Osteoporosis–Associated Alteration in the Signalling Status of BMP-2 in Human MSCs Under Adipogenic Conditions

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ABSTRACT

Postmenopausal osteoporosis is characterized by decreased bone quality and mineral density. Mesenchymal stem cells (MSCs) found in the bone marrow, are pluripotent cells able to differentiate into several phenotypes, including osteoblasts and adipocytes. In osteoporosis, MSCs′ commitment and differentiation into osteoblast/adipocyte is unbalanced, favoring adipocyte formation. The osteo and adipogenic processes are modulated by the bone morphogenetic protein-2 (BMP-2). This cytokine regulates the expression of transcription factors PPARγ and Runx 2, but its action on cells under adipogenic conditions is poorly understood. In this work we studied BMP-2 signaling in MSCs obtained from bone marrow of control or osteoporotic volunteer postmenopausal women. MSCs were cultured under basal, adipogenic (AD) or AD plus BMP-2 conditions. The protein content of PPARγ, p-PPARγ, Runx2, bone morphogenetic receptor IA (BMPR IA), phosphorylated Smad-1/5/8 (p-Smad) and Smad 4 were determined by specific western blots. mRNA level for BMPRs was determined by PCR and cell localization of p-Smad-1/5/8 were detected by immunocytochemistry. Control MSCs showed a differential response to both AD and AD plus BMP-2 treatments: BMP-2 exerted an anti-adipogenic effect increasing both transcription factors analyzed. Moreover, p-Smads-1/5/8 were detected in nuclei after short term BMP-2 treatment. Osteoporotic MSCs showed no response to exogenous added BMP-2, as shown by p-PPARγ/PPARγ ratio and Runx2 levels, although BMPR-IA level was significantly higher in osteoporotic than in control MSCs. In addition, staining for p-Smad-1/5/8 in o-MSCs was observed around nuclei at all experimental conditions. Taken together results demonstrate failure of BMP-2 signaling in osteoporotic MSCs. J. Cell. Biochem. 116: 1267–1277, 2015.   © 2015 Wiley Periodicals, Inc.

KEY WORDS: BMP-2; BMP RECEPTOR TYPE IA; OSTEOPOROSIS; HUMAN MESENCHYMAL STEM CELLS; ADIPOGENESIS

Osteoporosis is a bone disease characterized by low bone mass and a structural deterioration of bone tissue that leads to bone fragility and an increased susceptibility to fracture [Consensus Development Conference, 1993].

Fracture healing is dependent on the activity of mesenchymal stem cells (MSCs) with osteogenic potential. These are pluripotent cells residing in the bone marrow, able to differentiate into osteogenic, adipogenic, chondrogenic, and myogenic lineages. The MSCs′ fate is modulated by factors from the bone marrow microenvironment, which gather signals from other bone marrow cell phenotypes, extracellular matrix and systemic and local factors [Baksh et al., 2004]. One of the most potent osteoinductive cytokines is bone morphogenetic protein-2 (BMP-2), which has been associated with the fracture-healing process and the pathophysiology of osteoporosis. It has also been proposed as a potential therapeutic agent [Vögelin et al., 2005; Gautschi et al., 2007].

The intracellular effects of BMP-2 are mediated by type I and II receptors complexes; the type I receptor (BMPR-I) determining the final fate of progenitor cells. Seven different type I BMPRs have been reported. In cells of animal origin, BMPR-IA (ALK3), and BMPR-IB (ALK6) appear to command differential intracellular pathways driving mesenchymal cells towards adipocytes or osteoblasts, respectively [Chen et al., 1998]. However, studies in human MSCs suggest that their responses to BMPs may differ from those observed in commonly studied cell lines and rodent MSCs culture models [Osyczka and Leboy, 2005], for instance very low or no expression of BMPR-IB has been found in h-MSCs [Lavery et al., 2008].

Binding of BMPs to the cell membrane associated BMPRs activate the serine/threonine kinase function at type I BMPRs, which propels intracellular signaling through phosphorylation of specific receptor-regulated (R-) Smad proteins 1, 5, or 8. After phosphorylation, R-Smads form heteromeric complexes with the common mediator Smad (Co-Smad, Smad4). These Smad complexes migrate into the nucleus and activate the transcription of specific target genes [Shi and Massagué, 2003; Nohe et al., 2004]. In addition, BMPs may activate Smad-independent signaling pathways, such as p38 MAPK and phosphatidyl inositol 3-kinase [Miyazono et al., 2005; Osyczka and Leboy, 2005].

Differentiation of MSCs depends on the activation of lineage specific transcription factors, such as the osteoblast specific factor Runx2/Cbfa1 or adipocyte specific peroxisome proliferator-activated receptor transcription factor gamma (PPARγ2) [Tontonoz et al., 1994; Ducy et al., 1997; Komori et al., 1997; Karsenty, 2001; Rosen and Spiegelman, 2001]. Several local and systemic factors have been shown to regulate the lineage commitment and terminal differentiation of MSCs. Among the earliest, BMP-2 stimulates osteoblastic commitment of MSCs through Runx2- dependent and Runx2-independent pathways [Lee et al., 2002; Celil et al., 2005; Wang et al., 2007; Kang et al., 2009], thereby inhibiting adipogenesis [Muruganandan et al., 2009]. In addition to its osteogenic effects, several studies demonstrated that BMP-2 signaling, as other BMPs, are involved in the earliest stages of adipocyte differentiation increasing the expression of PPARγ, thus inducing MSCs' commitment into the adipogenic process [Sottile and Seuwen, 2000; Bowers and Lane, 2007; Haiyan et al., 2009; Kang et al., 2009; Muruganandan et al., 2009]. Further, it has been proposed that BMP-induced commitment of MSCs to osteogenic or adipogenic lineage is mutually exclusive [Kang et al., 2009].

The maintenance of normal bone homeostasis relies on adequate commitment and differentiation of MSCs in the osteoblast lineage. In conditions associated with bone loss such as aging, glucocorticoid treatment, increased cortisol production, and osteoporosis, a shift in MSCs differentiation has been shown favoring the adipocyte lineage over the osteoblast lineage formation, thus presenting increased bone marrow adiposity [Kirkland et al., 2002; Rosen et al., 2009]. Given the close association between adipocyte and osteoblast formation, the action of BMP-2 on MSCs may be critical for lineage divergence during cell commitment, particularly in bone loss conditions such as osteoporosis.

BMP-2 physiologically contributes to the early phase of fracture healing [Gerstenfeld et al., 2003; Dimitriou et al., 2005] and is clinically approved for the treatment of distinct fracture types [Govender et al., 2002]. Since fracture healing is reduced in osteoporosis, producing a high rate of complications during treatment [Stromsoe, 2004; Giannoudis and Schneider, 2006; Nikolaou et al., 2009], it is necessary to understand whether BMP-2 is effective in MSCs originated from osteoporotic patients. Only a recent study demonstrates osteoporosis-associated alteration in the BMP-2 signal transduction, though cells maintained their capacity for osteogenic differentiation [Prall et al., 2013]. On the other hand, there is no information on the effect of the cytokine on h-MSCs under adipogenic conditions, being this of interest because microenvironment-sustaining MSCs in bone marrow have been shown to be pro adipogenic, in osteoporotic condition [Pino et al., 2010].

We have previously shown several intrinsic functional alterations leading to poor osteogenic capability and increased adipogenesis in MSCs originated from the bone marrow of osteoporotic women [Rodríguez et al., 1999; Astudillo et al., 2008; Rodríguez et al., 2012]. The aim of the present report was to study direct effects of BMP-2 on MSCs from control and osteoporotic women, during the determination phase of in vitro adipogenesis. Our observations indicate that control and osteoporotic MSCs differ in their response to BMP-2 as shown by the protein level of Runx2 and active PPARγ, as osteogenic and adipogenic gene markers, respectively. Both cell types expressed functional BMPR-IA, but the receptor content was higher in osteoporotic compared to control cells. Results point to an osteoporosis-associated alteration in the signalling status of BMP-2 in hMSCs. Our results suggest that increased levels of BMPR-IA and impaired downstream signalling would distort triggering of the canonical BMP-2 response in h-MSCs originated from osteoporotic patients.

MATERIALS AND METHODS

SUBJECTS

Postmenopausal women aged 60–75 years old who required bone surgery because of bone fracture at the Trauma Section of Hospital Sótero del Río, Santiago, Chile, were invited to volunteer as bone marrow donors. Bone marrow was obtained by iliac crest aspiration during surgical procedures [Rodríguez et al., 1999]. Written informed consent was obtained from all subjects and ethical approval was obtained from ethics committees at the Hospital Sótero del Río and Instituto de Nutrición y Tecnología de los Alimentos (INTA). For each subject, bone mineral density (BMD) was measured within the 4 weeks following surgery using dual-energy x-ray absorptiometry (LUNAR, Prodigy, General Electric Medical Systems, Madison, WI). Donors were classified as control or osteoporotic according to their BMD value; control donors had BMD values higher than −2.5 standard deviation (SD) and osteoporotic donors had BMD lower than −2.5 SD and hip fracture [Raisz, 1997]. Control and osteoporotic donors considered themselves healthy, besides the fracture, and were not under glucocorticoid or estrogen replacement therapy.

CELL PREPARATION AND CULTURE METHODS

MSCs were classified as control (c-MSCs) or osteoporotic (o-MSCs) according to whether they derived from control or osteoporotic donors. MSCs were isolated from bone marrow according to Jaiswal et al. (1997). Briefly, 10 ml of bone marrow aspirate was added to 20 ml of Dulbecco's Modified Eagle medium high glucose (D-MEM) containing 10% fetal bovine serum (FBS) (basal medium); cells were sedimented by centrifugation, discarding the fat layer. Cells were resuspended in basal medium and fractionated on a 70% Percoll density gradient. The MSCs-enriched low-density fraction was collected, rinsed with basal medium and plated at a density of 1–2×10^7 nucleated cells/100 mm dishes. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. After 4 days in culture, non-adherent cells were removed and fresh culture medium was added. Basal medium was replaced by fresh medium twice weekly. When

culture dishes became near confluence, cells were detached by mild treatment with trypsin (0.25%, 5 min, 37 °C) and replated at 1/3 the original density to allow for continued passage. The experiments described here were performed after the fourth cell passage.

DIFFERENTIATION OF MSCS

MSCs obtained from control and osteoporotic donors were maintained in basal medium for at least 4 days before adding basal or adipogenic (AD) medium. The AD medium contained basal medium supplemented with 1 μM dexamethasone, 10 μg/ml insulin, 0.45 mM isobutyl-methyl-xanthine, and 0.1 mM indomethacin. The medium was replaced by fresh medium every four days.

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MSCs were cultured in 24 multiwell plates, on coverslips (12 mm) (5×10^3 cells/well), in different culture conditions. After selected times, cells were washed with PBS and fixed with 100% methanol at -20 °C during 15 min. Cells were then washed with PBS and subsequently treated with a 3% BSA solution (Rockland Immunochemicals Inc., Gilbertsville, PA) in TBS 1X (blocking solution) during 1 h. MSCs were incubated with primary specific antibody anti-BMPR-IA (E-16 goat-polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) or anti p-Smad1/5/8 (rabbit-polyclonal, Cell Signaling, Danvers, MA) at 1:200 dilution in blocking solution during 1 h at room temperature. Afterwards, cells were washed three times with PBS, and then were incubated with the secondary antibody anti-goat coupled to Cy3 (mouse-polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 or anti-rabbit coupled with FITC (mouse monoclonal, Sigma, Missouri) at a dilution of 1:750 in blocking solutions during one hour. Cells were washed three times with PBS and stained with 4′,6-diamino-2-phenylindole (DAPI) (Calbiochem, La Jolla) or Alexa Fluor® 594 Phalloidin (Life Technologies, Paisley, UK), and mounted (Fluorescent Mounting Medium, DakoCytomation, Glostrup, Denmark). Preparations were analyzed with an epifluorescence microscope (Nikon, modelo Eclipse 50i) and photographs were taken with a digital camera (Nikon coolpix p6000).

WESTERN BLOT ANALYSIS

At selected times, cells cultured under basal or adipogenic conditions were lysed in 500 μl of 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and a mixture of proteases inhibitors (aprotinin, p-methylsulphonylfluoride, and sodium orthovanadate) as described by Lecka-Czernik et al. [1999]. Proteins (20 μg) from the former fraction were denatured with sample buffer (2% SDS, 10% glycerol, 0.06 M Tris-HCl, 0.01%, bromophenol blue, 20% β-mercaptoethanol, pH 6.8) during 5 min at 100 °C, and separated by 8% SDS-PAGE. Then, samples were transferred to nitrocellulose or PVDF membranes (polyvinylidene fluoride; Perkin Elmer Life Sciences, Boston, MA), for 90 min at 100 V.

Membranes were blocked with 10% fat-free milk in 0.1% TBS-Tween-20, for 1 h at room temperature; after this, membrane was incubated with the corresponding primary antibody anti BMPR-IA (1:300, E-16 goat-polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), PPARγ (1:1000, E-8 mouse monoclonal, Santa Cruz

Biotechnology, Santa Cruz, CA), pPPARγ (1:1000 AW504 rabbit monoclonal, Millipore, Temecula, CA), Runx2 (1:2000, M70 Rabbit-polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), p-Smad 1/5/8 (1:1000 mouse monoclonal, Upstate Biotechnology, Lake Placid, NY), Smad 4 (1:500 Rabbit-polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), Smad 1 (1:300 mouse monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA) or actin (1:2000, AC-15 mouse monoclonal, Sigma, Missouri), during 1 h at room temperature. Membranes were washed three times during 10 min in 0.1% TBS–Tween-20 and subsequently incubated during 1 h at room temperature with the secondary antibody rabbit, mouse or goat anti-IgG, conjugated with peroxidase, at 1:5000 dilution in 0.1% TBS-Tween-20. Immunoreactive bands were visualized using an enhanced chemilumescent (ECL) kit (Pierce, Rockford, IL). Quantification was performed using Kodak Digital 1D software. β-actin or Smad 1 were used as a load control and its value was used for normalization.

RNA EXTRACTION AND RT–PCR ANALYSIS

Cells were released from the culture dishes by a mild treatment with trypsin; cells were collected and suspended with 1 ml of RNAWiz. The RNA was quantified in a spectrophotometer (MBE2000, Perkin Elmer, Boston, MA) and stored at -80 °C. RNA integrity was visualized by electrophoresis under denaturing conditions.

The complementary cDNA was synthesized using reverse transcriptase M-MLV, through a 5 min cycle at 70 °C, then at 4 °C in ice for 5 min, 90 min cycle at 42 °C, and an extension of 15 min at 70 °C. For PCR reactions, 300 ng of cDNA was amplified with 30 μL of reaction mix (1X PCR reaction buffer, 1.5 mM $MgCl_2$, 6 pmol of each primer, 0.2 mM dNTPs, 22.3 μl of nucleases-free water, and 2.5 U of Taq Polymerase). The primer sequences for BMPRIA were 5′-CAGCCTCCAGACTCACAGCAT-3′ and 3′-GGCAATTCAGTACCCA-GAGC-5′; and for BMPRIB 5′-CATGACCTAGTGCCCAGTGA-3′ and reverse 5′-AAGCAGGACGATGTTCAAGG-3′. The size of the PCR products were 399 bp and 345 bp, respectively. The PCR products were separated in 1.2% agarose gels. 18s RNA was used as housekeeping.

STATISTICAL ANALYSIS

Results were obtained studying at least three different cell samples. Comparison between control and osteoporotic groups were done by the two-sample Students t-test, using the GraphPad Prism software version 5.0, $P < 0.05$ was considered statistically significant.

RESULTS

EFFECT OF BMP-2 ON ADIPOGENIC AND OSTEOGENIC GENE MARKERS EXPRESSED BY MSCS CULTURED IN ADIPOGENIC CONDITIONS

Activation of the PPARγ transcription factor is essential in the determination phase of adipogenesis. PPARγ transcriptional activity is positively regulated by specific lipophilic ligands and negatively by phosphorylation on Ser 112/82. Several factors that inhibit AD differentiation cause p38 and/or MAPK-mediated phosphorylation of PPARγ, with a subsequent loss of its transcriptional activity. Therefore, here we determined phosphorylated PPARγ at serine

112/82 as a measure of inactive PPARγ protein, in addition to total PPARγ protein. A representative western blot of these proteins is shown in Figure 1A. Results evaluated the p-PPARγ / PPARγ protein ratio in c- and o-MSCs under basal, AD or AD plus BMP-2 condition, for 48 h. AD treatment for 48 h decreased the p-PPARγ/PPARγ ratio slightly in c-MSCs, as expected from cells committed in adipogenesis, while concurrent BMP-2 treatment significantly increased p-PPARγ/PPARγ ratio, deviating PPARγ to its inactive form (Fig. 1B). In contrast, o-MSCs had a similar p-PPARγ/PPARγ ratio under basal or stimulated conditions (Fig. 1C).

An early marker for MSCs osteogenic capacity is the expression of the transcription factor Runx2. Accordingly, the protein level of Runx2 was analyzed by western blot: As shown in Figure 2A, under basal conditions both control and osteoporotic cells presented similar Runx2 protein level which was not significantly modified by BMP-2 treatment. In contrast, the expression of Runx2 protein differentiated among these cells after basal, AD or AD plus BMP-2 treatment, as shown in the representative western blot of Figure 2B. AD treatment for 48 h decreased Runx2 level in c-MSCs, compared to untreated cells. However, in AD plus BMP-2 treatment, these cells increased the content of Runx2 by 2.5 and 5 times compared to the basal and AD conditions, respectively (Fig. 2C). In o-MSCs, the level of Runx2 was similar in all culture conditions tested (Fig. 2D).

EFFECT OF BMP-2 ON THE EXPRESSION OF BMP RECEPTORS TYPE IA AND IB ON MSCS

Several lines of evidence indicate that differential expression of BMP receptors type I is associated with adipogenic or osteogenic differentiation. To explore on the different responsiveness of c- and o-MSCs to BMP-2, both mRNA and proteins levels of BMPR-IA and BMPR-IB were measured in these cells treated in basal conditions for 48 h. Both, c- and o-MSCs expressed low mRNA level for BMPR-IB, while BMPR-IB protein level was not detected by western blot analysis (data not shown). In contrast, BMPR-IA was expressed by both c- and o-MSCs; immunofluorescence showed positive staining for BMPR-IA allocated homogeneously in both c- and o-MSCs under basal conditions for 48 h (Fig. 3A,B). Further studies by RT-PCR and western blot demonstrated that BMPR-IA content was 1.5 fold higher in o-MSCs than in c-MSCs, in both mRNA (Fig. 3C) or protein (Fig. 3D). Considering the former observations, only BMPR-IA was subsequently analyzed.

BMPR-IA level was determined by western blot in c- and o-MSCs maintained for 48 h in basal, adipogenic or adipogenic plus BMP-2 media (Fig. 4A). In control cells, adipogenic stimulation decreased the receptor level by 80%, compared to cells in basal medium, while cells treated with adipogenic medium plus BMP-2 maintained the receptor at level observed in basal condition (Fig. 4B). In contrast,
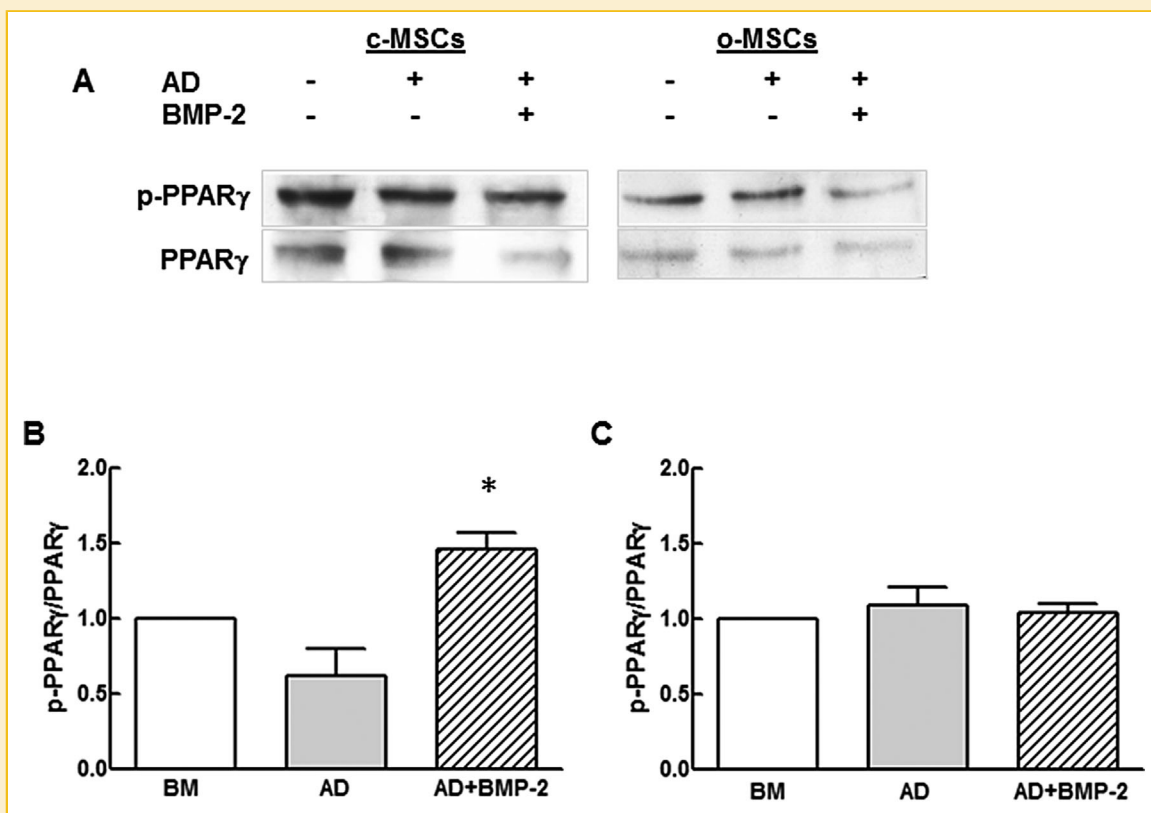

Fig. 1. Effect of BMP–2 on the relationship between p–PPARγ and PPARγ protein levels in control (c–MSCs) and osteoporotic (o–MSCs) MSCs. Cells were cultured for 48 h in basal or adipogenic (AD) medium supplemented with vehicle or 50 ng/ml BMP–2 (BMP–2). Protein extracts from c- and o-MSCs were separated by SDS–PAGE and subjected to immunoblot analysis using anti PPARγ antibody (lower row); the same membrane was reblotted with anti p–PPARγ antibody (upper row). Representative western blot analysis is shown in (A). Densitometric values from western blots expressed as the p–PPARγ/ PPARγ ratio are shown for control (B), and for osteoporotic MSCs (C). Values are means ± SD of at least three different samples. *$P < 0.05$ compared with control MSCs.
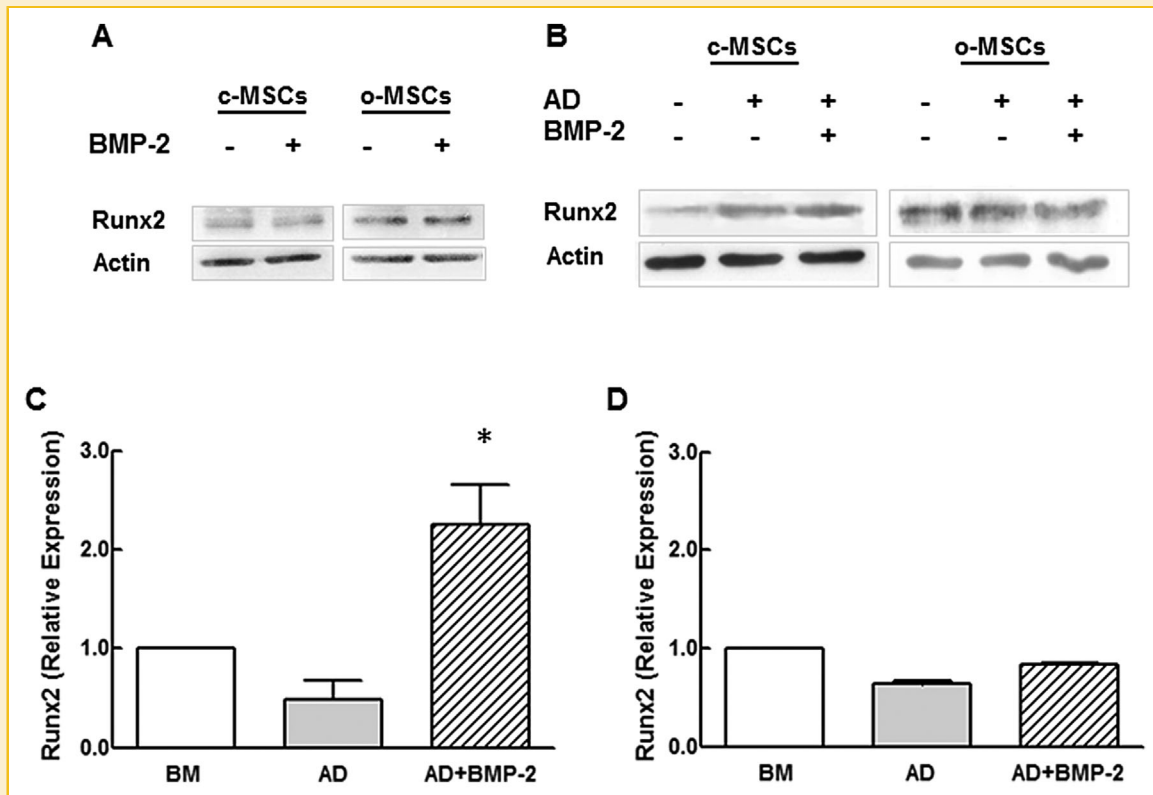
Fig. 2. Effect of BMP–2 on Runx2 protein levels in control and osteoporotic MSCs. Cells were cultured for 48 h in basal (BM) or adipogenic (AD) medium supplemented with vehicle or 50 ng/ml BMP–2. Representative western blot analysis of Runx2 in cells under basal medium (A), and in cells under basal or adipogenic condition (B). Densitometric values from western blots of control (C) and osteoporotic MSCs (D) are shown. Data were normalized to corresponding actin level. Values are means ± SD of at least three different samples. *$P < 0.05$ compared with basal medium.

adipogenic treatment of o-MSCs increased BMPR-IA level by 2–3 fold, as compared to the receptor content observed under basal condition, while under the adipogenic plus BMP-2 condition the increased BMPR-IA level was sustained (Fig. 4C).

ACTIVATION OF SMAD 1/5/8 AND SMAD 4

BMP-2 signaling downstream of the receptor was analyzed by measuring by western blots p-Smad 1/5/8 and Smad 4 proteins (Left panels Fig. 5A–C). Under basal conditions, BMP-2 significantly induced phosphorylation of Smad 1/5/8 in both control and osteoporotic cells (Fig. 5 A). On the other hand, p-Smad1/5/8 level was not modified by AD treatment of cells, but BMP-2 treatment increased pSmad 1/5/8 level in both c-MSCs and o-MSCs. (Fig. 5B). Finally, under basal conditions the level of Smad 4 showed significantly higher in c-MSCs than in o-MSCs. Adipogenic or adipogenic plus BMP-2 treatment decreased Smad 4 protein level in c-MSCs, only (Fig. 5C). Consequently, the level of Smad 4 in o-MSCs remained unchanged after treatments and showed lower than the corresponding values in c-MSCs.

After BMP-2 dependent activation of BMPRs, pSmads 1/5/8 associate to Smad4 and translocate to the nucleus. Therefore, the intracellular localization of pSmads 1/5/8 in response to BMP-2 was analyzed by immunocytochemistry in c- and o- MSCs. For this purpose, cells were incubated during 24 h in serum-free basal or adipogenic medium, then BMP-2 was added to medium for 45 min. Results in Figure 6 (panel A and C) show that pSmad 1/5/8 localized in the nucleus of c-MSCs, at both basal and adipogenic conditions. Treatment of these cells with BMP-2 increased the nuclear fluorescence intensity for p-Smad 1/5/8 protein (Fig. 6 panel B and D). On the other hand, staining for p-Smad 1/5/8 in o-MSCs was observed confined perinuclearly at basal or adipogenic conditions (Fig 6. panel E and G). The perinuclear distribution of p-Smad 1/5/8 did not change after BMP-2 stimulation (Fig. 6 panel F and H).

DISCUSSION

In this work we studied whether human MSCs derived from control or osteoporotic donors differ in their response to BMP-2 during the determination phase of in vitro adipogenesis. In this phase, commitment signals acting on responsive MSCs modify their gene expression patterns and/or activity of several key transcription factors [Tontonoz et al., 1994; Ducy et al., 1997]. The capacity of cells to develop appropriate response to commitment through this phase is of particular interest in conditions of bone lose like age-related osteoporosis because of the reciprocal relationship between decreased bone formation and increased adipogenesis.
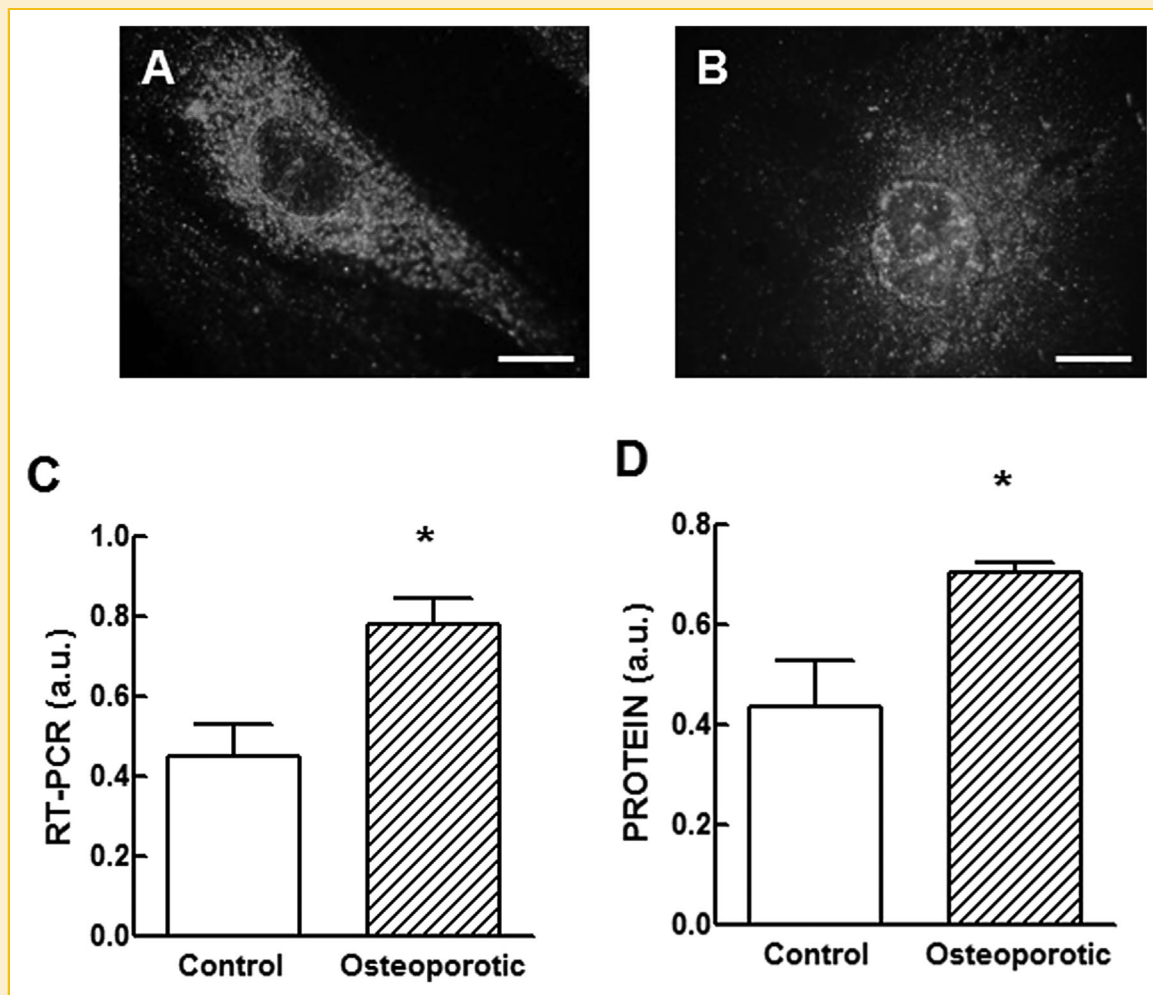
Fig. 3. Expression of BMP receptor type IA (BMPR–IA) by control and osteoporotic MSCs cultured for 48 h in basal medium. Representative immunocytochemistry for BMPR–IA in control (A) and osteoporotic MSCs (B). Cells were immunostained with specific BMPR–IA goat–polyclonal antibody. The secondary antibody was mouse–polyclonal anti–goat coupled to Cy3. Nuclei were stained with DAPI. Scale bar, 10 um. Histograms depict the densitometric values, expressed as arbitrary units, from RT–PCR (C) and from western blots (D) for BMPR–IA. RNA and protein values were normalized to 18 S RNA and to actin, respectively. Values are means ± SD of at least three different samples. *$P < 0.05$ compared with c–MSCs.

Results demonstrate differing BMP-2 signaling status in c- and o-MSCs at all levels analyzed: the effect on the activity of key transcription factors, the availability of BMPR-IA and of activated proteins downstream from the receptor. Control MSCs showed a differential response to both AD and AD plus BMP-2 treatments, thus BMP-2 exerted an anti-adipogenic effect only on these cells as revealed by increased p-PPARγ/PPARγ ratio and Runx2 level. Thus, although the level of BMPR-IA was significantly higher in osteoporotic than in control MSCs at both mRNA and protein levels, osteoporotic MSCs showed no response to exogenous added BMP-2, as detected from p-PPARγ/PPARγ ratio and Runx2 levels, and through the sub cellular distribution of Smad 1/5/8.

Several inhibitory factors on AD differentiation promote MAPK-mediated phosphorylation of the PPARγ protein and subsequent loss of its transcription activity [Hu et al., 1996; Reginato et al., 1998; Chan et al., 2001]. Previously, we described this kind of post-transcriptional regulation for the activity PPARγ protein in c- and

o-MSCs: hence, at basal or under AD conditions c-MSCs had much lower content of PPARγ protein than osteoporotic cells, while control and o-MSCs showed similar amount of PPARγ mRNA, before or through AD differentiation [Astudillo et al., 2008]. Since both PPARγ mRNA and protein have a short half-life [Waite et al., 2001], even slight changes in the amount of active/inactive PPARγ protein could affect the AD potential of cells. Our results demonstrate diminished phosphorylated PPARγ/PPARγ ratio in control cells under AD stimulation, compared to cells in basal conditions. Moreover, we found that BMP-2 added to control cells concurrently with AD medium significantly increased this relationship, implying an anti-adipogenic effect of BMP-2 early on adipogenesis. In contrast, osteoporotic cells maintained the level of phosphorylated PPARγ during AD differentiation either in the presence or absence of BMP-2, showing pro adipogenic active/inactive PPARγ protein relationship. Our results also show that, as expected, AD treatment diminished Runx2 protein level in c-MSCs compared with untreated
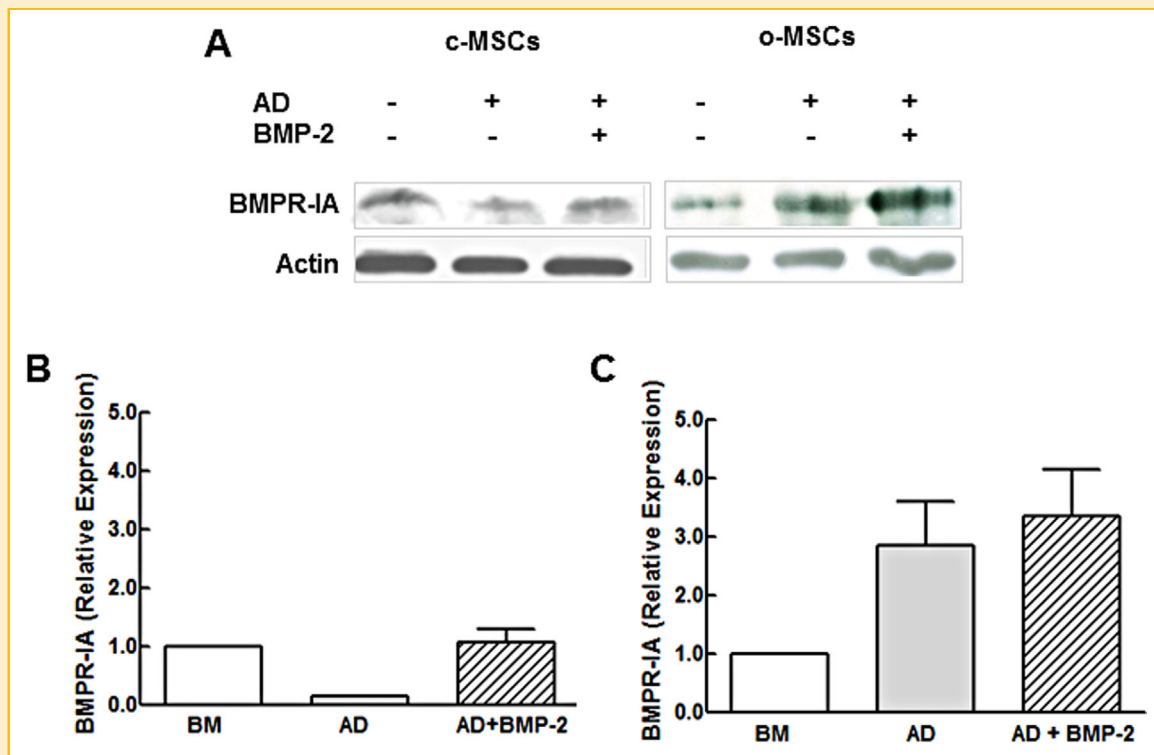
Fig. 4. Effect of BMP-2 on BMPR-IA protein level in control and osteoporotic MSCs under adipogenic condition. Cells were cultured for 48 h in basal (BM), adipogenic (AD) or adipogenic medium plus 50 ng/ml BMP-2 (AD + BMP-2). (A) Representative western blot analysis. Histograms depict the densitometric values from western blots of control (B) and osteoporotic MSCs (C). Data are expressed relative to the corresponding basal value and are means ± SD of at least three different samples. *$P < 0.05$ compared to basal condition. **$P < 0.05$ compared to AD condition.

cells, while BMP-2, in the presence of AD medium, significantly increased Runx2 protein level after 48 h. These findings imply a pro-osteogenic effect of the cytokine. Then, the increased phosphorylated PPARγ/PPARγ ratio observed in control cells in response to BMP-2 could result from their increased Runx2 protein level, since Runx2, among other factors, inactivates PPARγ transcription factor [Gimble et al., 1995]. On the other hand, o-MSCs showed apparent pro-adipogenic response, diminishing Runx2 protein level compared to untreated cells, both in the absence or presence of BMP-2. Thus, in c-MSCs, the favorable effect of BMP-2 on the Runx2 transcription factor is maintained even in the presence of AD stimulus, whereas o-MSCs showed an apparent pro-adipogenic over osteogenic Runx2 response both in the absence or presence of BMP-2. These observations are in agreement with the proposal that BMPs could develop pro- osteo or adipogenic effects depending on the expression and activity state of PPARγ in a given cell type [Sottile and Seuwen, 2000], implying that in o-MSCs, BMP-2 could exacerbate adipogenesis.

Since the former results could imply a hindered action of BMP-2 on o-MSCs, it was of interest to study the availability and function of the cytokine receptors. In agreement with former studies in human mesenchymal stem cells, the expression of BMPR-1B was not detected in c- or o-MSCs [Lavery et al., 2008], but both c- and o-MSCs characterized by their BMPR-IA level. Thus untreated o-MSCs had significantly higher BMPR-IA content than control cells, at both

mRNA and protein level. In addition, the results show that BMPR-IA is active in both c- and o-MSCs, but the response developed by each cell type is divergent. At least three observations support this conclusion. First, BMPR-IA western blot measurements demonstrated that in addition to their contrasting basal BMPR IA level, c- and o- MSCs diverged in their BMPR-IA protein level after AD or AD plus BMP-2 treatments for 48 h. Second, notwithstanding the availability of BMPR-IA was dissimilar in c- and o-MSCs, BMP-2 similarly activated Smad 1/5/8 at both basal and adipogenic conditions. Finally, immunocytochemical studies showed that pSmad 1/5/8 proteins remained in nuclear localization in c-MSCs, while its localization appeared restricted to nuclear border in o-MSCs. This observation could be explained in part by the decreased availability of Smad 4 protein observed in o-MSCs, compared to control cells.

The different expression of BMPR IA (at the mRNA and protein level) in untreated c- and o-MSCs could imply disparity in signaling through the receptor either in signal type, dose or timing. It could be argued that the protein level of BMPR IA in MSCs delineates its responsive status to BMPs throughout AD differentiation; hence enlarged BMPR IA protein content in o-MSCs could turn on unrestrained AD response by increasing cell susceptibility to factors acting through the receptor, or by alternative activation of pathways downstream from the receptor, for instance. In contrast, the limited BMPR IA protein level of c-MSCs apparently warrants
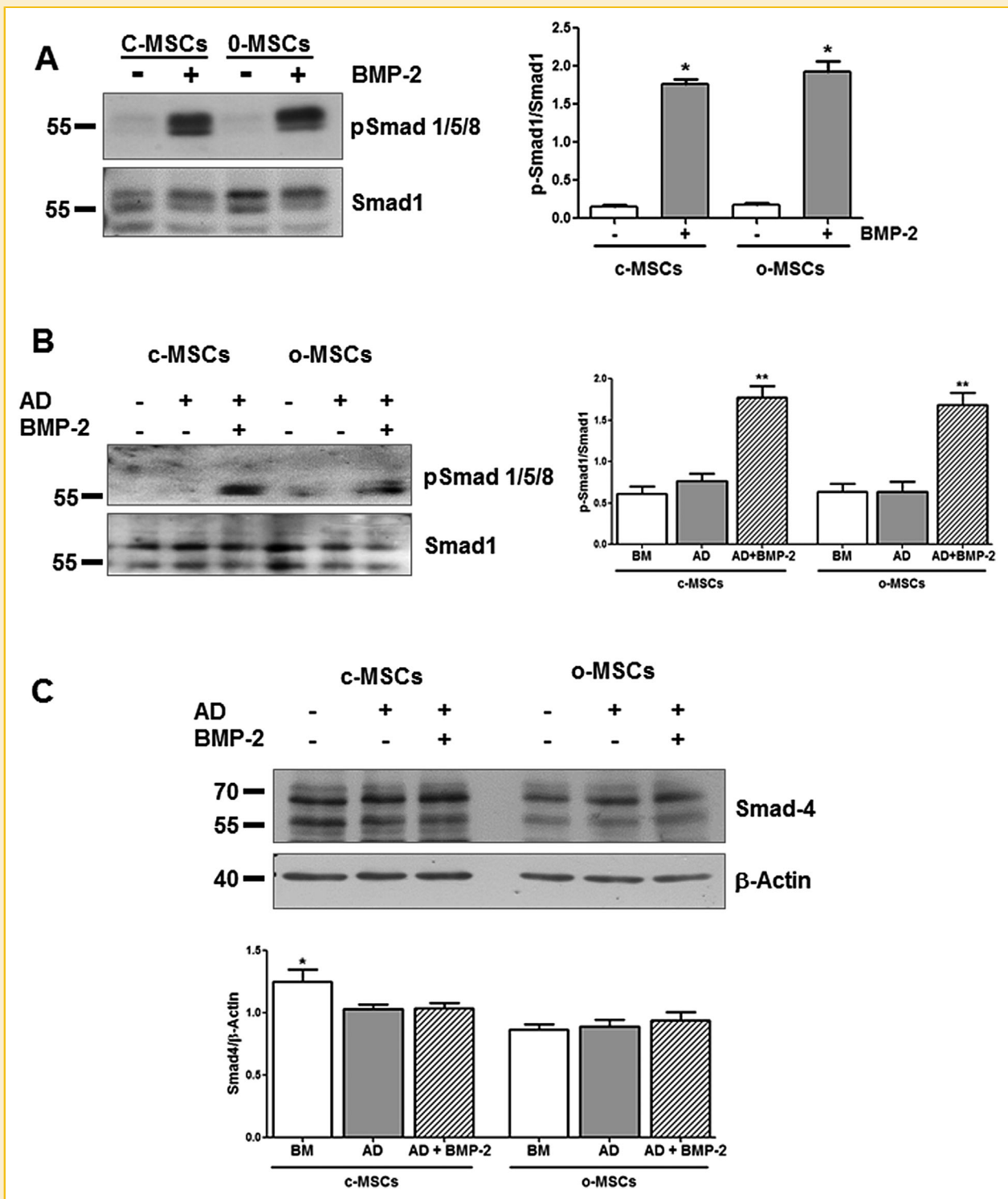
Fig. 5. Activation of Smad 1/5/8 and Smad4 in control and osteoporotic MSCs. Cells were cultured for 48 h in basal or adipogenic medium(AD), then cells were treated with vehicle or 50 ng/ml BMP-2 during 45 min (BMP-2). (A) Representative western blot of p–Smad and Smad proteins (left panel) and densitometric values from western blots expressed as the p–Smad1/ Smad1 ratio (right panel). (B) Representative western blot for p–Smad 1/5/8 (left panel) and densitometric values from western blots expressed as p–Smad 1/Smad1 ratio (right panel). (C) Representative western blot for Smad 4 protein; actin immunodetection is shown as a protein loading control and molecular weight markers are shown on the left. (C, up) Densitometric values from western blots normalized to corresponding actin level for c–MSCs and (C, bottom) for o–MSCs. Values are means ± SD of at least three different samples. *$P < 0.05$ compared to basal condition. **$P < 0.05$ compared to basal and adipogenic conditions.
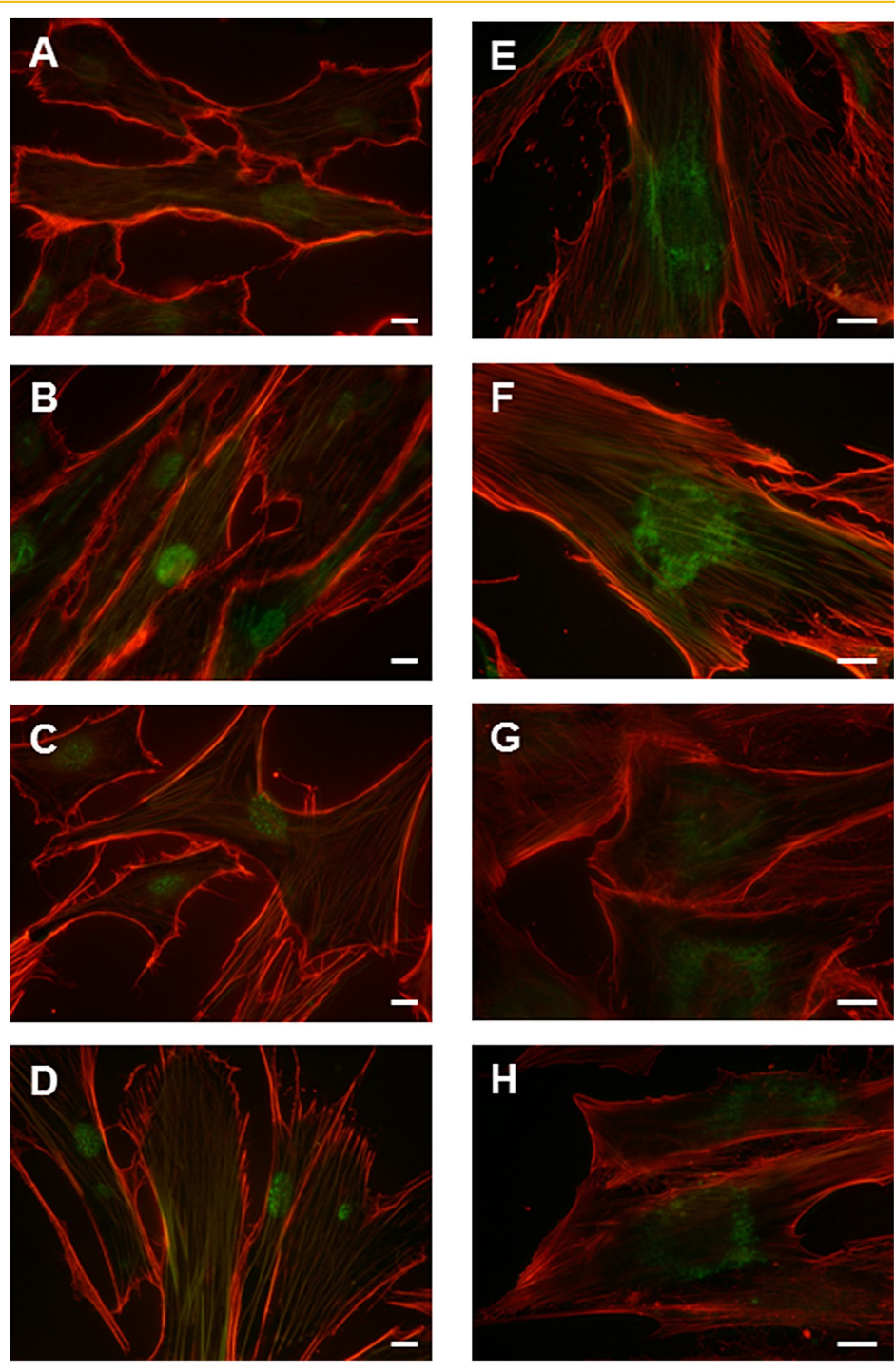
Fig. 6. Activation of Smad 1/5/8 by BMP-2. Cells were incubated in serum-free basal or adipogenic medium for 24 h prior to adding vehicle or 50 ng/ml of BMP-2 for 45 min. Representative immunocytochemistry for p-Smad 1/5/8 is shown for control (A–D) and osteoporotic MSCs (E–H). Panels A, E: basal medium; B, F: basal medium plus BMP-2; C, G: adipogenic medium; D, H: adipogenic medium plus BMP-2. Cells were immunostained using a specific rabbit polyclonal anti p-Smad1/5/8 as primary antibody followed by an anti-rabbit coupled with FITC as secondary antibody. Cells were counterstained with Alexa Fluor® 594 Phalloidin. Scale bar, 10 um.

responsiveness to canonical BMP-2 stimulation, restricting adipogenesis. Taken together our results suggest that increased levels of BMPR IA and impaired downstream signalling would distort triggering of the canonical BMP-2 response in o-MSCs. This is in line with observations in rodent MSCs and MSCs cell lines, proposing that the BMPs effect on MSCs commitment in the AD lineage is highly sensitive to the ligand type [Ahrens et al., 1993; Kang et al., 2009], dose [Wang et al., 1993], and to cell BMPR I level or combination of BMPRs I/II types [Chen et al., 1998; Muruganandan et al., 2009]. In addition, genetic variation in the BMPR IA gene has been related to human obesity, showing significantly increased mRNA expression of the gene in both the visceral and subcutaneous adipose tissue of overweight and obese, compared with lean, subjects [Böttcher et al., 2009].

In a recent study Prall et al. [2013] observed that BMP-2 signal transduction is partly impaired in o-hMSC, though cells sustained BMP-2-specific osteogenic differentiation capacity. Our observations extend the former, demonstrating an osteoporosis-associated alteration of BMP-2 signalling in hMSCs under adipogenic conditions. Such failure of BMP-2 signalling in o-MSCs may relate to decreased fracture healing in osteoporosis, which questions the efficacy of BMP-2 as the therapeutic approach in the treatment of osteoporotic fractures.

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