Original Paper

Connexin 43 Modulates Osteogenic Differentiation of Bone Marrow Stromal Cells Through GSK-3 β /Beta-Catenin Signaling Pathways

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Key Words

Connexin 43 • Osteogenic differentiation • Bone marrow stromal cells • GSK-3 β • Beta-catenin

Abstract

Background/Aims: Bone marrow stromal cells (BMSCs) are multipotent precursors that give rise to osteoblasts, and contribute directly to bone formation. Connexin 43 (Cx43) is the most ubiquitous gap junction protein expressed in bone cell types, and plays crucial roles in regulating intercellular signal transmission for bone development, differentiation and pathology. However, the precise role and mechanism of Cx43 in BMSCs are less known. Here, we investigate the function of Cx43 in osteogenic differentiation of BMSCs *in vitro*. **Methods:** BMSCs were isolated by whole bone marrow adherent culture. Knock down of Cx43 was performed by using lentiviral transduction of Cx43 shRNA. BMSCs were induced to differentiate by culturing in α -MEM, 10% FBS, 50 μ M ascorbic acid, 10 mM beta-glycerolphosphate, and 100 nM dexamethasone. Alkaline phosphatase (ALP) activity and alizarin red S staining were used to evaluate osteogenic differentiation in calcium nodules. Target mRNAs and proteins were analyzed by using real-time quantitative PCR (qPCR) and western blotting. **Results:** Cx43 expression markedly increased during osteogenic differentiation. Osteogenic differentiation was suppressed following lentiviral-mediated knockdown of Cx43 expression, as judged by decreased levels of Runt-related transcription factor 2 (Runx2), bone sialoprotein (BSP), osteocalcin (Bglap), Osterix (Osx), alkaline phosphatase (ALP) activity and the number of calcium nodules in response to osteogenic differentiation stimuli. Knock down of Cx43 reduced

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the level of phosphorylation of GSK-3 β at Ser9 (p-GSK-3 β), resulting in decreased beta-catenin expression and activation. Furthermore, treatment of Cx43-knockdown cells with lithium chloride (LiCl), a GSK-3 β inhibitor, reduced osteogenic differentiation and decreased GSK-3 β levels, as well as partially rescued levels of both total and activated beta-catenin. **Conclusion:** These findings indicate that Cx43 positively modulates osteogenic differentiation of BMSCs by up-regulating GSK-3 β /beta-catenin signaling pathways, suggesting a potential role for Cx43 in determining bone mass and bone mineral density by modulating osteogenesis.

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Introduction

BMSCs are multipotent adult stem cells that have self-renewing, proliferative potential and the ability to differentiate into a variety of cell types, including osteoblasts, adipocytes, chondrocytes, neurons and myoblasts [1, 2]. Differentiation of BMSCs into adipocytes and osteoblasts is regulated in an inverse manner [3, 4], and plays a key role in the fate of BMSCs and bone mass. Under normal conditions, the balance between adipogenic and osteogenic differentiation of BMSCs is weighted toward osteogenic differentiation to maintain normal bone formation [5-7]. However, the balance of BMSC differentiation can be disrupted under pathological circumstances [3, 7]. For example, BMSCs from osteoporotic women have a low growth rate and exhibit enhanced adipocyte differentiation that proceeds at the expense of osteogenesis, leading to low bone mass and a high marrow fat mass [8-10]. Many factors have been identified as key determinants in the process of osteogenic differentiation by BMSCs, and many studies have demonstrated that a change in the expression and/or activity of these factors is associated with bone formation or bone loss [11-13]. Therefore, understanding the cellular and molecular factors that influence BMSC fate may provide us with potential therapeutic targets for several diseases associated with bone loss and high marrow adiposity.

The cell surface contains gap junctions that consist of connexins, in particular Cx43, which mediate cellular communication and permit the passage of small molecules and ions between cells [14, 15]. Cx43, encoded by the Gja1 gene, is the most ubiquitous gap junction protein expressed in bone cell types, including chondrocytes, osteoblasts, osteocytes, osteoclasts, and BMSCs [14, 16]. Cx43 plays important roles in regulating signal transmission among different bone cell types and thereby regulates development, differentiation, modeling and remodeling, as well as pathologies of bone [16-19]. Cx43 is required for survival [20], proliferation [21] and differentiation [22-30] of different types of osteoblasts, for instance, MC3T3-E1 [28], UMR 106 [29], or mouse osteoblasts, and in 3D cultures [30], overexpression of Cx43 in BMSCs enhances both the magnitude and spatial distribution of gap junction intercellular communication and the expression of osteogenic markers [31]. However, the role of Cx43 in BMSCs remains obscure, and the mechanism by which Cx43 enhances osteogenic differentiation has not yet been established.

Loiselle et al. reported that deletion of Cx43 in osteoblasts/osteocytes results in delay of bone formation and bone remodeling, and impairs mechanical properties [32], and the mechanisms by which healing is impaired are related to altered activity of GSK-3 β , and therefore beta-catenin expression, during fracture healing [31]. Several signaling pathways have been shown to participate in the regulation of osteogenesis [13]. The Wnt/beta-catenin signaling pathway is an essential pathway for BMSC commitment to osteogenic differentiation [33-35]. Without Wnt ligands, cytoplasmic beta-catenin undergoes phosphorylation and degradation by a destruction complex composed of GSK-3 β , adenomatous polyposis coli and axin [36]. In contrast, when Wnt ligands bind to their cell surface receptors, inactivation of GSK-3 β occurs, leading to beta-catenin stabilization and translocation to the nucleus, where it binds to the T-cell factor/lymphoid-enhancer factor and activates transcription of Wnt target genes, such as axin 2 and cyclin D1 [36, 37]. Inhibitors of GSK-3 β can cause stabilization and accumulation of cellular beta-catenin to initiate the expression of target

genes, resulting in an increase in osteogenic differentiation of BMSCs [35, 37]. These give us clues about mechanisms by which Cx43 regulates osteogenic differentiation of BMSCs. Therefore, we characterized the role of Cx43 in osteogenic differentiation of BMSCs and tested the hypothesis that Cx43 modulates osteogenic differentiation of BMSCs through GSK-3 β /beta-catenin signaling pathways.

Materials and Methods

Animals

All experiments using laboratory animals were conducted according to the guidelines of the institutional animal care committee and were approved by the Animal Use Committee of Shantou University Medical College. Sprague-Dawley rats were housed in the Experimental Animal Center of Shantou University Medical College, and were maintained at an indoor temperature of 22°C, relative humidity of 18% to 22%, and a 12-h light/dark cycle. Ad libitum access to standard rodent chow and water was allowed during the entire study.

Isolation and culture of BMSCs

Primary BMSCs were isolated from four or more randomly selected 6-week-old Sprague-Dawley rats, weighing approximately 100 to 150 g, according to an established protocol described previously [38, 39]. After SD rats were euthanized, tibias and femurs were immediately removed, and attached muscle and tissue were removed using a scalpel under sterile conditions. Then the metaphysis of the bone was cut off, and bone marrow was repeatedly flushed into a 10 cm Petri dish by using a 5 ml injection syringe with basal medium (α -MEM (Gibco, #C12571500BT, abbreviated as BAM) supplemented with 10% (v/v) fetal bovine serum (Gibco, #12664025), and 1% (v/v) penicillin-streptomycin solution (Hyclone, #SV30010)). The cell suspension was plated equally into three 10 cm petri dishes and cultured in a humidified incubator at 37°C, with 5% CO₂ and 95% air. BAM was refreshed 2 days later to remove non-adherent cells, and changed 3 or 4 times a week. After 7 to 9 days in culture, when the adherent cells reached 80–90% confluence, cells were initially passaged at a split ratio of 1:2 using 0.25% trypsin (AMRESCO, #0458). Cells after passage 1 were subcultured at a split ratio of 1:3 about every 3 days, or plated for subsequent experiments.

Lentivirus infection

Lentiviral vectors (H1/GFP&Puro), expressing GFP and either a Cx43 short-hairpin RNA (shCx43) or a scrambled shRNA, were both generated by GenePharma (Suzhou, China). The sequence targeting the coding region of rat Cx43 mRNA was: 5'-GCAATTACAACAAGCAAGCTA-3' [40], and the scrambled sequence used as the negative control (NC) was: 5'-ACTACCGTTGTTATAGGTG-3'. The virus titer used for infection was 10⁹ pfu/ml. Passage 1 BMSCs were inoculated into 6-well plates at a density of 8×10⁴ to 10⁵ cells per well and allowed to attach overnight. Then cells were refed with BAM containing 10 μ g/ml polybrene (Sigma, #H9268) and incubated for one hour prior to addition of lentivirus at a multiplicity of infection between 50 to 300. The incubation medium was replaced with fresh BAM at 24 hours after infection, and live BMSCs were obtained following selection in growth medium, containing 2 μ g/mL puromycin (Sigma, #P8833), for two days. GFP fluorescence was detectable using an inverted fluorescence microscope (Nikon, #ECLIPSE TE2000-S).

Osteogenic differentiation

Passage 3 BMSCs were plated at a density of 5000–10000 cells/cm² and incubated in osteogenic induction medium (OIM) consisting of α -MEM, 10% FBS, 50 μ M ascorbic acid (Sigma, #A4544), 10 mM beta-glycerophosphate (Sigma, #G9422), 100 nM dexamethasone (Sigma, #D1756), and 1% (v/v) penicillin-streptomycin solution. Cultures were gently refed every 2–3 days with OIM until cells were harvested for experiments. Since the process of osteogenic differentiation of BMSCs is progressive, we assayed biochemical parameters at early and later phases corresponding to 7 days and 14 days of osteogenic differentiation, respectively.

Quantitation of ALP activity

ALP activity is elevated early following induction of differentiation, but decreases at later stages, so we choose the 9-day time point to determine ALP activity. Activity was measured using an ALP measurement kit (Beyotime, # P0321). Briefly, cells were lysed with 1% Triton X-100 and centrifuged at 4°C, 1000 rpm. Supernatants were transferred to a clean tube, then subjected to the ALP assay according to the manufacturer's protocol. Absorbance was measured at 405 nm by a 96-well plate microplate reader (Thermo Scientific).

Alizarin red S staining and quantification of calcium nodules

Cells were differentiated by continuous incubation in OIM for 14 days in 12-well plates, then gently washed twice with phosphate-buffered saline (PBS), fixed with absolute ethanol for 15 minutes, rinsed once with double-distilled water, and then incubated in 0.1% alizarin red S solution (Panera, #AAPR122-A1) for 30 minutes. After washing cells twice with double-distilled water and drying completely, the stained calcium nodules were photographed under a light microscope. To quantify the calcium nodules, dye was eluted in cetyl pyridinium chloride (Sigma, # 588393) in 10 mM sodium phosphate for 30 min, and the absorbance was then measured at 540 nm with a 96-well plate microplate reader (Thermo Scientific). The entire process was conducted at room temperature.

RNA extraction and qPCR

At 7 or 14 days of differentiation with OIM, total RNA was extracted with an RNA extraction kit (TaKaRa, #9767) according to the manufacturer's instructions, and total RNA concentration was determined with a SmartSpec Plus spectrophotometer (BIO-RAD). First-strand cDNA was synthesized using a PrimeScript™ RT Master Mix (TaKaRa, #RR036A) from 1000 ng total RNA in a 20 µl total reaction volume. qPCR was performed with SYBR® Premix Ex Taq™ (TaKaRa, #RR420A) using a CFX96 qPCR Detection System (BIO-RAD Laboratories) in a total reaction volume of 25 µL. Primer sequences are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, and relative gene expression levels were calculated by means of the 2-ΔΔCt method. Each experiment was performed at least three times independently.

Protein preparation and western blotting analyses

After differentiation with OIM for 7 or 14 days, cells were washed with PBS and total protein was extracted. In brief, cells were prepared by lysing with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, #P0013B) containing 1% (v/v) protease inhibitor cocktail (Biotool, #B14001) and 1% (v/v) phosphatase inhibitor cocktail (Biotool, #B14001). After centrifugation for 15 minutes at 4°C, 15000 rpm, protein concentrations of supernatants were determined using the Bradford method (Bio-Rad Laboratories). Approximately 50 µg of protein per lane was separated by 8% or 10% SDS-PAGE, then transferred onto polyvinylidene fluoride membranes (Millipore, #IPVH00010). After blocking in 5% nonfat milk for 1 hour at room temperature, membranes were incubated with rabbit anti-Cx43 (1:1000, Cell Signaling Technology, #3512), rabbit anti-GSK-3β (1:1000, Cell Signaling Technology, #9315), rabbit anti-phospho-GSK-3β (1:1000, Cell Signaling Technology, #9322), rabbit anti-beta-catenin (1:1000, Cell Signaling Technology, #9581), rabbit anti-active beta-catenin (1:1000, Cell Signaling Technology, #4270), or rabbit anti-GAPDH (1:1000, Panera, #SF-PA005) primary antibodies overnight at 4°C. The following day, membranes were washed three times in TBST (10 min each time), and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, #111-035-003) antibodies for 1 h at room temperature. Then

Table 1. Primer sequence of qPCR

Gene	Forward primer sequence	Reverse primer sequence
Cx43	TGCTTGGGATAGCTGGCGGA	TGGGGCAGAGAGAGAAAGCCC
Runx2	GGGACCGTCCACTGTCACTTT AATA	CAAGTGGCCAGGTTCAACGA
BSP	GCTATGAAGGCTACGAGGGTC AGGATTAT	GGGTATGTTAGGGTGGTTAGCAATGGTGT
Bglap	AGGTGGTGAATAGACTCCG	GCTGTGCCGTCCACTACTTT
Osx	GGAGGCACAAAGAAGCCATA	GGGAAAGGGTGGGTAGTCAT
Axin 2	GACCGACGATTCATGTCC	CCAGCTCCAGTTTCAGCTTC
Cyclin D1	GCTGGCGTTTGAAGTAG	CCTCAGTTTGGATGGCTC
GAPDH	TCCTGCACCACCACTGCTTAG	AGTGGCAGTGATGGCATGGACT

membranes were washed three times in TBST (10 min each time), and protein bands were visualized using an enhanced chemiluminescence reagent (Thermo Scientific), imaged with Bio-Rad ChemiDoc-XRS+, and analyzed using Image Lab software (Bio-Rad). Protein values were normalized to GAPDH.

Statistical analysis

Each assay was performed more than three times independently. All values are expressed as the fold change over the control, and presented as means $\pm$ standard deviation (SD). Statistical analysis was calculated using either two-way or one-way analysis of variance, and for experiments with three groups SPSS 19.0 software (SPSS Inc., Chicago, IL) was used. Differences were regarded as significant when $P < 0.05$.

Results

Cx43 expression is upregulated during osteogenic differentiation

Exposure of BMSCs to BAM or OIM for 7 and 14 days resulted in cells increasingly undergoing osteogenic differentiation with time, as revealed by alizarin red S staining (Fig. 1A and B). We analyzed the changes of the Cx43 mRNA and protein in BMSCs by qPCR and western blotting. Results indicated that Cx43 mRNA increased upon differentiation, as compared to undifferentiated cells, at both 7 and 14 days (Fig. 1C and D), as well as the level of Cx43 protein (Fig. 1E-H). These data suggest that up-regulation of Cx43 occurs upon differentiation, and that Cx43 may be involved in osteogenic differentiation of BMSCs.

Knock down of Cx43 attenuates osteogenic differentiation of BMSCs

To further confirm the role of Cx43 in BMSC osteogenic differentiation, we established Cx43 knockdown BMSCs by infection with lentiviruses expressing shCx43. Viral efficacy

Fig. 1. Upregulation of Cx43 expression during osteogenic differentiation. (A), Calcium nodules were stained with alizarin red S, then photographed. (B), alizarin red staining was quantified. (C and D), Expression of Cx43 mRNA was measured by qPCR at 7 or 14 days after initiation of osteogenic differentiation. (E-H), Western blot analysis of Cx43 at 7 or 14 days following induction of osteogenic differentiation. Fold change in Cx43 protein expression following normalization to the values of BAM-cultured cells. Data are expressed as the mean $\pm$ SD of the fold change from three separate experiments (* $p < 0.05$, ** $p < 0.01$).

Fig. 2. Knockdown of Cx43 in BMSCs with lentivirus expressing Cx43 shRNA. (A), Bright field and GFP expression following virus infection at an MOI of 50, 100, 150, 200, 250 and 300 in passage 1 BMSC cells. Bar indicates 100 μ m. (B and C), qPCR and western blotting analysis of Cx43 mRNA and protein levels in shCx43 BMSCs after lentivirus transduction at 300 MOI. Data are expressed as the mean $\pm$ SD of the fold change following normalization to CON. Values represent analysis from three separate experiments (** $p < 0.01$).

was tested using MOIs of 50, 100, 150, 200, 250 and 300 to infect passage 1 BMSC cells. The virus had no effect on cell viability and approximately 90% of BMSCs were GFP-positive at 72 hours after lentivirus transduction at 300 MOI (Fig. 2A). Therefore, for knockdown studies, lentivirus transduction was carried out at 300 MOI, and stably transduced cells were selected by culture in puromycin-containing medium for 2 days, followed by continuous culture in BAM for 14 days. The mRNA expression was lower in shCx43 cells than that in both uninfected cells (as control: CON) and NC cells (Fig. 2B). In accordance with the mRNA level, western blotting showed that the expression of CX43 protein was almost completely abrogated by lentivirus transduction (Fig. 2C and D).

To evaluate the ability of representative CON, NC and shCx43 BMSCs to undergo osteogenic differentiation, cells were cultured in BAM or OIM for the indicated times. After a 9-day differentiation period, Cx43-silenced cells displayed an approximately 50% decrease in ALP activity, as assessed by enzymatic measurement (Fig. 3A). We measured mineralization using alizarin red S staining following induction of differentiation for 14

Fig. 3. Knockdown of Cx43 attenuates osteogenic differentiation of BMSCs. (A), ALP activity of BMSCs was observed in BMSCs at 9 days following differentiation. (B), alizarin red S staining was performed 14 days after induction, then photographed under bright field. Bar indicates 100 μ m. (C), alizarin red S staining was quantified. (D and E), Expression of Runx2, BSP, Bglap and OSX was measured by real-time RT-PCR after 7 and 14 days of induction, respectively. Data are expressed as the mean fold change $\pm$ SD of the values in CON. Values represent analysis from three separate experiments (* p <0.05, ** p <0.01).

days. Knockdown of Cx43 prior to osteogenic differentiation of BMSCs resulted in a >80% decrease in mineralization (Fig. 3B and C) compared with CON and NC.

To investigate the molecular mechanism induced by Cx43 knockdown, the mRNA levels of marker genes for osteogenesis, specifically, Runx2, bone sialoprotein (BSP), Bglap, and OSX, were examined after 7 or 14 days of exposure to BAM or OIM. Consistent with the reduction of ALP activity and mineralization observed in shCx43 BMSCs, when compared with CON and NC cells, the mRNA levels of the osteogenesis marker genes in shCx43 BMSC were decreased by approximately 20%-90%, regardless of whether cells were induced to differentiate for 7 or 14 days (Fig. 3D and E). Taken together, these results suggest that ablation of Cx43 suppresses osteogenic commitment, as judged by inhibition of osteogenesis marker gene expression.

The GSK-3 β /beta-catenin pathway is involved in suppression of Cx43-mediated osteogenic differentiation of BMSCs

To investigate whether the GSK-3 β /beta-catenin pathway is affected by Cx43 during osteogenic differentiation of BMSCs, we examined alterations in this pathway in cells cultured with OIM for 7 and 14 days. Western blot analysis showed that the levels of p-GSK-3 β (inactive form phosphorylated at Ser9) were decreased by more than 40% during the early period (7 days) and by almost 40% during the later period (14 days) in shCx43 BMSCs following osteogenic differentiation, compared with levels in CON and NC cells (Fig. 4A-D). Moreover, in response to Cx43 knockdown, total beta-catenin progressively decreased from the early period (nearly 15%) to the later period (almost 80%) following induction of osteogenic differentiation (Fig. 4A, B, E and F). Correspondingly, the activation of beta-

Fig. 4. GSK-3β/β-catenin pathway is involved in suppression of osteogenic differentiation of BMSCs caused by Cx43. (A and B) Total protein was isolated after 7 and 14 days after start of induction, and the expression of phospho-GSK-3β, total GSK-3β, active β-catenin, and total β-catenin was analyzed by western blotting. The p-GSK-3β/total GSK-3β ratio (C and D), total β-catenin (E and F) and active β-catenin/total β-catenin ratio (G and H) were quantified. (I and J), GSK-3β/β-catenin pathway target gene mRNAs for axin 2 and cyclin D1 were measured by real-time RT-PCR after 7 and 14 days of induction. Data are expressed as fold change of the mean ± SD following normalization to CON. Values represent analysis from three separate experiments (*p<0.05, **p<0.01).

catenin was inhibited, being about 50% at 7 days, and approximately 90% at 14 days (Fig. 4 A, B, G and H). As evidenced by qPCR (Fig. 4I and J), mRNA expression of the GSK-3β/β-catenin pathway target genes, including axin 2 and cyclin D1, was markedly reduced in shCx43 BMSCs compared to those in CON and NC cells after 7 or 14 days of osteogenic induction. Taken together, these results thus support the view that Cx43 acts as a positive regulator of the GSK-3β/β-catenin pathway.

LiCl partially rescues the inhibitory effects of Cx43 on BMSC osteogenic differentiation by the GSK-3β/β-catenin signaling pathway

To further confirm the role of the GSK-3β/β-catenin pathway in osteogenesis observed in shCx43 BMSCs, cells underwent osteogenic differentiation with OIM or were treated with OIM in the presence of 5 mM LiCl, an inhibitor of GSK-3β. ALP was quantified by an enzymatic assay. After differentiation for 9 days, ALP activity was almost 40% higher in LiCl-treated shCx43 BMSCs compared with untreated cells (Fig. 5A). We then

Fig. 5. LiCl partly rescues the attenuation by Cx43 on BMSC osteogenic differentiation by the GSK-3β/β-catenin signaling pathway. ShCx43 BMSCs were cultured in with OIM or were treated with OIM in presence of 5 mM LiCl. (A), ALP activity of BMSCs was measured at 9 days after differentiation. (B), Osteogenesis was assessed by alizarin red S staining after 14 days of differentiation. Bar indicates 100 μm. (C), Quantification of alizarin red S staining. (D and E), Expression of Runx2, BSP, Bglap and OSX was measured by real-time RT-PCR after 7 and 14 days of induction. (F and G), Expression of p-GSK-3β, total GSK-3β, active β-catenin, and total β-catenin was analyzed by western blotting after 7 and 14 days of induction. Phospho-GSK-3β/total GSK-3β (F and G), total β-catenin (H and I) and active β-catenin/total β-catenin (J and K) were quantified. (N and O), Axin 2 and cyclin D1 mRNA levels were measured by real-time RT-PCR after 7 and 14 days of induction. Data are expressed as the mean ± SD of the fold change of the values in shCx43 BMSCs. Values represent analysis from three separate experiments (*p<0.05, **p<0.01).

performed alizarin red S staining to measure mineralization at 14 days following osteogenic differentiation. Compared to untreated cells, there was a nearly 7-fold increase in the total extent of mineralization following LiCl treatment (Fig. 5B and C). Following differentiation for 7 days in the presence or absence of LiCl, LiCl treatment elevated the mRNA levels of Runx2, BSP, Bglap, and OSX (Fig. 5D), and similar results were obtained after 14 days of differentiation (Fig. 5E). Moreover, western blot analysis showed that the level of p-GSK-3 β was markedly increased in response to LiCl treatment, being 40% elevated after 7 days of differentiation (Fig. 5F and H), and about 3-fold elevated after 14 days of differentiation, compared to cells without LiCl treatment (Fig. 5G and I). Consistent with this, LiCl treatment increased the level of total beta-catenin when examined either at 7 days or 14 days following induction of differentiation (Fig. 5F, G, J and K). Similarly, a dramatic increase of active beta-catenin was also observed, both after 7 days (Fig. 5L) and 14 days (Fig. 5M) following osteogenic differentiation with OIM containing LiCl. In addition, LiCl treatment resulted in a pronounced increase in the mRNA levels for axin 2 and cyclin D1 after 7 and 14 days of differentiation (Fig. 5N and O). Thus, our findings indicate that LiCl partially rescues the inhibitory effects of Cx43 knockdown on BMSC osteogenic differentiation by enhancing the GSK-3 β /beta-catenin signaling pathway.

Discussion

In the present study, we demonstrate that Cx43 expression markedly increases during osteogenic differentiation of BMSCs, and knockdown of Cx43 results in decreased mRNA levels for osteogenesis marker genes, cellular ALP enzymatic activity and levels of calcium nodules. Furthermore, we show that attenuated osteogenesis involves decreased levels and activity of beta-catenin due to increased GSK-3 β activity. These findings demonstrate that connexin 43 modulates osteogenic differentiation of bone marrow stromal cells through the GSK-3 β /beta-catenin signaling pathway.

Knock down of Cx43 in mice results in perinatal death as a result of impaired cardiac function [41, 42], and causes low bone mass and osteoblast dysfunction [43]. Oculodentodigital dysplasia is a developmental disorder associated with mutations in the Cx43 gene *Gja1*, leading to a skeletal phenotype characterized by syndactyly of the hands and feet, hypoplasia or aplasia of the phalanges and craniofacial abnormalities [44]. Many Cx43-deficient animal models, generated by means of gene mutation or conditional gene knockout, exhibit different extents of destructive cortical bone modeling and trabecular bone remodeling, or deteriorated bone quality. It has been established by *in vitro* studies that Cx43 expression is required for normal osteoblastic gene expression and function. For instance, overexpression of Cx43 in MC3T3-E1 osteoblasts increases the transcriptional activity of a Runx2 reporter in response to fibroblast growth factor-2 (FGF-2) treatment [28], whereas siRNA-mediated knockdown of Cx43 suppresses Runx2-dependent transcription, including *Col1a1*, *Bglap*, and *Osx* [45]. Similar consequences are shown by human fetuses upon loss of Cx43, resulting in decreased alkaline phosphatase (ALP) activity, and *Bglap*, osteopontin and *Cbfa1* expression [46]. Stable transfection with Cx43 significantly promotes proliferation and differentiation, and mineralization, as well as expression of ALP [21], osteocalcin and bone sialoprotein [28] in UMR 106 osteoblasts. Furthermore, Cx43-deficient osteoblasts from Cx43-null mice causes impaired differentiation and delayed mineralization compared to osteoblasts from wild-type mice [29]. These findings imply a role for Cx43 in the cellular differentiation and mineralization processes of osteoblasts. Several types of agents, such as transforming growth factor (TGF)- β and BMP2, contribute to maintain a dynamic balance between osteogenesis and adipogenesis in the bone marrow [47]. In the present study, we find that Cx43 expression increases during BMSC differentiation, and lentivirus-mediated knockdown of Cx43 expression diminishes osteogenesis of BMSCs, as judged by decreased ALP activity, mineralization and expression of osteogenesis marker genes. Therefore, our results further indicate that Cx43 could be a regulatory factor of osteogenesis in BMSC

differentiation.

Here, we examine the effects of Cx43 on osteogenic differentiation of BMSCs by characterizing ALP enzymatic activity and the expression levels of the Runx2, BSP, Bglap, and OSX genes. ALP is a bone-specific enzyme and is considered a vital marker for osteogenic differentiation, the presence of which is essential for bone formation [48, 49]. Runx2, an important transcription factor necessary for osteoblast differentiation and bone formation, controls expression of the Bglap and osteopontin genes [50, 51]. Numerous *in vitro* and *in vivo* studies have shown that early osteogenic differentiation at the level of Runx2 is among the primary mechanisms by which Cx43 exerts its action on bone cells [52-57]. Our results are consistent with a previous report showing inhibition of Cx43 decreases the expression of Runx2 during differentiation of hBMSC into osteoblasts [53]. Osterix is a novel zinc finger-containing transcription factor that is essential for osteoblast differentiation and bone formation [58]. BSP is an acidic, non-collagenous glycoprotein abundantly expressed in mineralized tissues, and is frequently used as a marker of osteoblast differentiation [59]. The absence of any osteogenic differentiation marker gene leads to a lack of cell differentiation and mineralization of bone tissue [49-51, 58, 59]. Overexpression of connexin 43 (Cx43) has been shown to enhance both the magnitude and spatial distribution of GJIC and osteogenic differentiation markers in 3D culture [30]. Consistent with this, knockdown of Cx43 attenuates expression of osteogenesis marker genes in both early and later periods of BMSC osteogenesis. These results clearly indicate that Cx43 promotes BMSC osteogenic differentiation by up-regulation of osteogenic marker gene expression.

Activation of Wnt/beta-catenin signaling suppresses adipogenesis and promotes osteogenesis of BMSCs [33, 34]. GSK-3beta is a key component of the Wnt/beta-catenin signaling pathway and can directly control beta-catenin activity. Beta-catenin suffers proteasomal degradation due to phosphorylation at Ser33, Ser37, and Thr41 by GSK-3beta, and consequently the transcription of beta-catenin responsive osteogenic genes is suppressed [60]. Beta-catenin is stable and active when dephosphorylated at Ser33, Ser37, and Thr41. The activity of GSK-3beta can be inhibited by phosphorylation at Ser-9 [61]. Furthermore, the impact of Cx43 on beta-catenin activity and GSK are also well established [56, 57, 62], whereas the mechanism by which the defects in Cx43 cause skeletal development deficiency has not been shown [17]. Our research involves the roles of Cx43 in skeletal development. We show that inhibition of Cx43 enhances GSK-3beta activity, and diminishes not only the level of total beta-catenin, but also the level of active beta-catenin and expression of Wnt target genes axin 2 and cyclin D1. Interestingly, treatment with LiCl, an inhibitor of GSK-3beta [60], partially rescues the inhibition of osteogenic differentiation resulting from Cx43 depletion, and reduces GSK-3beta and total beta-catenin levels, as well as activates beta-catenin and Wnt target genes. Although the link between Cx43 and GSK-3beta needs to be further clarified in future investigation, these results indicate there is a Cx43/GSK-3beta/beta-catenin signaling axis that regulates osteogenic differentiation of BMSCs.

However, it is noteworthy the effects of Cx43 deficiency in different contexts involves diverse interactions of Cx43 with the Wnt/beta-catenin pathway [63]. In contrast, Cx43-deficient ulnae and Cx43-silenced MLO-Y4 osteocyte-like cells elevate basal beta-catenin protein levels to respond to mechanical forces, and is accompanied by increases in the levels of the beta-catenin/Wnt target genes axin 2 and Sfrp4 [64]. In addition, similar correlativity is exhibited in cardiac myocytes [65], neural progenitor cells [66], seminiferous epithelium [67] and mammary epithelial cells [68]. Lloyd and colleagues have shown that Cx43 deficiency leads to enhanced beta-catenin, and have suggested that Cx43 serves as a target of Wnt/beta-catenin to regulate a negative feedback loop responsible for bone cell responses to anabolic stimuli [69]. Thus, Cx43 is increased in the context of loading, which in turn sequesters beta-catenin, leading to a decrease in beta-catenin-mediated stimulation of Cx43 expression, thus closing the negative feedback loop [64]. Furthermore, consistent with our study, downregulation of Cx43 in failing hearts from spontaneously hypertensive rats is related to reduced inactivating phosphorylation of GSK-3beta [70]. On the other hand, Cx43 deficiency results in alterations in the mineralization and remodeling phases of fracture

healing, and is characterized by delayed expression of osteogenic marker genes, increases in GSK-3 β mRNA and active GSK-3 β , consistent with a pronounced decrease in β -catenin expression. At the same time, alterations in healing of fractures, in Cx43 deficiency, can be rescued by inhibiting GSK-3 β activity using LiCl [32]. BMSCs play an important role in fracture repair, and BMSC commitment to the chondrogenic or osteogenic lineages improves healing, after homing to the site of fracture, by increasing bone formation [71, 72]. An increase or activation of β -catenin could promote osteogenesis of BMSCs and bone formation [73]. Thus, our data combined with previous reports suggests that upregulation of Cx43 could promote fracture repair by raising and activating GSK-3 β / β -catenin signaling, which is associated with enhancement of osteogenesis of BMSCs.

In summary, we demonstrate that Cx43 plays an essential role in regulating osteogenic differentiation of BMSCs. Moreover, we substantiate the GSK-3 β / β -catenin pathway to be involved in downstream signaling of Cx43 to enhance osteogenic differentiation of BMSCs, and serves as part of the mechanism by which Cx43 regulates osteogenic differentiation of BMSCs. This sheds new light that Cx43 has a potential therapeutic role as an osteogenesis modulator through modulating bone mass and bone mineral density during osteogenic differentiation of BMSCs.

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Disclosure Statement

The authors declare that they have conflict of interests.

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