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Connexin43 intercellular communication drives the early differentiation of human bone marrow stromal cells into osteoblasts

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Abstract

Although it has been demonstrated that human bone marrow stromal cells (hBMSCs) express the ubiquitous connexin43 (Cx43) and form functional gap junctions, their role in the early differentiation of hBMSCs into osteoblasts remains poorly documented. Using *in vitro* assays, we show that Cx43 expression and gap junctional intercellular communication (GJIC) are increased during the differentiation of hBMSCs into osteoblasts, both at the protein and mRNA levels. Two independent procedures to reduce GJIC, a pharmacological approach with GJIC inhibitors (18α-glycyrrhetinic acid and Gap27 peptide) and a molecular approach using small interfering RNA against *Cx43*, demonstrated that the presence of Cx43 and functional junctional channels are essential to the ability of hBMSCs to differentiate into osteoblasts *in vitro*. In addition, a reduced GJIC decreases the expression of *Runx2*, the major transcription factor implicated in the control of osteoblast commitment and early differentiation of hBMSCs into osteoblasts, suggesting that GJIC mediated by Cx43 is implicated in this process. Together our results demonstrate that GJIC mediated by the Cx43 channels plays a central role throughout the differentiation of hBMSC into osteoblasts, from the early stages to the process of mineralization.

Introduction

Bone formation is a regulated process that takes place during embryonic development, growth, remodeling and repair. It begins with the commitment of mesenchymal stem cells (MSC) into the osteoblastic differentiation program, continues with the early differentiation of stem cells into osteoblast progenitors and the subsequent differentiation of these osteoblast progenitors into immature osteoblasts, and finally the late differentiation of these immature osteoblast into mature osteoblasts (Augello 2010; Vaananen 2005; Neve et al., 2011). These MSC, located mainly in the bone marrow stroma, can also differentiate into other mesenchymal cell lineages such as fibroblasts, chondrocytes and bone marrow stromal cells including adipocytes, depending on the activated signaling transcription pathways (Friedenstein et al., 1987; Owen 1988; Marie 2008). At the end of the osteogenic differentiation process, mature osteoblasts produce many markers such as alkaline phosphatase (ALP), collagen type I (COL1), bone sialoprotein (BSP), osteopontin and osteocalcin (OCN) (Wennberg et al., 2000). This process results in the formation of an extracellular organic matrix which will be mineralized.

Connexins, the structural proteins of gap junctions, are a family of transmembrane proteins that oligomerize into hemichannels containing six connexin subunits (Sosinsky 2005). Upon reaching the cell surface, two hemichannels pair to complete an intercellular gap junction channel, which directly links the cytoplasm of neighboring cells and mediates the exchange of low-molecular-mass molecules (<1000 Da), including cAMP, inositol trisphosphate, and Ca^{2+} (Sohl and Willecke, 2004; Nielsen et al., 2012).

Multiple lines of evidence have established that Cx43, the most abundant connexin in bone cells, and subsequent intercellular channels play a major role in skeletal development and for the function of osteoblasts (Stains and Civitelli 2016; Plotkin and Stains, 2015). Cx43 gene mutations have thus been directly associated with abnormalities of skeletal development observed for exemple in oculodentodigital and cranometaphysal dysplasias (Paznekas et al., 2003; Laird, 2014). Specifically, it has been demonstrated that Cx43 expression is required for osteoblastic gene expression and function in primary and cultured osteoblasts (Lecanda et al., 1998; Lecanda et al., 2000). For exemple, it has been shown that gap junctional communication is required for the maturation process of osteoblastic cells in culture and for the stimulation of osteoblast

mineralization in response to parathyroid hormone (Schiller et al., 1992; Schiller et al., 2001a; Schiller et al., 2001b). In addition, *in vivo* experiments using either Cx43$^{-/-}$ or Cx43$^{fl/-}$;Col1a.1-2.3 kb-Cre mice have demonstrated that Cx43 play a crucial role in osteoblastic differentiation and function (Plotkin and Stains, 2015).

However, although it was shown that human MSC (hMSC) express Cx40, Cx43 and Cx45 (Valiunas et al., 2004, Talbot et al., 2013), and form functional gap junctions, the role of connexins in the *in vitro* differentiation of human MSCs into osteoblast progenitors remains poorly documented. In this context, the aim of the present work was to determine the specific role of Cx43 in the early differentiation of hMSCs into osteoblasts and throughout the process of osteoblast differentiation by using small interfering RNA against Cx43 as well as via a pharmacological approach with GJIC inhibitors (18α-glycyrrhetinic acid and Gap27 peptide).

Materials and methods

Ethics statement

Bone marrow aspirates were obtained from healthy volunteers (age=46±12; range=36-67) during orthopaedic surgical procedures after exposure of the iliac crest in the orthopaedic department of the University Hospital of Tours (France). The written consent of informed patients was obtained for the use of tissue samples for anonymized research, in accordance with French law (Art. L. 1245-2 du code de la santé publique, Loi n° 2004-800 du 6 août 2004 Journal Officiel du 7 août 2004). The donors had no significant medical history. The study followed the ethical guidelines of the University Hospital of Tours and was approved by the ethics committee "Comite de protection des personnes" (Tours - Region Centre Ouest).

Cell culture

Human bone marrow stromal cells (hBMSCs) were isolated by Ficoll density gradient separation as previously described (Chang et al., 2009). Non-adherent cells were removed by replacing the culture medium and hBMSCs were then cultured in medium composed of Dulbecco's Modified Eagle Medium (Lonza, Belgium), 10% fetal bovine serum (Hyclone Perbio, France) with 1 ng/ml basic fibroblast growth factor (bFGF; R&D systems, UK). Adherent cells were characterized by flow cytometry (CD45$^-$, CD34$^-$, CD105$^+$, CD73$^+$, CD90$^+$ and CD146$^+$, purity ≥ 99%) prior to further experiments. The expression of each marker was not changed in the presence of bFGF.

Osteoblast differentiation

hBMSCs were seeded at 1×10^4 cells/cm^2 in 24-well plates in culture medium without bFGF. After 3 days, when the cells were confluent, the medium was replaced by fresh medium supplemented with vitamin D3 (10^{-8} M; Hoffmann-La Roche, Switzerland) and dexamethasone (10^{-8} M; Sigma, France), referred to as "osteogenic medium" in the text. Three days later, ascorbic acid (50 µg/ml; Sigma) and β-glycerophosphate (10^{-7} M; Sigma) was added to allow mineralization. Alizarin red staining was used to detect the mineralized nodules formed, as previously described (Chipoy et al., 2004). Briefly, around day 15, cells were fixed in ice-cold 70% ethanol for 1h and incubated with Alizarin red S (40 mM, pH 7.4; Sigma) for 10 min at room temperature. After washing, images were

captured using a stereo microscope (Stemi 2000-C; Zeiss, Germany), and mineralized surfaces were quantified using the Qwin software (Leica, Germany). This software quantifies the red pixel intensity of the whole well surface and expresses these values in arbitrary units. Two gap junction inhibitors were used: the Cx43 mimetic peptide Gap27 (250 μM; GeneCust, France) and the chemical agent 18α-glycyrrhetinic (50 μM; Sigma). Medium in presence or absence of GJIC inhibitors was replaced by fresh medium every 24h. Control cultures were treated with the solvent (DMSO, Sigma) only.

Western Blot

Cells were lysed in a lysis buffer (1% SDS, 10mM Tris pH 7.4, 1 mM Sodium orthovanadate) and protein concentration was determined by BCA kit (Sigma). 10 μg of total protein extracts in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) were separated by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon-P membranes (Millipore, France). Membranes were immunoblotted with mouse monoclonal anti-connexin43 (dilution 1/1000, Sigma) or mouse monoclonal anti-GAPDH (dilution 1/2000, Abcam, France). Antibody binding was visualized with the enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate, ThermoSientific, France). For quantification, luminescence was detected with a Charge Couple Device (CCD) camera and analyzed using the GeneTools program (Syngene, UK).

Real-time polymerase chain reaction

Total RNA was extracted using NucleoSpin®RNAII (Macherey Nagel, France). 1 μg of total RNA was used for first strand cDNA synthesis using ThermoScript RT-PCR System (Invitrogen, France). DNase I treatment (25 units, 10 min) of total RNA was performed to eliminate genomic contamination. Real-time PCR was performed with a Chromo4 instrument (Biorad, France) using SYBR Green Supermix reagents (Biorad). Primer sequences are provided in Table 1. Calibration curves with different amounts of cDNA were used to validate the primers according the MIQE guidelines (Bustin et al., 2009). Cyc1 (cytochrome c-1) and Hprt 1 (Hypoxanthine guanine phosphoribosyl transferase 1) were used as internal controls to normalize the data to determine the relative expression of the target genes using the $2^{-\Delta\Delta Ct}$ method.

Plasmid constructs, transient cell transfections and reporter assays

The *Cx43* promoter/gene reporter construct -2400Cx43-lux was a gift from GI Fishman, The Leon H. Charney Division of Cardiology, New York University School of Medicine, New York, NY and has been described previously (De Leon et al., 1994). Transient cell transfections were performed with jetPEI[TM] (Polyplus-transfection, France). The phRLMLP-*Renilla* luciferase expression vector was cotransfected in every experiment to monitor transfection efficiencies. Luciferase activity was determined with the Dual-Luciferase reporter assay system (Promega, France).

RNA interference

Modified hBMSCs cells were obtained by lentiviral cell transduction as previously described (Trichet et al., 2006). Oligonucleotides were designed and cloned into pSUPER to produce the short hairpin RNA shRNACx43 directed against the human Cx43 gene. The targeted sequence was TGCTGCGAACCTACATCAT. The cloned oligonucleotides were controlled by sequencing (Genome Express) before subcloning them with the upstream H1 promoter into the vector pFG12. As a control, a vector pFG12 was developed to produce shRNAs targeting the LacZ gene. The resulting constructs were used for lentivirus production following the protocols provided with the ViraPower Lentiviral Expression System (Invitrogen) to transduce hBMSCs.

Parachute assay

Gap junctional intercellular communication (GJIC) was determined by "parachute" assay, as previously described (Ziambaras et al., 1998, Talbot et al., 2013; Tellez-Gabriel et al., 2017). Cell cultures were divided into "acceptor" and "donor" cell groups. Adherent acceptor cells were loaded with DiI (Sigma; 5 µM), a nontransferable membrane dye, for 1h, and confluent donor cells were dyed with calcein-acetoxymethylester (calcein-AM; Sigma; 1 µM), a gap junction permeant dye, for 45 min. After loading, cells were washed in PBS to remove the excess dye. Then, the calcein-AM-dyed cells were trypsinized and parachuted on the DiI-dyed acceptor cell layer at a cell ratio of 1:10 and incubated at 37°C for 2h to allow calcein transfer via gap junctions. Dye coupling was analyzed using a FACS FC500 cytometer (Beckman Coulter). For the analysis of results, gates were established on the day of each experiment using non-dyed cells, cells dyed with calcein-AM,

and cells dyed with DiI.

Cell proliferation assay (test XTT)

Cell viability was determined using a 2,3- bis(2 methoxy-4 nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent assay kit (Roche Molecular Biomedicals, Germany). Two thousand cells per well were plated into 96-well plates. At confluence, cells were cultured for 17 days in culture medium in the presence or absence of GJIC inhibitors.

Statistical analysis

Analyses were performed using GraphPad Prism 6.0 software (GraphPad Software). The unpaired t-test was used for the comparison of two conditions. An ANOVA test was used for the multi-group comparisons of more than two samples. A two-way ANOVA test was used for multi-group comparisons of data that compare two variables. The results are given as mean ± SD. Results with $p < 0.05$ were considered significant.

Results

Increased production of Cx43 during the differentiation of hBMSCs into osteoblasts in vitro

We first analyzed the *in vitro* differentiation of hBMSC into osteoblasts under our experimental conditions (see materials and methods). As shown in Figure 1A, incubation of hBMSCs in monolayer cultures with osteogenic medium over 2 weeks resulted in osteoblastic differentiation process as demonstrated by a marked increase in mineral deposition revealed by Alizarin red staining. Concurrently, there was an increase in the expression of genes associated with osteoblast differentiation (Figure 1B). The expression of *Runx2*, the main transcriptional factor involved in the osteoblast commitment and the early differentiation of hBMSCs into osteoblast progenitors (Marie, 2008), was increased quickly after stimulation of hBMSCs with osteogenic medium. Indeed, quantitative real-time PCR demonstrated that *Runx2* mRNA levels increased from day 1 until day 3 and then decreased. Quantitative real-time PCR demonstrated that the levels of transcripts encoding *Alkaline Phosphatase* (ALP), a gene associated with the differentiation of osteoblast progenitors into immature osteoblasts, increased from day 3 to day 7, and then slowly returned to basal level. The transcript levels of *Bone Sialoprotein* (BSP) and *Osteocalcin* (OCN), two genes associated with the late differentiation of immature osteoblasts into mature osteoblasts, gradually increased starting at day 13 and day 7, respectively, until the end of the experiment. As expected, the expression of *Sox9*, a transcription factor implicated in chondrocyte differentiation was not modified after stimulation of hBMSCs with osteogenic medium (data not shown).

We secondly analyzed the production of Cx43 during hBMSC differentiation into osteoblasts under our experimental conditions. Interestingly, Western-Blot analysis showed an increase in Cx43 protein level after treatment of hBMSCs with osteogenic medium for 9 days, which remained stable until day 15 (Figure 2A). To determine whether the induction of Cx43 production during hBMSC differentiation occurred via modulation of the corresponding gene, the *Cx43* mRNA steady-state levels following treatment with osteogenic medium were measured by real-time PCR. As shown in Figure 2B, induction of *Cx43* mRNA was observed in response to osteogenic medium after 9 days of treatment, and *Cx43* levels remained elevated until day 17. By contrast,

no change in the expression of *Cx45* was observed during this process of hBMSC differentiation (Figure 2B). Although the presence of Cx40 has been demonstrated in hMSC (Valiunas et al., 2004), its expression level remains very low (under the limit of capacity of detection by PCR in our experimental conditions) throughout the differentiation process (data not shown). To further explore whether the effect of osteogenic medium on *Cx43* gene expression takes place at the transcriptional level, transient cell transfections were performed with the *Cx43* promoter/gene reporter construct -2400Cx43-lux. As shown in Figure 2C, osteogenic medium induced a 6.5-fold transactivation of the *Cx43* promoter in hBMSCs. To determine whether the increase in *Cx43* gene expression in hBMSCs during osteogenic differentiation is associated with gap junctional intercellular communication changes, GJIC was analyzed by FACS using the parachute assay. GJIC was significantly enhanced (26-fold increase) after 17 days of incubation of hBMSCs with osteogenic medium (Figure 2D).

Together, these results demonstrated an increase in Cx43 production and subsequent cell-cell communication during the differentiation of hBMSCs into osteoblasts.

Small interfering RNA against Cx43 and treatment with 18α-glycyrrhetinic acid or Gap27 peptide efficiently reduce GJIC between hBMSCs

To investigate the role of Cx43 and GJIC during the early differentiation of hBMSCs into osteoblast progenitors and throughout the process of hBMSC differentiation into mature osteoblasts, Cx43 was stably knocked down in hBMSCs by transduction with a lentiviral vector or the cells were treated with the GJIC inhibitor 18*α*-glycyrrhetinic acid or with the peptide Gap27.

As shown in Figure 3A, shCx43 cells were characterized by a significant (70%) reduction in *Cx43* mRNA levels compared to shLacZ or parental cells used as control. This decrease was confirmed at the protein level (Figure 3B). The knocked down of Cx43 does not change the *Cx45* gene expression in hBMSCs (Figure 3A). In addition, GJIC analysis using the parachute assay demonstrated that GJIC was significantly decreased (by 50%) in shCx43 cells compared to shLacZ cells or parental hBMSCs (Figure 3C). Of note, the Cx43 knock-down does not affected the proliferation rate of the cells (data not shown). As shown in Figure 3D both, 18α-glycyrrhetinic (50 μM) acid and Gap27 peptide (250 μM) significantly reduce GJIC between hBMSCs by respectively 70% and 60% after 24h incubation of the cells. Of note, in the experimental

conditions used, the viability of cells was not affected in the presence of 18α-glycyrrhetinic acid or Gap27 peptide throughout the experiment (Figure 3E).

Together these results demonstrated that small interfering RNA against Cx43 or treatment with two GJIC inhibitors efficiently reduce GJIC between hBMSCs

Cx43 knocked down blocks the differentiation of hBMSCs into osteoblasts

We next investigated the effect of the Cx43 knocked down on the osteogenic differentiation of hBMSCs. As shown in Figure 4A, an early decrease in *Runx2* expression was observed in shCx43 cells in response to osteogenic medium compared to that measured in shLacZ cells or in parental cells. Indeed, *Runx2* mRNA steady-state levels were increased by approximately 3-fold in parental and shLacZ cells after treatment of hBMSCs with osteogenic medium for 1 day, but only by 1.7-fold in shCx43 cells. Furthermore, a significant decrease in *ALP*, *BSP* and *OCN* expression levels was observed in shCx43 cells in response to osteogenic medium compared to those measured in shLacZ or in parental cells. *ALP* mRNA steady-state levels were increased by approximately 9-fold in parental and shLacZ cells, but only by 2-fold in shCx43 cells after 7 days of incubation with osteogenic medium (Figure 4A). *OCN* and *BSP* mRNA steady-state levels were increased by approximately 180- and 16-fold, respectively, in parental and shLacZ cells, and only by 120- and 8-fold in shCx43 cells after treatment of hBMSCs with osteogenic medium for 17 days (Figure 4A). Interestingly, after 17 days of treatment with osteogenic medium, a marked decrease (60%) in mineral deposition was measured in shCx43 monolayer cultures compared to that measured in shLacZ or in parental cultures (Figure 4B). Finally, the expression level of *Cx45* was evaluated by quantitative real-time PCR throughout the process of differentiation. As shown in Figure 4C, the expression level of *Cx45* is not modified by the knocked down of Cx43 in hBMSC and remains unchanged throughout the process of differentiation. In contrast and as expected, the expression level of *Cx43* was decreased after hBMSC infection with the shRNA against Cx43 and remains very low throughout the process of differentiation.

Together, these results indicate a crucial role of Cx43 during the early differentiation of hBMSCs into osteoblasts and throughout the process of osteoblast differentiation.

Treatment with GJIC inhibitors inhibits the differentiation of hBMSCs into osteoblasts

To investigate the role GJIC, and not only the role of Cx43 expression, during the early differentiation of hBMSCs into osteoblast progenitors and throughout the process of hBMSC differentiation into mature osteoblasts, the cells were treated with 18α-glycyrrhetinic acid or Gap27 peptide.

As shown in Figure 5A, a significant decrease in *Runx2* expression level in response to osteogenic medium was observed in the presence of 18α-glycyrrhetinic acid or Gap27 peptide compared to hBMSCs cultured without inhibitor. Treatment of hBMSCs with osteogenic medium for 1 day induced a 3.6-fold increase in *Runx2* mRNA steady-state levels. By contrast, *Runx2* level increased by only 1.4-fold and 1.6-fold in the presence of 18α-glycyrrhetinic acid or Gap27 peptide. A significant decrease in *ALP, BSP* and *OCN* expression levels in response to osteogenic medium was also observed in the presence of 18α-glycyrrhetinic acid or Gap27 peptide compared to hBMSC controls (Figure 5A). *ALP* mRNA steady-state levels were increased by 8.6-fold in the absence of a GJIC inhibitor, while treatment with 18α-glycyrrhetinic acid or Gap27 peptide induced only a 2.2-fold and 3.2-fold increase in mRNA levels, respectively, after 7 days of incubation of hBMSCs in osteogenic medium. Treatment of hBMSCs with osteogenic medium for 17 days induced an increase in *OC* and *BSP* mRNA steady-state levels of 137- and 10-fold, respectively. By contrast, *OCN* and *BSP* levels increased by only 30- and 2.3-fold in the presence of 18α-glycyrrhetinic acid, and by 63- and 4.7-fold in the presence of Gap27 peptide. In agreement with these data, a significant decrease in mineral deposition was measured in the presence of 18α-glycyrrhetinic acid or Gap27 peptide compared to cells cultured in the absence of GJIC inhibitors. After 17 days of treatment with osteogenic medium, hBMSCs treated with 18α-glycyrrhetinic acid or Gap27 peptide showed significantly less Alizarin red S staining (95% and 60%, respectively) compared to cells cultured without GJIC inhibitor (Figure 5B).

Together, these results indicate an important role GJIC during the early differentiation of hBMSCs into osteoblasts and throughout the process of osteoblast differentiation.

Discussion

Previous *in vitro* studies have demonstrated the importance of Cx43 in osteoblast function and during the late stages of osteoblast differentiation specifically during the differentiation of immature osteoblasts into mature osteoblasts able to mineralize (Plotkin anf Bellido, 2013; Stains and Civitelli, 2016). *In vivo* experiments using either Cx43$^{-/-}$ or Cx43$^{fl/-}$;Col1a.1-2.3 kb-Cre mice have demonstrated that Cx43 play a crucial role in osteoblastic differentiation (Plotkin and Stains, 2015). However, the role of Cx43 in the early differentiation of hBMSCs into osteoblasts *in vitro* remains poorly documented.

In the present study, we first demonstrate that Cx43 expression is increased at the transcriptional level during the late differentiation of hBMSCs into mature osteoblasts. Many transcription factors control osteoblastogenesis. Among them, activator protein-1 (AP-1), a dimeric transcription factor formed by Fos (c-Fos, Fra-1, Fra-2, FosB) and Jun proteins (c-Jun, JunB, JunD) is required for bone formation and remodeling (Eferl and Wagner 2003). For example, most Fos proteins are implicated in the proliferation and differentiation of osteoblasts (Grigoriadis et al., 1993). Interestingly, one AP-1-binding site has been identified in the human Cx43 proximal promoter P1 (approximately 150 nucleotides up- and downstream of the transcription initiation site), suggesting that this AP-1 response element could be involved in the increased expression of Cx43 during hBMSC differentiation into osteoblasts.

Additionally, we demonstrate that Cx43 and subsequent GJIC play an important role throughout the differentiation of hBMSCs into osteoblasts from the early stages to the process of mineralization. It is well recognized that gap junctional communication plays an important role in the control of differentiation into different cell types and tissues (Duval et al., 2002). Specifically, it has been shown that Cx43 and GJIC are crucial in the regulation of self-renewal and maintenance of pluripotency in embryonic stem cells (Todorova et al., 2008). With regard to hBMSC differentiation, osteoblastogenesis may be schematically defined by three phases (Marie, 2008): 1) the osteoblast commitment of hBMSCs and the early differentiation of hBMSCs into osteoblast progenitors, 2) the differentiation of these osteoblast progenitors into immature osteoblasts, and 3) the differentiation of immature osteoblasts into mature osteoblasts able to mineralize. All these three phases are characterized by sequentially expressed genes. The first stage is characterized

by the expression of *Runx2*, the earliest and the most specific transcription factor of mesenchymal stem cell commitment toward osteoblastic lineage (Wagner and Karsenty, 2001). Runx2 is indeed expressed in all mesenchymal condensations before osteoblast differentiation during early murine skeleton development at E10.5-E12.5 (Ducy et al., 1997). Our *in vitro* results showed that Runx2 is induced very early, 1 day after stimulation of hBMSCs with osteogenic medium. This early increase in Runx2 expression precedes the increased expression of ALP (expression peak at day 7), a gene specific to the differentiation of osteoblast progenitors into immature osteoblasts (the second stage of the differentiation process). In this work, we specifically demonstrated that the early increase in Runx2 expression observed after stimulation of hBMSCs with differentiation medium is inhibited both by the use of an shRNA against Cx43 or by GJIC inhibitors, suggesting that Cx43 as well as GJIC play a role during the commitment of hBMSCs toward osteoblastic lineage or at least during the early stages of hBMSC differentiation into osteoblast progenitors. In addition, using hBMSCs rather than osteoblast progenitors used in previous studies, our results also demonstrated that Cx43 plays a central role during the differentiation of osteoblast progenitors into immature osteoblasts and later during the differentiation of these immature osteoblasts into mature osteoblasts.

Regarding the convergence of Cx43 and Runx2 expressions, it has been previously demonstrated that the overexpression or knockdown of *Cx43* is able to regulate the expression and/or the transcriptional activity of Runx2 in several cells such as MC3T3-E1, mouse calvaria osteoblastic, human fetal osteoblastic or longitudinal ligament cells (Stains et al., 2003; Li et al., 2006; Thi et al., 2010; Niger et al., 2011; Niger et al., 2012; Yang et al., 2016). The studies on the molecular mechanism underlying this process have identified two effectors, the extracellular signal-regulated kinase (ERK) and the protein kinase C delta (PKC), responsible for *Cx43*-regulation of *Runx2* expression and/or *Runx2* transcriptional activity (Lima et al., 2009; Niger et al., 2013; Niger et al., 2012; Li et al., 2015; Tu et al., 2016).

Gap junctions can mediate the passage of nucleotides, amino acids, ions and second messengers between coupled cells allowing ionic and metabolic signaling communication (Harris, 2007). Among these messengers able to cross through gap junctional channels, there is evidence that the spatial and temporal patterns of calcium are crucial in the regulation of the differentiation process. Both the stable increase in Ca2+ intracellular concentration ([Ca2+]), and the oscillations

of [Ca2+] are a mode of signaling in both excitable and non-excitable cells. In bone marrow-derived stem cells, spontaneous [Ca2+] oscillations were shown to depend on Inositol P3-mediated Ca2+ release followed by Ca2+ influx from the extracellular compartment (Kawano et al., 2002). In this context, it has been shown that [Ca2+] oscillations can be regulated by intercellular coupling through gap junctions (Dupont et al., 2007). We can thus hypothesize that Cx43-mediated gap junctional intercellular communication drives the differentiation of hBMSCs into osteoblasts via its ability to propagate [Ca2+] oscillations. However, future studies are required to establish the nature of the signals, Ca2+ or others, which drive the hBMSC differentiation process.

Legends of Figures

Figure 1: *In vitro differentiation of hBMSCs toward osteoblasts*

hBMSCs were cultured in the presence or absence of osteogenic medium up to 17 days. (**A**) At each time point, cultures were fixed and stained with Alizarin red S. Results show one representative experiment of three independent experiments. (**B**) *Runx2, Alkaline Phosphatase* (ALP), *Bone Sialoprotein* (BSP) and *Osteocalcin (*OCN) mRNA steady-state levels were determined by quantitative RT-PCR, and are expressed relative to the control culture in absence of osteogenic medium. Bars indicate mean ± SD of three independent experiments performed in duplicate.

Figure 2: *Increase of Cx43 production during the differentiation of hBMSCs into osteoblasts.*

hBMSCs were cultured in the presence or absence of osteogenic medium (Diff) up to 17 days. (**A**) Cx43 production was detected by Western Blot analysis. Result shown is representative of three independent experiments. (**B**) *Cx43* and *Cx45* mRNA steady-state levels were determined by quantitative RT-PCR, and expressed relative to the control culture in absence of osteogenic medium. Bars indicate mean ± SD of three independent experiments performed in duplicate. (**C**) hBMSC cells were incubated in the presence or absence of osteogenic medium for 9 days. After incubation, cell cultures were transfected with the -2400Cx43-lux construct, and incubation continued for another 48h. Bars indicate mean ± SD of three independent experiments performed in duplicate. (***p<0.01) (**D**) FACS analysis of calcein transfer in hBMSCs treated or not with osteogenic medium during 17 days. Histogram plots (left panel) represent the mean of fluorescence recorded in FL1 channel, which corresponds to calcein-positive cells. Control peak corresponds to cells dyed with DiI only, and parachute peak to cells double-labeled. Histograms (right panel) indicate mean of the relative transfer-ratio of calcein between cells, which represents a direct measure of the degree of dye coupling. Bars indicate mean ± SD of three independent experiments performed in duplicate. (***p<0.01)

Figure 3: *Small interfering RNA against Cx43 and GJIC inhibitors efficiency block GJIC in hBMSCs*

(**A**) *Cx43* and *Cx45* mRNA steady-state levels were determined by quantitative RT-PCR in parental (P), shLacZ and shCx43 cells as indicated. Bars indicate mean ± SD of three independent experiments performed in duplicate. (****p<0.05**) (**B**) Cx43 production was detected by Western Blot analysis in parental, shLacZ and shCx43 cells as indicated. Result shown is representative of three independent experiments. (**C**) FACS analysis of calcein transfer in parental, shLacZ and shCx43 hBMSCs treated or not with osteogenic medium (Diff) for 17 days. Bars indicate mean ± SD of three independent experiments performed in duplicate. (*****p<0.01**). (**D**) FACS analysis of calcein transfer in hBMSCs treated or not with 18α-glycyrrhetinic acid (50 μM) or Gap27 peptide (250 μM) for 24h. Bars indicate mean ± SD of three independent experiments performed in duplicate. (*****p<0.01**). (**E**) hBMSCs viability, treated or not with 18α-glycyrrhetinic acid (50 μM) or Gap27 peptide (250 μM), was determined using a 2,3- bis(2 methoxy-4 nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent assay kit. Bars indicate mean ± SD of three independent experiments performed in duplicate.

Figure 4: *Small interfering RNA against Cx43 inhibits the differentiation of hBMSCs into osteoblasts.*

(**A**) Parental, shLacZ and shCx43 cells were cultured in the presence or absence of osteogenic medium. The expression of *Runx2* was determined by quantitative RT-PCR after incubation of hBMSCs with osteogenic medium for 1 day. The expression of *ALP* was determined by quantitative RT-PCR after incubation of hBMSCs with osteogenic medium for 7 days. The expression of *BSP* and *OCN* mRNA steady-state levels were measured by quantitative RT-PCR after incubation of hBMSCs with osteogenic medium for 17 day. Bars indicate mean ± SD of three independent experiments performed in duplicate. (****p<0.05, ***p<0.01**). (**B**) Parental, shLacZ and shCx43 hBMSCs were cultured in the presence or absence of osteogenic medium. 17 days after induction of osteogenesis, cultures were fixed and stained with Alizarin red S. Results show one representative experiment of three independent experiments. (**C**) Parental, shLacZ and shCx43 cells were cultured in the presence or absence of osteogenic medium. The expression of *Cx43* and *Cx45* were determined by quantitative RT-PCR throughout the differentiation process. Results indicate mean ± SD of three independent experiments performed in duplicate.

Figure 5: *GJIC inhibitors reduce the differentiation of hBMSCs into osteoblasts.*

(**A**) hBMSCs were cultured in the presence of GJIC inhibitors (α-glycyrrhetinic acid or Gap27) as indicated. The expression of *Runx2* was determined by quantitative RT-PCR after treatment with osteogenic medium for 1 day. The expression of *ALP* was determined by quantitative RT-PCR after treatment with osteogenic medium for 7 days. 17 days after treatment of the cells with osteogenic medium (Diff), *BSP* and *OCN* mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean ± SD of three independent experiments performed in duplicate. (**p<0.05; ***p<0.01). (**B**) Cultures were fixed and stained with Alizarin red S 17 days after induction of osteogenesis in the presence or absence of 18α-glycyrrhetinic acid (50 μM) or Gap27 (250 µM). Results show one representative experiment of three independent experiments.

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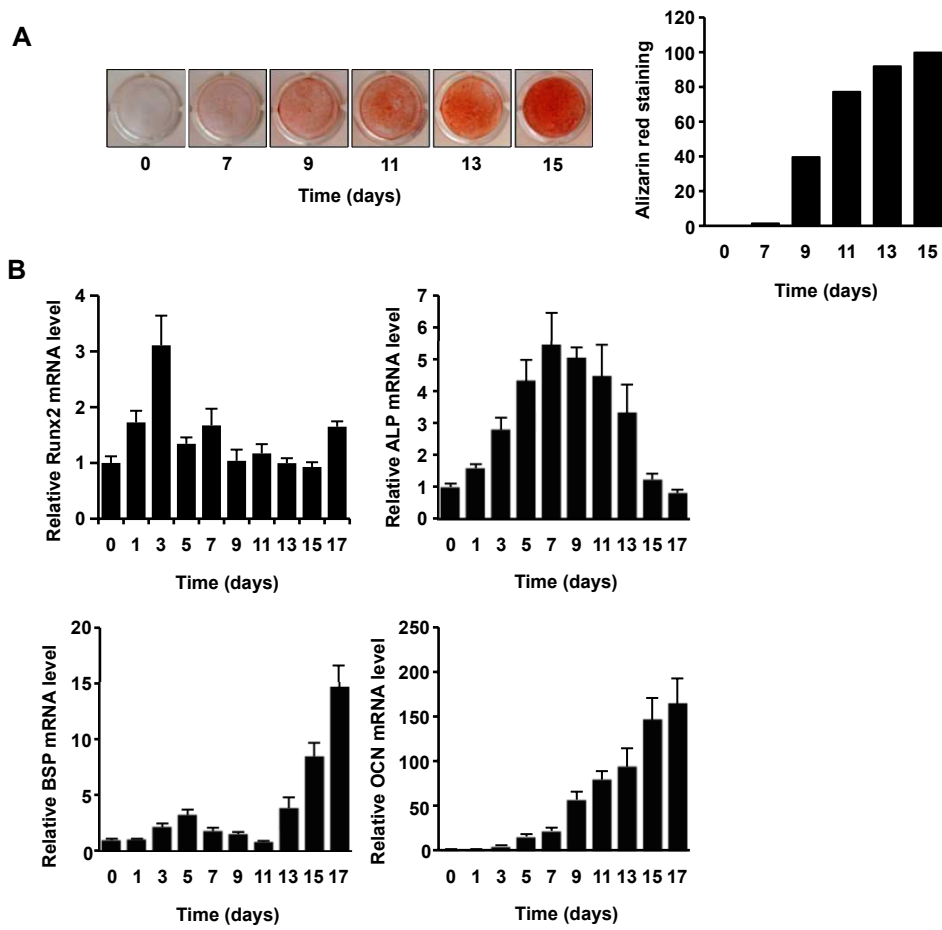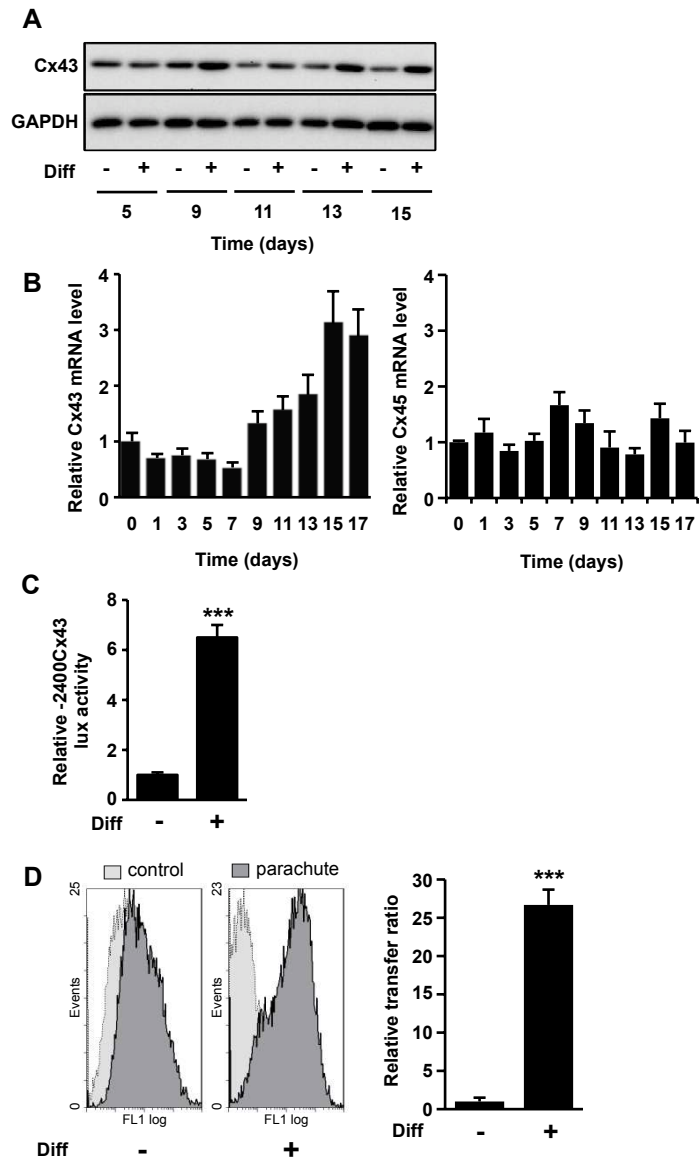**A**

B

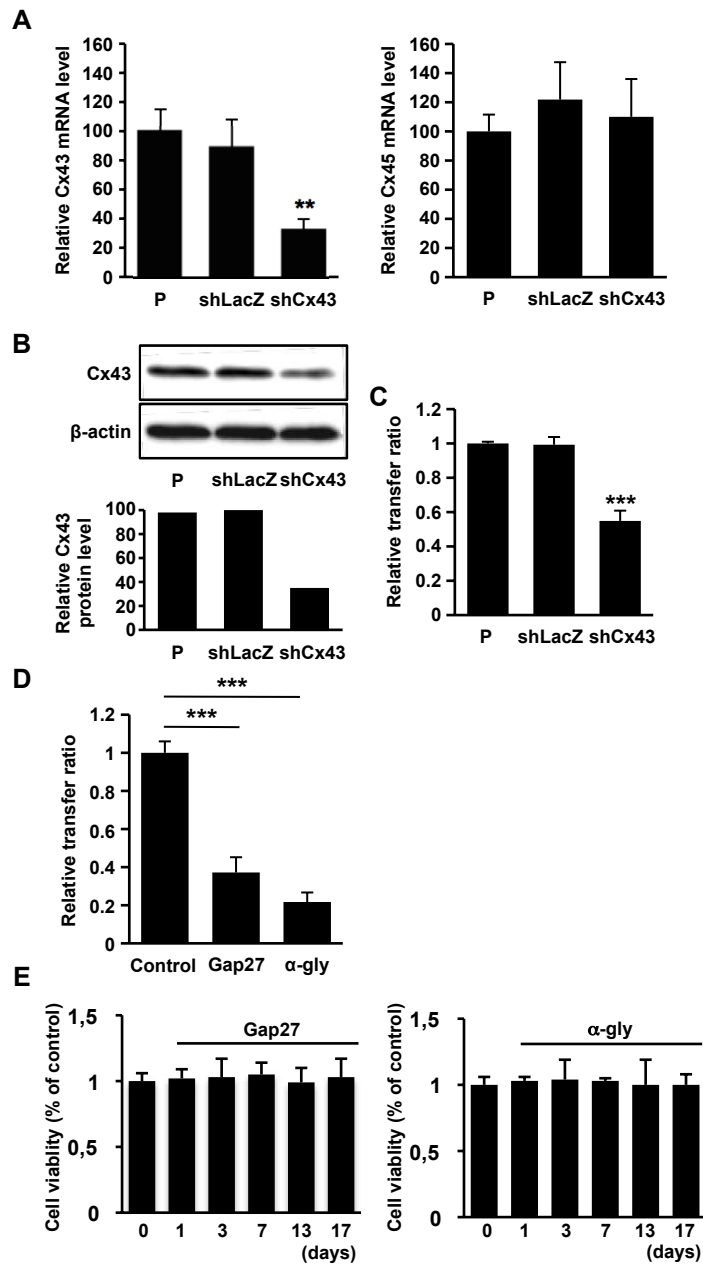**Figure 1**

A

Cx43

GAPDH

Diff   - +   - +   - +   - +   - +

5   9   11   13   15

Time (days)

B

Relative Cx43 mRNA level

Time (days)
0 1 3 5 7 9 11 13 15 17

Relative Cx45 mRNA level

Time (days)
0 1 3 5 7 9 11 13 15 17

C

Relative -2400Cx43 lux activity

***

Diff   -   +

D

control   parachute

Events   Events

FL1 log   FL1 log

Diff   -   +

Relative transfer ratio

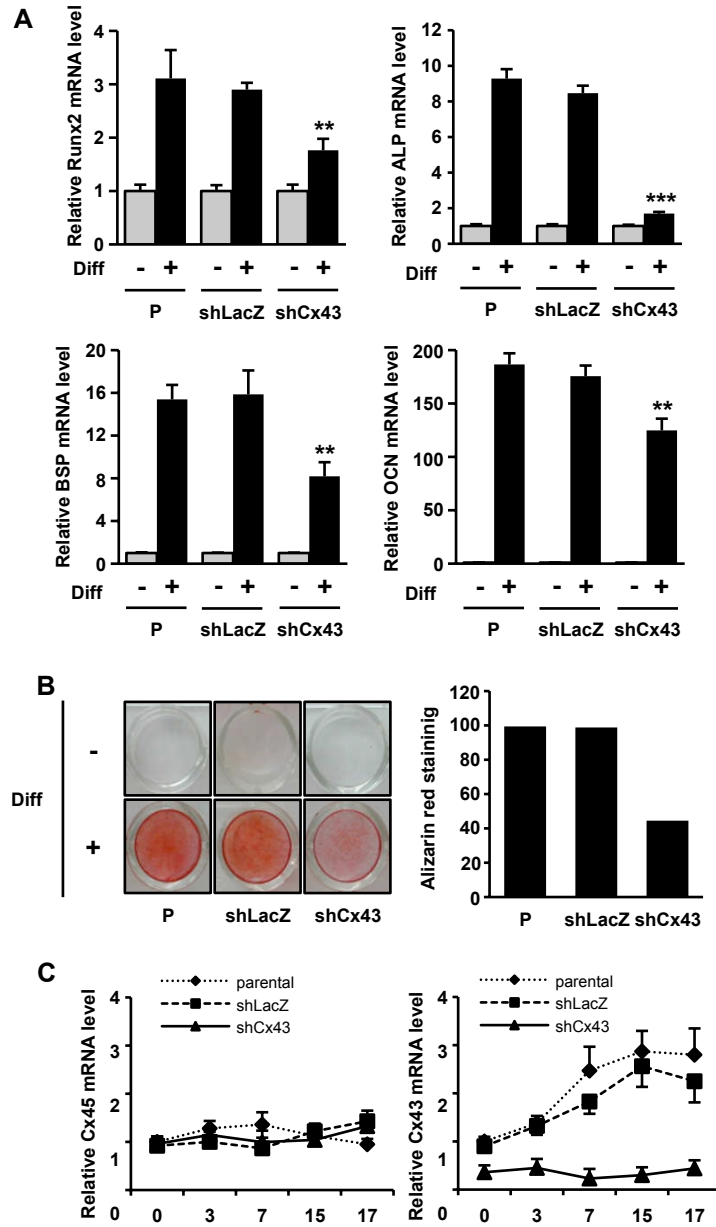***

Diff   -   +

Figure 2

Figure 3

Figure 4

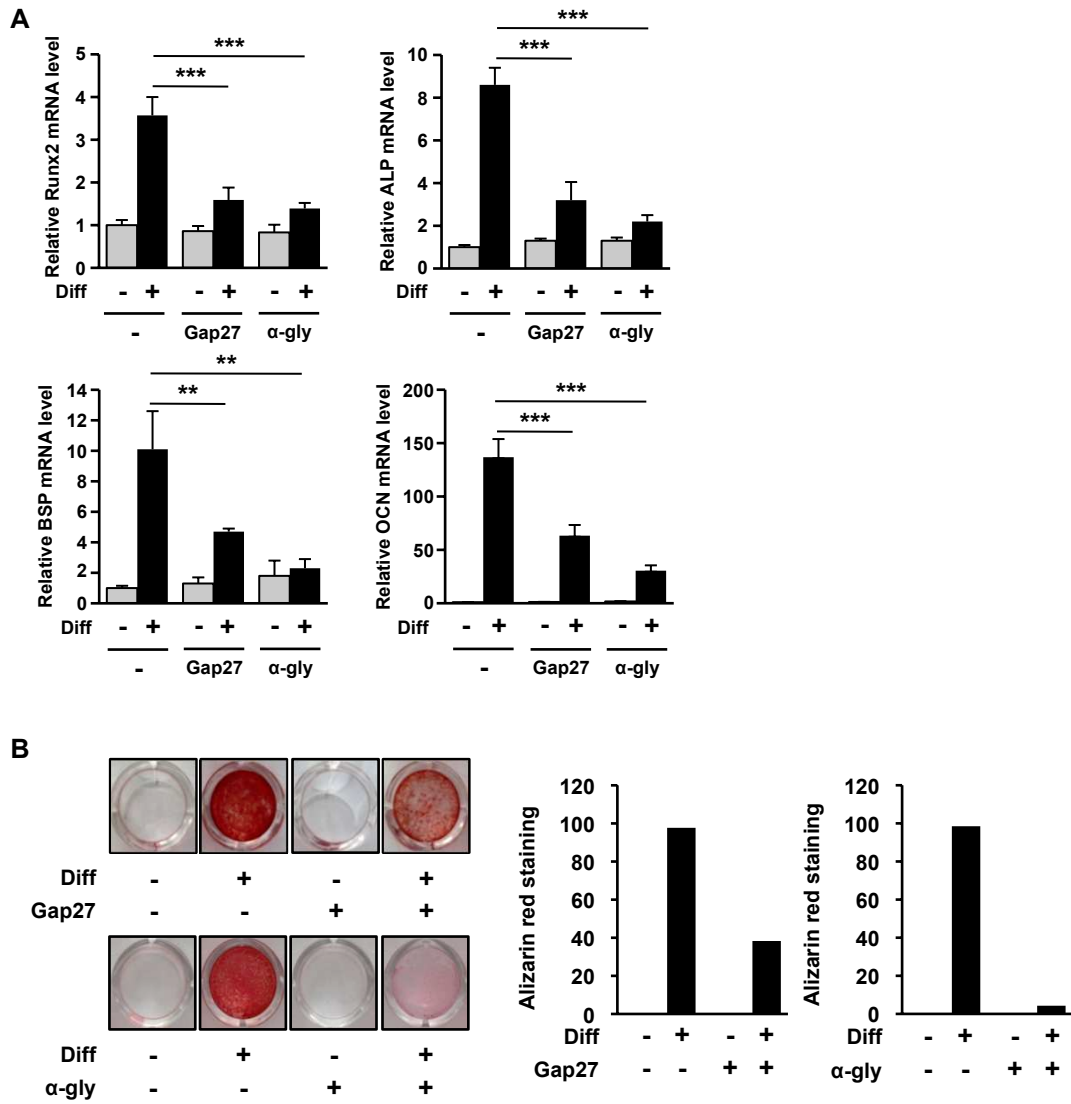**Figure 5**

Table 1. Primer sequence for quantitative RT-PCR

Gene	Gene (full name)	Accession number	Forward primer	Reverse primer
Cx43	Connexin43	NM_000165.3	gcctgaacttgccttttcat	ctccagtcacccatgttgc
OC	Osteocalcin	NM_199173.2	ggcgctacctgtatcaatgg	tcagccaactcgtcacagtc
BSP	Bone sialoprotein	NM_004967	cgaatacacgggcgtcaatg	gtagctgtactcatcttcataggc
ALP	Alkaline phosphatase	NM_000478.3	aacaccacccaggggaac	ggtcacaatgcccacagatt
Runx2	Runt-related transcription factor 2	NM_004348.3	gcctaggcgcatttcaga	gctcttcttactgagagtggaagg
SOX9	SRY (sex determining region Y)-box9	NM_000346.3	gtacccgcacttgcacaac	tcgctctcgttcagaagtctc
Cx45	Connexin 45	NM_005497.3	tgggtaaccgaagttctggaca	gaacagtgagccagatcttccc
Hprt 1	Hypoxanthine guanine phosphoribosyl transferase 1	NM_000194.1	tgaccttgatttattttgcatacc	cgagcaagacgttcagtcct
Cyc1	Cytochrome c-1	NM_001916	gcatggtggtgaggactacg	ggccaggaaagtaggggttg