Received: 10 October 2017 | Accepted: 17 January 2018

DOI: 10.1002/jcp.26493

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Autocrine signals increase ovine mesenchymal stem cells migration through Aquaporin-1 and CXCR4 overexpression

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Funding information
Funds of Research of the Department of Advanced Biomedical Sciences, University of Naples, Federico II, Italy

Sheep is a relevant large animal model that is frequently used to test innovative tissue engineering (TE) approaches especially for bone reconstruction. Mesenchymal stem cells (MSCs) are used in TE applications because they represent key component of adult tissue repair. Importantly, MSCs from different species show similar characteristics, which facilitated their application in translational studies using animal models. Nowadays, many researches are focusing on the use of ovine mesenchymal stem cells (oMSCs) in orthopedic preclinical settings for regenerative medicine purposes. Therefore, there is a need to amplify our knowledge on the mechanisms underlying the behaviour of these cells. Recently, several studies have shown that MSC function is largely dependent on factors that MSCs release in the environment, as well as, in conditioned medium (CM). It has been demonstrated that MSCs through autocrine and paracrine signals are able to stimulate proliferation, migration, and differentiation of different type of cells including themselves. In this study, we investigated the effects of the CM produced by oMSCs on oMSCs themselves and we explored the signal pathways involved. We observed that CM caused an enhancement of oMSC migration. Furthermore, we found that CM increased levels of two membrane proteins involved in cell migration, Aquaporin 1 (AQP1), and C-X-C chemokine receptor type 4 (CXCR4), and activated Akt and Erk intracellular signal pathways. In conclusion, taken together our results suggest the high potential of autologous CM as a promising tool to modulate behaviour of MSCs thus improving their use in therapeutically approaches.

KEYWORDS
AQP1, CXCR4, ovine mesenchymal stem cells

1 | INTRODUCTION

Mesenchymal stem cells (MSCs) represent an optimal tool for tissue regeneration in a clinical setting due to their multipotency, being able to differentiate into various cells (bone, cartilage, fat, muscle, and tendon) under appropriate conditions. This particular property together with their low immunogenicity and the ability to secrete bioactive factors (cytokines, chemokines, and growth factors) in response to local microenvironmental cues, makes them an ideal candidate for the treatment of different pathologies (Dalal, Gandy, & Domen, 2012; Gnecchi, Danieli, & Cervio, 2012; Liu, Shu, Kenny, Chang, & Leung, 2014; Park, Kim, Lee, Park, & An, 2015).

Antonella Zannetti and Arturo Brunetti contributed equally to this work.

In particular, MSCs can migrate toward the injured tissues in response to different factors and here they can secrete molecules involved in the repair of damage. These secreted factors can be found in the medium where MSCs are cultured; thus, the medium is called conditioned medium (CM) (Pawitan, 2014). The CM, regulating multiple physiologic functions, must be taken into due consideration in therapy for several advantages (rapid collection, freeze-dried, packaged, and transported more easily) compared to the use of MSCs (Angoulvant et al., 2011; Huang et al., 2015). Furthermore, the use of CM could overcome rejection problems linked to the compatibility between the donor and the recipient.

The improvement of several MSCs-based therapies, as well as, the development of new strategies for measuring the efficacy of MSCs in preclinical studies, has increasingly required the use of large animal models (Cibelli et al., 2013). Notably, these animal species can provide significant advantages when modelling specific human disease conditions and testing stem cell therapies (Harding, Roberts, & Mirochnitchenko, 2013).

Among the large animals, sheep are an ideal model for bone tissue engineering (Guo et al., 2004; Pobloth et al., 2016; Sanjurjo-Rodríguez et al., 2017) and have been proposed for a wide range of applications in biomedical research (Adamzyk et al., 2016; Delling, Brehma, Ludewigb, Winterc, & Jülkec, 2015; Scheerlinck, Snibson, Bowles, & Sutton, 2008; Sill et al., 2011). Sheep are used to test the efficacy of MSCs in different bone's pathologies because evidence proves that their skeletal structure has a higher resemblance to the human one as compared to mice and rabbits (Kalaszczynska et al., 2013; O'Loughlin et al., 2008; Pearce, Richards, Milz, Schneider, & Pearce, 2007; Petite et al., 2000).

Despite a considerable number of reports employing ovine MSCs (oMSCs) in tissue engineering (Al Faqeh, Nor Hamdan, Chen, Aminuddin, & Ruszymah, 2012; Boos et al., 2011; Di Bella et al., 2010; Dozza et al., 2017; Lucarelli et al., 2005; Mrugala et al., 2008; Scharf et al., 2015), thereare limited studiesregarding their morphological and biochemical characterization (Burk et al., 2017; Desantis et al., 2015; Lyahyai et al., 2012, McCarty, Gronthos, Zannettino, Foster, & Xian, 2009; Mrozik et al., 2010; Rentsch et al., 2010; Zannettino, Paton, Itescu, & Gronthos, 2010). Differently from human MSCs, oMSCs are not well studied for their physiological activity and behaviour in tissue repair.

It is well known that regulation of MSCs migration is a complex phenomenon that requires the contribution of numerous regulatory factors. Stromal cell-derived factor (SDF) and its receptor C-X-C chemokine receptor type 4 (CXCR-4) certainly represent an important pathway involved in mediating cell migration. Recently, we demonstrated the ability of conditioned medium (CM) from human bone-marrow mesenchymal stem cells (hBM-MSCs) to promote migration and invasion of osteosarcoma and hepatocellular carcinoma cells through CXCR4 signalling (Fontanella et al., 2016). In addition, it has been demonstrated that the overexpression of CXCR4 accelerates MSCs mobilization in the infarcted myocardium (Zhang et al., 2008).

Many studies report that aquaporins (AQPs), a family of 13 water-transporting membrane channel proteins known for their key role in fluid transport in various tissues (Verkman, 2005) play a crucial role in different biological processes related to cell migration (Papadopoulos, Saadoun, & Verkman, 2008), proliferation, apoptosis, and cell volume regulation (Conner et al., 2012). Importantly, it has been observed that AQP1 promotes MSC migration in physiological and pathological conditions (Meng et al., 2014; Pelagalli, Nardelli, Fontanella, & Zannetti, 2016). The aim of this study was to evaluate whether CM produced by ovine MSCs (oMSCs) could affect, through autocrine signals, their capacity to migrate and proliferate. Furthermore, we investigated the possible involvement of AQP1 and CXCR4, as well as, the activation of downstream signals such as PI3K/Akt and MAPK/Erk pathways in mediating CM effects on oMSCs.

2 | MATERIALS AND METHODS

2.1 | Isolation, culture, and immunophenotypic characterization of ovine bone-marrow mesenchymal stem cells (oMSCs)

Ovine bone marrow aspirates were obtained according to the isolation protocol previous described by Dozza et al. (2011). In particular, heparinized aspirates were obtained from iliac crests of five 18–24 month sheep at Rizzoli Orthopaedic Institute. Isolation and culture expansion of ovine bone marrow mesenchymal stem cell (oMSCs) was performed as previously described (Dozza et al., 2011) through gradient separation and plastic adherence. Ovine MSCs were characterized using FC500 flow cytometer (Beckman Coulter, Brea, CA) for staminal markers and they resulted positive for CD44, CD73, CD90, CD105, CD146, and negative for CD34 and CD45 (Beckman Coulter, Fullerton, CA). The MSC-enriched interface fraction was collected and plated in a 10 cm dish containing culture medium (a-MEM; Sigma Chemical, St. Louis, MO) with 10% FBS and 1% v/v penicillin/streptomycin/fungizone (PSF). The medium was changed every 4 days. When the cells reached 80% confluence, they were trypsinized and passaged into new 10 cm dishes at a cell density at 5×10^5 cells/dish. According to the method well described from Dozza et al. (2011), ovine MSCs were sub-cultured until P3 (passage 3). P3 cells were then seeded at designated condition and subjected to experiments, and the remaining cells were stored for later use. The isolated and characterized ovine MSCs, were grown in medium with 10% FBS.

2.2 | Preparation of oMSCs conditioned medium

Conditioned medium (CM) was obtained from oMSCs (passage 2 and at 80–90% confluency) grown for 24 hr in medium with 1% FBS. CM was collected, centrifuged at 1000*g* for 5 min to remove debris, filtered, and stored in aliquots at −80 °C.

2.3 | Western blot analysis

In order to evaluate protein expression, oMSCs were cultured for 24 hr with medium supplemented with 1% FBS, 10 % FBS, or in presence of CM. Western blot analysis was carried out, as described previously with

some modifications (Zannetti et al., 2008, 2012). Whole-cell lysates were prepared by homogenization in lysis buffer (40 mM Hepes, pH 7.5, 120 mM NaCl, 5 mM MgCl2, 1 mM EGTA, 0,5 mM EDTA, 1% Triton X-100) with protease inhibitors cocktail (complete tablets-EDTA free, Roche, Basel, Switzerland) and phosphatase inhibitors (20 mM α-glycerol-3-phosphate, 2,5 mM Na-pyrophosphate). Protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Equal proteins samples (50 μg) were loaded and separated by on NUPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) using MES-SDS running buffer. Then, proteins were transferred to nitrocellulose membrane using the iBlot gel transfer System Invitrogen for immnuoblot. After blocking with 5% not fat milk powder in Tris-buffered saline 0.1% Tween 20, the membranes were incubated overnight at 4 °C with primary antibodies: anti-AQP1 (rabbit polyclonal orb10122 Biorbyt, dilution 1:500) and anti-CXCR4 (rabbit polyclonal IMG-537 Imgenex, San Diego, CA, dilution 1:500).

To investigate the intracellular signal pathways activated by CM in oMSCs, the levels of Akt/p-Akt, Erk/p-Erk, and FAK/p-FAK were also evaluated. The antibodies used were: rabbit polyclonal anti-Akt (CST-9272, Cell Signaling, Leiden, the Netherlands), rabbit polyclonal anti p-Akt (Ser473) (CST-9271, Cell Signaling), rabbit polyclonal anti-Erk (CST-9102, Cell Signaling), rabbit polyclonal anti p-Erk (CST-9101, Cell Signaling), rabbit polyclonal anti-FAK (Cell Signaling, 3285), rabbit anti p-FAK (Y397) (pFAK, Cell Signaling, 3283), and mouse anti-β-actin (A4700, Sigma–Aldrich Corp, St Louis, MO).

After rinsing with TBS-T, the membranes were incubated with HRP-conjugated secondary anti-rabbit and anti-mouse antibodies (mouse: 115-035-003, rabbit 111-005-003 Jackson ImmunoResearch, Baltimore, PA, dilution 1:2000) for 1 hr at room temperature. To visualize the immunoreactive proteins bands, an ECL detection kit (Advansta, Menlo Park, CA) was used. Band acquisition was performed by using the C-Digit Blot Scanner (Li-Cor). The level of expression of different proteins was analyzed by using the public domain software ImageJ (a Java-based image processing tool inspired by National Institutes of Health's Image for windows).

2.4 | Transwell migration assay

Cell migration assay was performed using 24-well Transwell chambers (Corning, Inc., Corning, NY) containing inserts with an 8 μm polycarbonate pore membrane. Briefly, oMSCs were placed in the upper chamber (2.5×10^5 cells/well) in serum free α-MEM. Medium containing 1% FBS, 10% FBS, and CM were added to the lower chamber as chemoattractants. After incubation in a humidified incubator with 5% $C0_2$ at 37 °C for 24 hr, oMSCs that had not migrated through the filter were removed using a cotton wool swab. Migration of cells to the lower surface of the membrane insert was evaluated by staining with 0.05% crystal violet in 25% methanol and imaged under a microscope. Stained cells were extracted by eluting crystal violet with 1% sodium dodecyl sulphate and reading the absorbance at 595 nm by a Thermo Scientific Multiskan, Thermo Scientific, Helsinki, Finland, FC microplate reader. For each migration assay three identical replicate were performed. To block AQP1 and CXCR4 the cells were incubated with 100 μM of tetraethylammonium chloride (TEA) (Müller, Hub, Grubmüller, & de Groot, 2008) and AMD3100 (10 μM), respectively.

2.5 | Wound healing assay

The wound healing assay was performed as previously described (Pelagalli et al., 2016). Briefly, oMSCs were seeded into six-well dishes at a density of 1×10^5 cells/well and grown until confluent. To these monolayers a linear wound was gently created using a sterile pipette tip (200 μl). Then, each well was washed with medium to remove cell detached. Wounded cell cultures were then incubated for 24–48 hr in the presence of medium supplemented with 1% FBS, 10% FBS, or oMSC-CM. The migration of cells to close the wound was observed and images captured at different time points (0, 24, and 48 hr) using a phase contrast microscopy (Leica) equipped with a digital camera (Canon). The surface areas of the cell-free zones were measured and the percentage scratch closure was quantified with TScratch software (Gebak, Schulz, Koumoutsakos, & Detmar, 2009). Results are expressed as the percentage of wound closure at 24 hr and 48 hr respect to time 0.

2.6 | Statistical analysis

Data are reported as the mean ± standard deviation (SD). The Student's *t*-test was used for comparison of the mean values between different groups. All *p*-values were two-tailed, and $^*p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Expression of AQP1 and CXCR4 in oMSCs

In order to assess the autocrine effect of CM on oMSCs, these cells were cultured in presence of medium supplemented with 1% FBS, 10% FBS, or CM for 24 hr and the levels of AQP1 and CXCR4, were analysed. As shown in Figure 1a, oMSCs express both AQP1 and CXCR4 proteins and their levels are increased when cells were grown in presence of 10% FBS or in presence of CM respect to 1% FBS. These results are confirmed by densitometric analysis of AQP1 and CXCR4 bands normalized for beta actin used as a protein loading control (Figure 1b). Interestingly, the effect of CM caused a 1.8 and 1.63 folds increase of AQP1 and CXCR4 levels, respectively, in comparison with the control (1% FBS), thus confirming the autocrine signal of CM.

3.2 | Intracellular signaling pathways activated by CM in oMSCs

To elucidate the mechanism underlying of CM effect on oMSCs we analysed AQP1 and CXCR4 downstream signaling. We investigated the involvement of intracellular pathways such as the phosphatidyl-3-kinase (PI3K)/Akt and extracellular signal-regulated kinase 1/2 (Erk1/2) (Hayashi, Tsuchiya, Nakayama, Takayuki, & Nishida, 2008). We found that CM increased phosphorylation either Akt and Erk in oMCSs

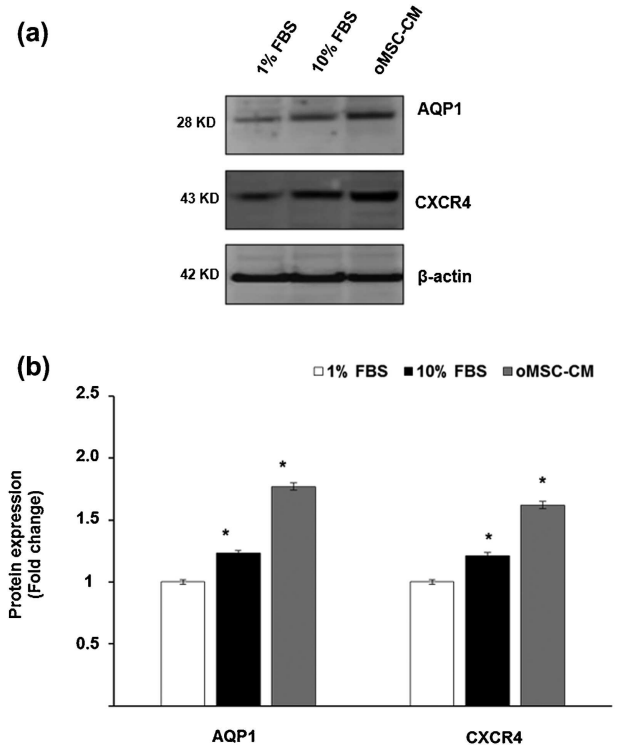

FIGURE 1 Expression of AQP1 and CXCR4 in oMSCs. (a) Western blot analysis of AQP1 and CXCR4 expression in oMSC grown for 24 hr in presence of 1% FBS, 10% FBS and oMSC-CM. Equal loading was confirmed by immunoblot with β-actin antibody. Molecular weights of indicated proteins are reported. Depicted results represent one of three typical experiments performed; (b) The histogram indicates the fold of densitometric signals, reported as relative to 1% FBS, arbitrarily set to 1. Data are expressed as mean ± SD from three independent experiments. *$p < 0.01$

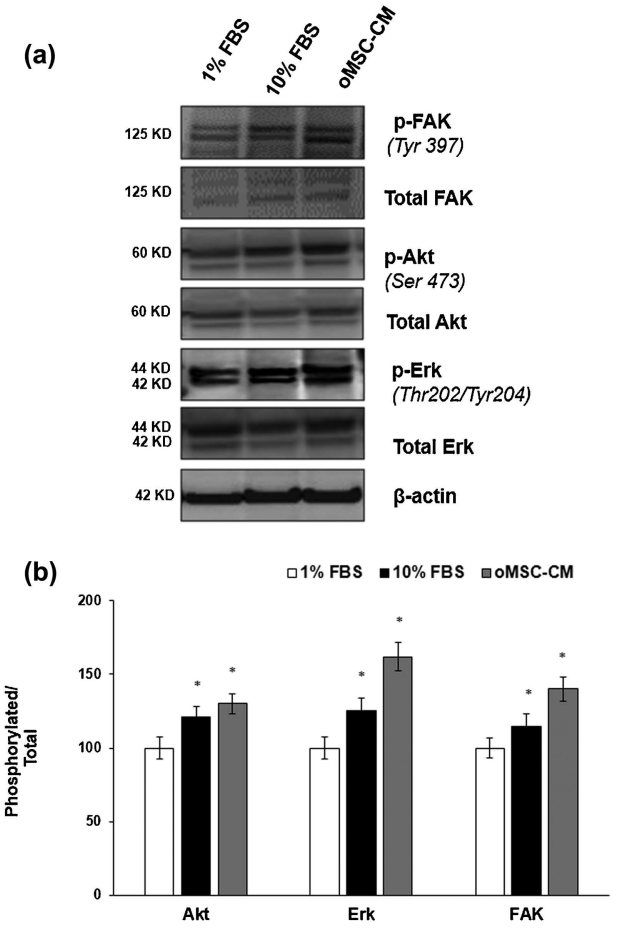

FIGURE 2 Intracellular signalling pathways activated by CM in oMSCs. (a) Representative Western blots of phosphorylated and total Akt, Erk, and FAK in oMSCs grown for 24 hr in presence of 1% FBS, 10%FBS, and oMSC-CM. Equal loading was confirmed by immunoblot with β-actin antibody. Molecular weights of indicated proteins are reported. Depicted results represent one of three typical experiments performed. (b) The histogram indicates, pErk/Erk, pAkt/Akt pFAK/FAK ratio of densitometric signals, reported as relative to 1% FBS-treated cells, arbitrarily set to 100%. Results of densitometric analysis of Akt, Erk, and FAK activation normalized to β-actin, performed from three different Western blotting. Data are expressed as mean ± SD from three independent experiments, *$p < 0.01$

compared to that observed in cells grown in medium supplemented with 1% FBS (control), whereas no increase was observed in Akt and Erk levels (Figure 2a). In particular, CM caused an higher increase of pAkt/total Akt and pErk/total Erk protein ratio (130 ± 8.34; 162 ± 8.03), respectively, respect to 1% FBS (considered as 100%) (Figure 2b). The induction of pAkt and pErk caused by CM was also higher than that elicited by medium supplemented with 10% FBS (121 ± 7.42; 125 ± 8.73, respectively). Furthermore, in accord with other studies (Meng et al., 2014) that demonstrated the involvement of AQP1 in human MSC migration through FAK pathway, we found that CM caused an increase of FAK phosphorylation of 140 ± 8.54 respect to control (1% FBS) (Figures 2a and 2b).

3.3 | Autocrine effect of CM on oMSC migration and wound healing

It has been known that MSCs with high levels of AQP1 and/or CXCR4 show a strong ability to migrate to the site of injury and regulate the repair process. To assess the CM effect on oMSCs migration a transwell chamber was used. As shown in Figures 3a and 3b, a significant increase of cell migration was observed when oMSCs were exposed to CM or medium supplemented with 10% FBS, added to the lower chamber as chemoattractants. The cell migration to medium supplemented with 1% FBS was considered 100% and used as control (*$p < 0.001$). The inhibition of CXCR4 using AMD3100 (10 μM) and of AQP1 using TEA (100 μM) dramatically suppressed the migration of oMSCs that was promoted by CM ($^{\#}p < 0.05$).

Furthermore, we observed that the treatment of oMSCs with CM induced a significant enhancement in wound closure both at 24 hr and 48 hr compared to control (1% FBS) (49.3% ± 2.45 vs 35.33 ± 3.5 at 24 hr; 76.07% ± 3.07 vs 52.07 ± 3.06 at 48 hr) (*$p < 0.001$). Treatment of cells with AQP1 inhibitor (TEA) or CXCR4 inhibitor (AMD3100) caused a significant delay in wound closure compared to cells grown in

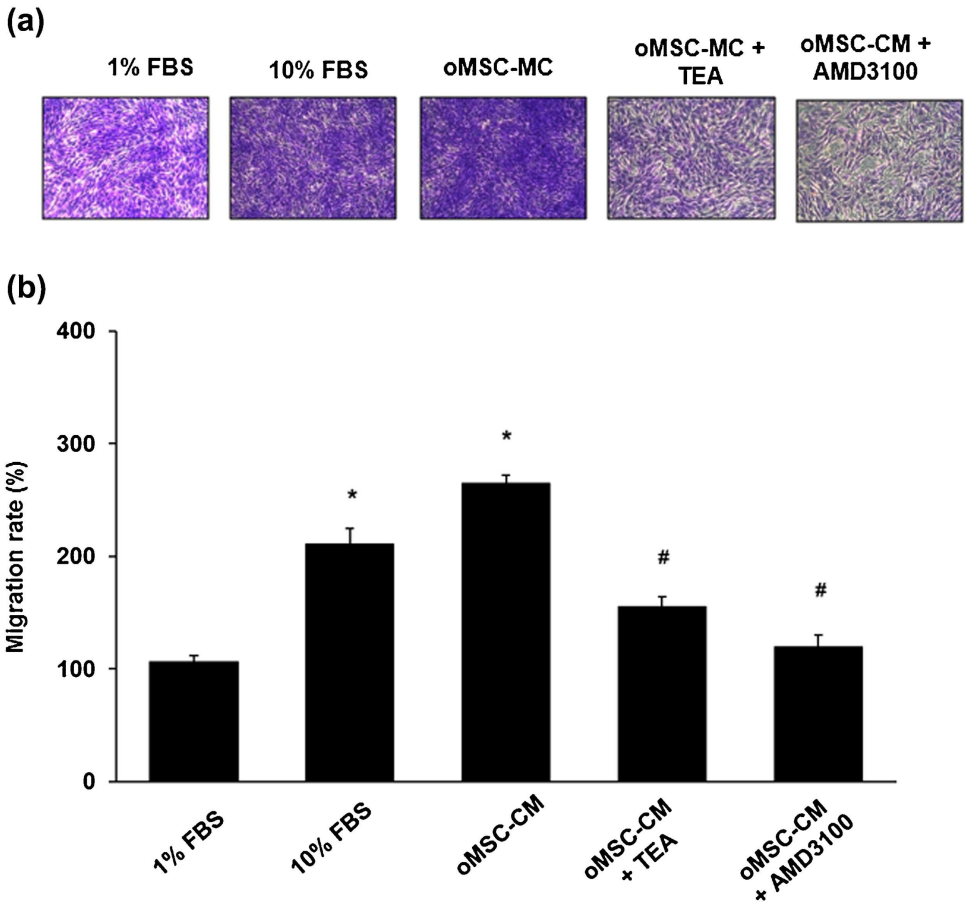

FIGURE 3 Autocrine effect of oMSCS-CM on oMSC migration. (a) Representative images of oMSCs migration in response to 1% FBS, 10% FBS, and CM, used as chemoattractants analysed using transwell assay. (b) The cell migration to medium supplemented with 1% FBS was considered 100% and used as control (*$p < 0.001$). The effects on oMSC migration of CXCR4 inhibitor, AMD3100 (10 μM) and of AQP1 inhibitor, TEA (100 μM) are evaluated respect to CM ($^{\#}p < 0.05$)

the presence of CM alone (TEA: 33.07 ± 2.5 vs 49.3% ± 2.45 at 24 hr, 59.38 ± 2.51 vs 76.07% ± 3.07 at 48 hr; AMD3100: 26.02 ± 2.74 vs 49.3% ± 2.45 at 24 hr; 39.5 ± 3.26 vs 76.07% ± 3.07 at 48 hr. $^{\#}p < 0.05$) (Figures 4a and 4b). Taken together, these results suggest the crucial role played by AQP1 and CXCR4 in promoting oMSC migration.

4 | DISCUSSION

Nowadays, many studies are focused on paracrine effects elicited by CM of the MSCs whereas the autocrine mechanism has not yet been entirely elucidated.

Conditioned medium form MSCs, containing a cocktail of different factors (growth factors, cytokines, interleukins, and C-C ligands etc.), represents a promising tool in regenerative medicine. The ability of MSCs to repair damaged tissues depends on their migration attitude needed to move from residing niches to injured tissues (Rennert, Sorkin, Garg, & Gurtner, 2012). Migration and proliferation of MSCs are controlled by complicated signal networks. For such purposes, a good knowledge regarding MSC biochemical characterization is necessary in order to better clarify their potential use in therapy. Recently, numerous studies have been carried out on therapeutic application of autologous oMSCs in models of injury of cartilage and bone (McCarty et al., 2010). Furthermore, Chang et al. (2015) studied the possible use of conditioned medium on endogenous stem cells to enhance their ability to repair tissue injuries.

Here, we report for the first time that the autocrine loop of CM produced from oMSCs on oMSCs themselves causes an enhancement of migration, wound healing closure, as well as, expression of AQP1 and CXCR4 (Figure 5). Our results demonstrated an inhibitory effect of oMSC migration by TEA and by AMD3100. TEA is a specific inhibitor of AQP1 permeability, has been recently studied to investigate its involvement in proliferation and migration of human MSCs (Ding et al., 2012; Pelagalli et al., 2016). AMD3100 is a well-known CXCR4 antagonist used to modulate the receptor activity in different physiological and pathological states (Cashen, Nervi, & Di Persio, 2007; Fontanella et al., 2016).

Many researches focused their attention on the role of aquaporins family on tumor cell migration (Jiang, 2009; Monzani, Shtil, & La Porta, 2007; Papadopoulos & Saadoun, 2015) and metastatic process (Hu & Verkman, 2006). It has been hypothesized that these pore-channel proteins might provide the major pathway for water entry into

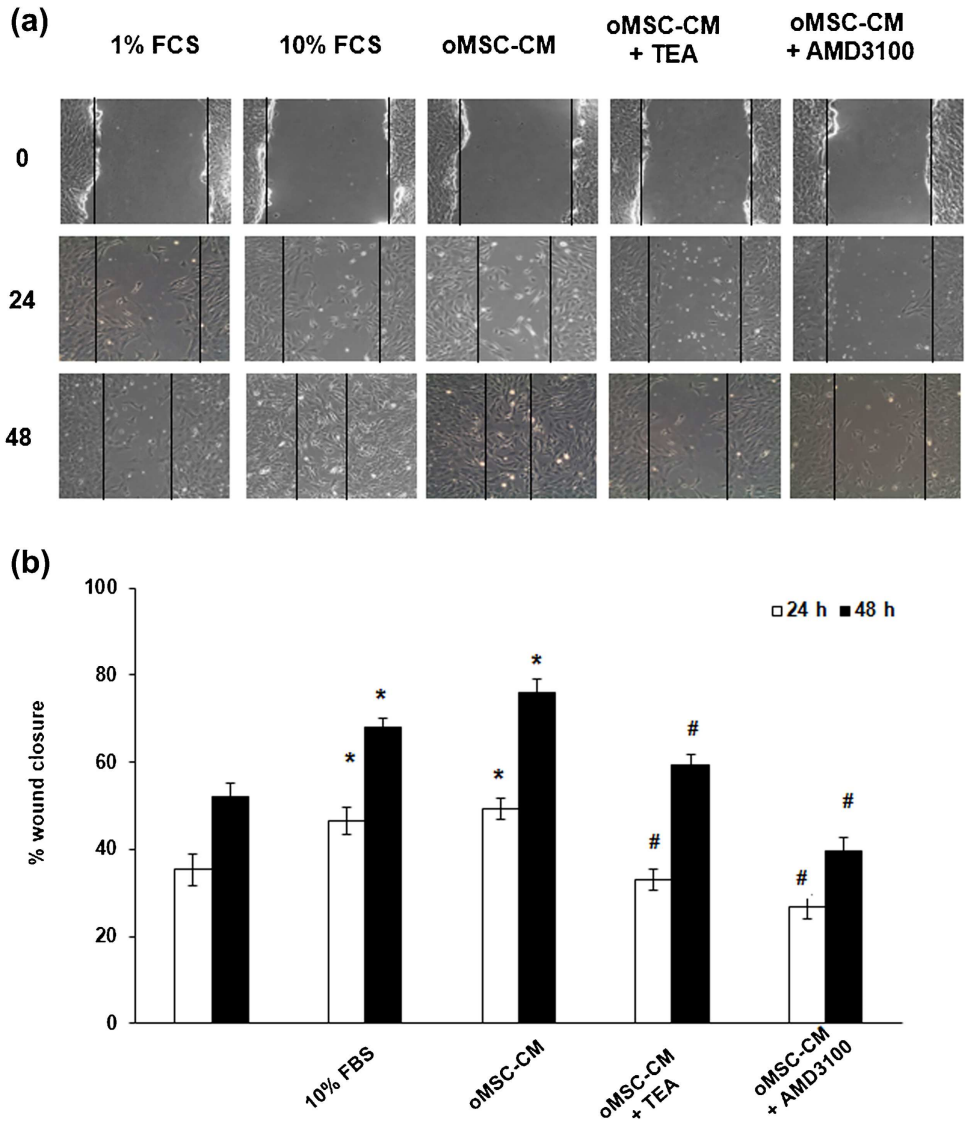

FIGURE 4 Autocrine effect of oMSCS-CM on oMSC wound healing. (a) Representative images of oMSC scratch wounds after cells were grown for 24 and 48 hr in medium containing 1% FBS or 10% FBS or oMSCS-CM in the presence of the CXCR4 inhibitor AMD3100 (10 μM) or AQP1 inhibitor, TEA (100 μM). The images were captured using a digital camera attached to phase contrast microscopy. (b) The extent of wound closure was quantified with TScratch software. Data are expressed as mean ± SD from three independent experiments. $^{*}p < 0.001$ versus medium with 1% FBS; $^{\#}p < 0.001$ versus oMSC-CM

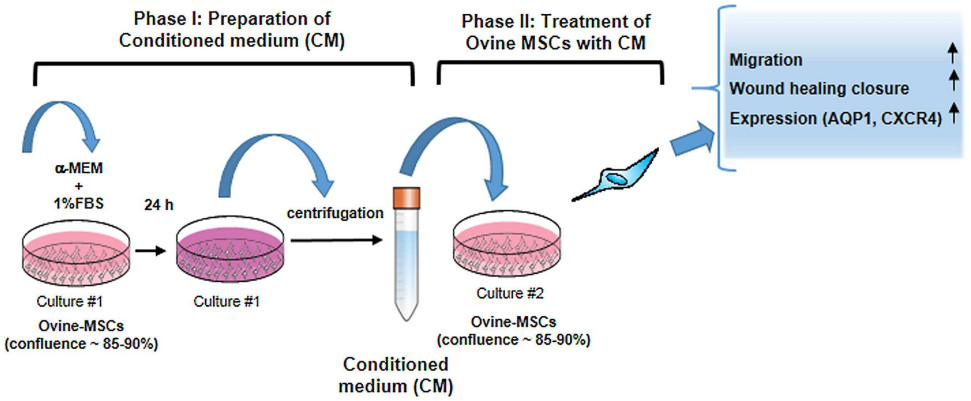

FIGURE 5 Schematic illustration of Ovine MSC-CM autocrine effect on oMSC behaviour and signal pathways involved

lamellipodia (Verkman, 2005) driven by the local osmotic gradient (Saadoun, Papadopoulos, Hara-Chikuma, & Verkman, 2005), whereas Papadopoulos et al. (2008) reported that the AQP1 could act by polarization facilitating water movements that mediate rapid changes in cell volume.

CXCR4 represents a receptor of SDF-1α motif chemokine axis, with a pivotal role for the migration and homing of multiple cell type to specific tissues (Li, Luo, & Sun, 2015; Santagata et al., 2017). Mognetti, La Montagna, Perrelli, Pagliaro, and Penna (2013) showed that among the many factors produced in vitro by BM-MSCs, SDF1α is the main modulator of cell migration. Many studies reported that CXCR4 activates several G-protein-mediated downstream signalling pathways after its stimulation (Ganju et al., 1998; Kang et al., 2015) including PI3K/Akt and MAPK network.

In accordance with our recent findings, in this study we observed an enhancement of phosphorylated forms of Akt and Erk correlated with MSC migration (Camorani et al., 2017). Furthermore, we detected also a p-FAK increase in oMSC stimulated by CM, similar findings were reported by Meng et al. (2014) that showed the ability of AQP1 to modulate MSC migration mainly through the FAK pathway.

However, further studies will be needed to clarify the autocrine activity of oMSC-CM, as well as, to characterize completely its composition.

In conclusion, our findings suggest CM as possible autocrine modulator of MSC behaviour in tissue repair and regeneration. Importantly, the use of CM could bring several advantages in orthopaedic field including cost effectiveness, efficiency and low invasiveness. Furthermore, this study corroborates the importance of mesenchymal stem cell-derived secretome and microvescicles as a cell-free therapeutic that can overcome the listed pitfalls related to cell-based therapeutics (Hill, Pelagalli, Passaro, & Zannetti, 2017; Kim, Choi, & Kim, 2013).

ACKNOWLEDGMENTS

This work was supported by funds of research of the Department of Advanced Biomedical Sciences, University of Naples, Federico II, Italy. We thank Billy Samuel Hill for helpful discussions.

CONFLICTS OF INTEREST

The authors declare that no conflict of interest exists.

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How to cite this article: Pelagalli A, Nardelli A, Lucarelli E, Zannetti A, Brunetti A. Autocrine signals increase ovine mesenchymal stem cells migration through Aquaporin-1 and CXCR4 overexpression. *J Cell Physiol*. 2018;1–9. https://doi.org/10.1002/jcp.26493