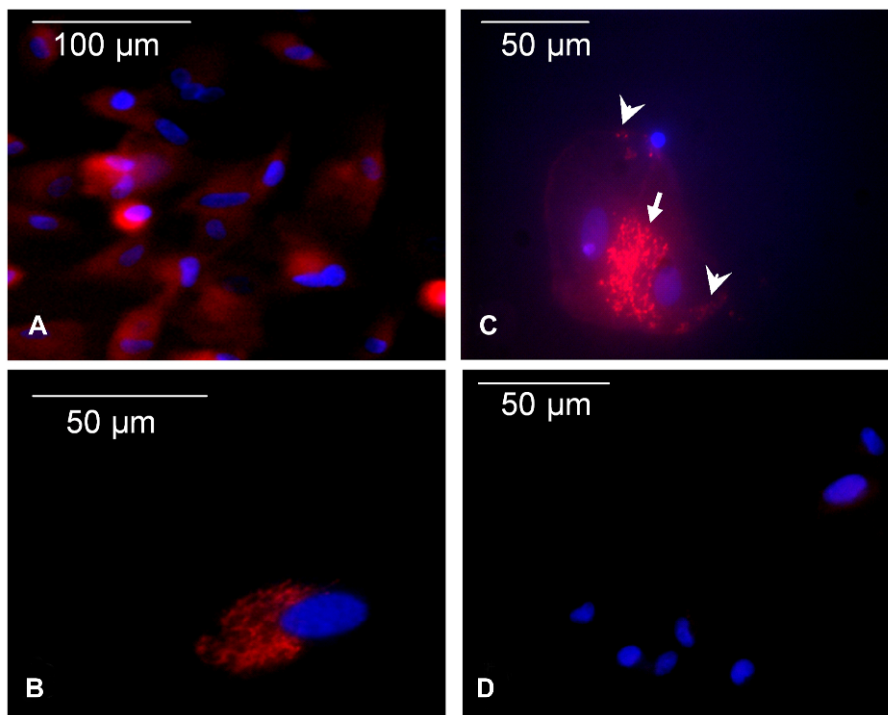


Fig. 2. The localization of TRPC1 in bone marrow mesenchymal stem cells (MSC) assessed via anti-TRPC1 immuno-fluorescence. A-B – TRPC1 staining of resting MSC at low and high magnification. C – Localization of TRPC1 staining at the intracellular interface of dividing cells (arrow). The arrowhead indicates dispersed membrane-like material. D – Staining with a secondary antibody in the absence of the anti-TRPC1 antibody was used as a negative control. Nuclei stained with Hoechst.

localization (Fig. 2C, arrows). It should be noted that the convergent Western blot results indicated the presence of the TRPC1 protein in the membrane and the cytosolic fractions. Such a membrane and intracellular distribution of TRPC1 already reported for the other types of differentiated and non-differentiated cells supported our observation [9]. It is admitted that the TRPC membrane channels represent heterotetramers where TRPC1 may be associated with other TRPC proteins, such as TRPC4 and TRPC5 [9], which is not found in MSC, or with TRPC6, which is detected on the membrane of mesangial cells [28]. In addition, the joint expression of the TRPC1 and TRPC6 proteins was identified in pulmonary vascular smooth muscle cells [19] and human hepatoma cells [29]. The expression of TRPC2 and TRPC6 in the MSC and the preponderant distribution of TRPC1 in an ER-like space lead us to consider the probability of the formation of TRPC2/TRPC6 heteromultimers, as described for the membrane of primary erythroid cells [8, 23] and homotetramers coupled to an IP3 cascade as observed in HEK293 cells [9] or DT40 B lymphocytes [30].

Further immunocytochemical studies of protein distribution and evaluation of microfluorimetric calcium fluxes should give an exact demonstration of the role of the detected TRPC proteins in the MSC, especially as the same protein may be addressed to the plasma membrane and to the ER [8, 23]. Given the weak documentation concerning the peripheral tissue distribution of TRPC2, revealing its expression and role in the MSC is particularly interesting. Since no specific pharmacological TRPC1 blockers are available to date, we inactivated the production of this protein by RNA interference in order to evaluate its effect on cell proliferation. In comparison to scramble siRNA, the intensity of the TRPC1 apparent band was significantly decreased, indicating a dose-dependent inhibition of TRPC1 expression by specific siRNA used at concentrations of 100 nM (Fig. 3A, lane 3; $p < 0.02$) and 150 nM (Fig. 3A, lane 4; $p < 0.01$). Consequently, this reduction in TRPC1 gene expression was accompanied by a significant downregulation of MSC proliferation (Fig. 3B), corresponding to a 42% diminution in comparison to the scramble ($p < 0.05$).

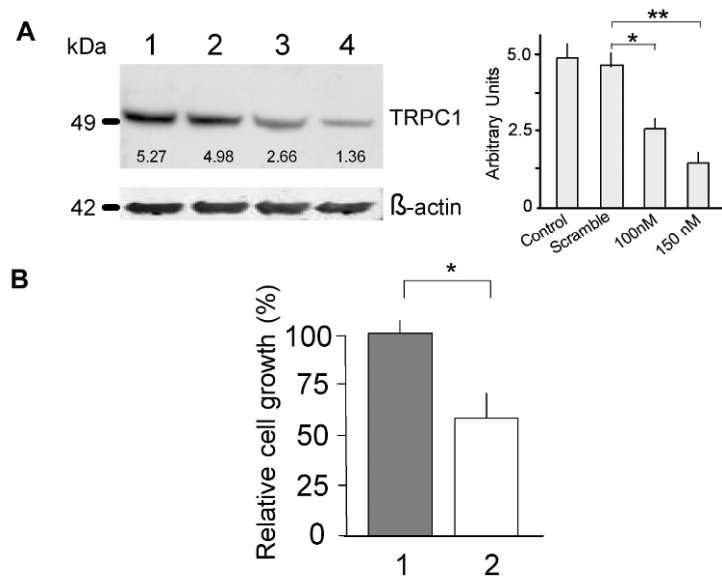


Fig. 3. The functional role of TRPC1 in rabbit bone marrow mesenchymal stem cells (MSC). A – Western blot detected an inhibition of TRPC1 expression in the MSC by TRPC1-specific siRNA (100 nM, lane 3; 150 nM, lane 4) in comparison with controls representing non-transfected MSC (lane 1) and MSC transfected with scramble siRNA (150 nM, lane 2). Each lane contains 10 μ g of proteins from total lysate. The densities of the blot bands, expressed in arbitrary units, are indicated below the bands. B – The inhibition of the proliferation of MSC transfected with scramble (100 nM, histogram 1) or TRPC1 siRNA (100 nM, histogram 2).

Our results for MSC indicate that the TRPC1 protein also plays an important role in the proliferation of stem cells, in accordance with recent results for breast cancer cells [31] and osteoblasts [32]. It should be noted that the hepatoma cell

proliferation depended on the expression of the TRPC6 gene but not on that of the TRPC1 gene [29], indicating the necessity of a detailed study of the localization and functionality of the two proteins in the MSC.

Our data suggests that: i) the MSC express genes encoding TRPC1, 2, 4 and 6; ii) the TRPC1 protein is present in both the membrane and cytosolic fractions of the MSC; and iii) TRPC1 plays an important role in the proliferation of MSC.

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