Connexin43 Potentiates Osteoblast Responsiveness to Fibroblast Growth Factor 2 via a Protein Kinase C-Delta/Runx2-dependent Mechanism

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In this study, we examine the role of the gap junction protein, connexin43 (Cx43), in the transcriptional response of osteocalcin to fibroblast growth factor 2 (FGF2) in MC3T3 osteoblasts. By luciferase reporter assays, we identify that the osteocalcin transcriptional response to FGF2 is markedly increased by overexpression of Cx43, an effect that is mediated by Runx2 via its OSE2 cognate element, but not by a previously identified connexin-responsive Sp1/Sp3-binding element. Furthermore, disruption of Cx43 function with Cx43 siRNAs or overexpression of connexin45 markedly attenuates the response to FGF2. Inhibition of protein kinase C delta (PKC δ) with rottlerin or siRNA-mediated knockdown abrogates the osteocalcin response to FGF2. Additionally, we show that upon treatment with FGF2, PKC δ translocates to the nucleus, PKC δ and Runx2 are phosphorylated and these events are enhanced by Cx43 overexpression, suggesting that the degree of activation is enhanced by increased Cx43 levels. Indeed, chromatin immunoprecipitations of the osteocalcin proximal promoter with antibodies against Runx2 demonstrate that the recruitment of Runx2 to the osteocalcin promoter in response to FGF2 treatment is dramatically enhanced by Cx43 overexpression. Thus, Cx43 plays a critical role in regulating the ability of osteoblasts to respond to FGF2 by impacting PKC δ and Runx2 function.

INTRODUCTION

Bone formation and remodeling is a tightly organized and dynamic process that requires the coordinated action of osteoblasts, osteocytes, and osteoclasts to maintain bone homeostasis. It is hypothesized that osteoblasts and osteocytes coordinate their activities, at least in part, through direct cell-to-cell communication through gap junctions. Gap junctions are composed of connexins, a family of transmembrane proteins that constitute the intercellular gap junction channels (Beyer *et al.*, 1990). Six connexins assemble to make up the gap junction hemichannel on the plasma membrane of one cell, which docks with a hemichannel on an adjacent cell to form an aqueous gap junction channel. The resultant gap junctions provide direct conduits for the passage of ions and other low molecular weight molecules, including second messengers, among cells.

The gap junction protein connexin43 (Cx43) is abundantly expressed in both osteoblasts and osteocytes, where it has been hypothesized to transmit hormonal signals, mechanical load, and growth factor cues among cells in order to coordinate the synthesis of new bone (reviewed in Stains and Civitelli, 2005a; Jiang *et al.*, 2007). Genetic ablation of *gja1*, the gene encoding Cx43, in mice leads to a severe delay in the ossification of both intramembranous and endochondral

derived skeletal elements during embryonic development (Lecanda *et al.*, 2000). The bones of these animals are remarkably brittle, and the osteoblasts isolated from the Cx43 null animals are dysfunctional, with reduced osteogenic and mineralizing capacity (Lecanda *et al.*, 2000). These Cx43-deficient mice die at birth because of a defect in cardiac function (Reaume *et al.*, 1995). Recently, conditional deletion of Cx43 in cells of osteogenic lineage has revealed an important role for Cx43 in postnatal skeletal homeostasis as well (Chung *et al.*, 2006). These conditional Cx43 knockout mice, which use a 2.3-kb collagen I promoter-driven Cre-recombinase to excise a floxed Cx43 allele in cells of the osteoblast lineage, exhibit severe osteopenia as a result of defective osteoblast function. Further, the skeletons of these animals have been shown to be refractory to the anabolic effects of intermittent PTH administration, indicating a critical role of Cx43 in coordinating cell function in response to anabolic cues.

Relatively little is known about the precise molecular role of gap junctions in sensing and responding to biological cues during bone formation, though in the past decade critical insights have been gained into the importance of gap junctions in processes such as response to growth factors and hormones (Schiller *et al.*, 1992; Chiba *et al.*, 1994; Van der Molen *et al.*, 1996; Civitelli *et al.*, 1998; Schiller *et al.*, 2001), mechanical load (Jorgensen *et al.*, 1997, 2000; Ziambaras *et al.*, 1998; Romanello and D'Andrea, 2001; Saunders *et al.*, 2001, 2003; Cherian *et al.*, 2003, 2005; Taylor *et al.*, 2007), bisphosphonates (Plotkin *et al.*, 2002, 2005), and interaction with other cells (Villars *et al.*, 2002). Importantly, less still is known regarding the biologically relevant second messengers that osteoblast and osteocyte gap junctions communicate among cells, and many molecular mechanisms have yet to be deduced.

Previously, we have shown that disruption of Cx43 can reduce transcription of the osteocalcin gene in response to

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Abbreviations used: ChIP, chromatin immunoprecipitation; DAG, diacylglycerol; Cx43 and Cx45, connexin43 and 45, respectively; FGF2, fibroblast growth factor 2; FGFR, fibroblast growth factor receptor; IOD, integrated optical density; PKC δ , protein kinase C delta.

treatment with serum by modulating signal transduction (Stains *et al.*, 2003; Stains and Civitelli, 2005b). Ultimately, we showed that disruption of Cx43 function results in a decrease in the magnitude of the extracellular signal-regulated kinase (ERK) activation, resulting in a shift in the transcription factor occupancy of the osteocalcin promoter, decreasing osteocalcin transcription. From this work, we proposed a model by which cells can utilize gap junction communication to coordinate and amplify the cellular response to growth factor exposure. Specifically, osteoblasts respond to a growth factor via the classic receptor–ligand interaction, which we termed the gap junction-independent primary response, and if the cells are coupled to an adjacent cell by gap junctions, then the adjacent cell may respond to the primary response as a result of second messengers passing through the Cx43 gap junction, initiating a gap junction-dependent secondary response. Thus, by utilizing gap junction intercellular communication, cells that may not directly sense an extracellular cue are capable of responding in a coordinated way. For example, the osteocytes sensing mechanical load, could activate surface osteoblasts via gap junctions, or populations of cells that have heterogeneous receptor expression for a growth factor would still be able to respond synchronously.

To expand on our knowledge of the molecular mechanisms by which gap junctions amplify osteoblast responses and to move closer toward identifying biologically relevant second messengers that pass through the Cx43 gap junctions in osteoblasts, we have examined the influence of Cx43 on the activation of osteoblast transcription by FGF2. FGF2 is an important regulator of skeletal tissue. FGF2 can act at several stages of osteoblast differentiation to differentially affect osteoblast function (Marie, 2003). In vivo, short-term administration of recombinant FGF2 has been shown to increase bone formation in intact rats (Nakamura *et al.*, 1995; Nagai *et al.*, 1995) and to exhibit bone anabolic effects on ovariectomized rats (Liang *et al.*, 1999), presumably through the recruitment and expansion of osteo-progenitor populations. Genetic ablation of FGF2 results in impaired trabecular bone formation associated with decreased mineral apposition and bone formation rates, and stromal cells isolated from FGF2 null mice have a markedly reduced mineralization potential (Montero *et al.*, 2000). In contrast, overexpression of FGF2 in transgenic mice leads to impaired intramembranous and endochondral ossification (Sobue *et al.*, 2005). Mutations in fibroblast growth factor receptors (FGFRs) in humans leads to numerous skeletal dysmorphic syndromes (Ornitz and Marie, 2002; Ornitz, 2005).

Treatment of osteoblasts with FGF2 has been shown to stimulate osteocalcin transcription (Schedlich *et al.*, 1994; Boudreaux and Towler, 1996; Newberry *et al.*, 1996; Xiao *et al.*, 2002). Furthermore, the osteocalcin promoter is a widely studied gene in osteoblasts, as osteocalcin expression is relatively osteoblast specific (Hauschka *et al.*, 1989). Indeed, the transcription factor Runx2, a master regulator of osteoblast differentiation, was highlighted based on its binding to an osteoblast-specific element in the osteocalcin promoter (OSE2; Ducky and Karsenty, 1995; Ducky *et al.*, 1997). Moreover, the activity of FGF2 on the osteocalcin promoter is relatively well studied, having been shown to be mediated by either Runx2 phosphorylation and recruitment to the promoter (Xiao *et al.*, 2002; Kim *et al.*, 2003; Kim *et al.*, 2006) and/or a novel Ku-antigen transcription complex that regulates the recruitment of Runx2 to the osteocalcin promoter (Willis *et al.*, 2002; Xiao *et al.*, 2002; Kim *et al.*, 2003, 2006; Sierra *et al.*, 2004;).

In this study, we examine the role of Cx43 in amplifying osteoblast responses to the osteogenic growth factor FGF2. We further characterize the molecular mechanisms controlling the gap junction-mediated impact on osteoblast transcription. By doing so, we hope to gain insight into how osteoblasts utilize gap junctions to coordinate function in response to osteogenic cues and to move closer to identifying the biologically relevant signals that are being propagated through the Cx43 gap junction channels.

MATERIALS AND METHODS

Chemicals and Reagents

Routine molecular biology reagents and chemicals were purchased from Promega (Madison, WI) and Sigma (St. Louis, MO), respectively, unless specified. FGF2 was from R&D Systems (Minneapolis, MN). The PKC δ inhibitor rottlerin and antibodies against phospho-protein kinase C delta (PKC δ ; Thr505) were from Cell Signaling Technology (Beverly, MA). Anti-PKC δ , anti-PKC ϵ , anti-Runx2 (PEBP2 α A), and anti-GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-serine and anti-Cx43 antibodies were purchased from Sigma.

Cell Culture

A clonal line of osteoblast-like MC3T3-E1 (clone 4) cells derived from newborn mouse calvaria (Sudo *et al.*, 1983; Wang *et al.*, 1999) were purchased from the ATCC (Manassas, VA). These cells were cultured in DMEM containing 10% fetal bovine serum (FBS), penicillin, and streptomycin. Growth-arrested cells were obtained by culturing in DMEM containing 0.1% FBS and 0.3% bovine serum albumin (BSA) for 24 h. FGF2 was administered at 5 ng/ml and maintained on the cells for 16 h, unless stated otherwise. Cell viability was routinely tested under all culture conditions used in these experiments with a colorimetric CCK-8 assay and/or by trypan blue exclusion. The number of viable cells under all conditions was consistently above 95% and did not statistically differ among groups.

Plasmid Constructs

The –199 osteocalcin promoter-luciferase reporter construct (–199OCN LUC) contains the –199 to +32 5' flanking sequence of rat osteocalcin gene, relative to the transcriptional start site and spans a single Runx2-binding OSE2 element (Towler *et al.*, 1994). This promoter construct was provided by Dr. Dwight Towler (Washington University, St. Louis, MO). The p6xOSE2-luciferase reporter construct contains six tandem repeats of the OSE2 element of the mouse OG2 promoter (Ducky and Karsenty, 1995). The p6xOSE2mut construct contains a mutation in the Runx2-binding region of the OSE2 element (Ducky and Karsenty, 1995). Both p6xOSE2– and p6xOSE2mut-luciferase constructs were provided by Dr. Gerard Karsenty (Columbia University, New York, NY). The pSFFV-Cx43 construct (Beyer *et al.*, 1987), which contains a full-length cDNA for rat Cx43 in the pSFFV-neo backbone plasmid (described in Fuhlbrigge *et al.*, 1988), was provided by Dr. Thomas Steinberg (Washington University, St. Louis, MO). This construct has previously been shown to increase electrical (Veenstra *et al.*, 1992) and chemical (Steinberg *et al.*, 1994) coupling among cells. The pcDNA-cCx45 plasmid was generated by subcloning the chicken Cx45 (cCx45) coding sequence into EcoRI site of pcDNA3 (Invitrogen, Carlsbad, CA).

Transfections and Luciferase Reporter Assays

MC3T3-E1 clone 4 cells were seeded at 4×10^5 cells/well in 12-well plates. Twenty four hours later, the cells were transfected with the appropriate plasmid with either Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or FuGene 6 (Roche, Indianapolis, IN), according to manufacturer's directions. Reporter plasmids were used at 0.8 μ g/well and expression plasmids at 1.6 μ g/well in each transfection. Total DNA content was kept constant in all experiment by addition of pcDNA. After 72 h, the cells were rinsed in Hanks' balanced salt solution (HBSS) and lysed in 1 $\times$ Passive Lysis Buffer (Promega). Luciferase activity was monitored using a Berthold Centro LB 960 luminometer (Oak Ridge, TN). Transfection efficiency was monitored by cotransfection with a β -galactosidase reporter (SV- β -gal) and β -galactosidase activity determined by a colorimetric assay. Using these transfection conditions, we routinely observe ~40% transfection efficiency, as determined by observation of green fluorescent protein (GFP) expression by fluorescence microscopy. Confirmation of the transfection of expression vectors were confirmed either by immunoblotting or real-time PCR. All experiments were performed in triplicate and repeated a minimum of three times.

Short Interfering RNA Construction

Short interfering RNA (siRNA) constructs (Cx43 and scrambled) were generated using the Silencer siRNA Construction kit (Ambion, Austin, TX) following the manufacturer's directions, using AATACAGCGAAAGCCAGACTGCCTGTCTC

and AACAGTCTGCCTTCGCTGTACCTGTCTC oligonucleotides as the sense and antisense Cx43 templates, respectively. The scrambled siRNAs were generated from the following sense and antisense oligonucleotide templates: AATCA-GACGAGAGGACCTAGCCTGTCTC and AACTAGGTCTCTCGTCTGACCTGTCTC. Plasmids encoding short hairpin RNA to knockdown PKC δ expression (pHUSH-PRKCD) and a scrambled control (pHUSH-30003) were purchased from Origene (Rockville, MD).

Cell Treatments with FGF2

Growth-arrested MC3T3-E1 clone four cells were treated with FGF2 in 1 ml of DMEM containing 0.1% FBS and 0.3% BSA for the indicated periods. FGF2 was reconstituted in a filtered solution of 0.5% BSA, 1 mM DTT, and 10% glycerol in HBSS. FGF2 was used at 5 ng/ml unless indicated otherwise. When we tested the effect of rottlerin on the FGF2 pathway, the cells were pretreated with 5 μ M rottlerin for 1 h before stimulation with FGF2.

Western Blotting and Immunoprecipitations

Whole extract from MC3T3-E1 cells were prepared using modified RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 10 mM β -glycerophosphate, 1 mM EGTA, 1 mM EDTA, 2 mM sodium vanadate, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 $\times$ protease inhibitor cocktail). Insoluble material was pelleted, and the supernatants were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% non-fat dry milk, probed with the indicated primary antibodies overnight, and detected with the appropriate horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence detection reagents (Pierce, Rockford, IL). Blots were acquired and analyzed using aEpiChem gel documentation system and Labworks 4.0 software (UVP Bioimaging Systems, Upland, CA).

Immunoprecipitations were performed using 500 μ g of whole cell extract, 2 μ g anti-Runx2 and/or anti-phosphoserine antibodies. Immunoprecipitated material was collected using protein A/G plus beads. Beads were washed five times in modified RIPA buffer. The bound protein was eluted from the beads in SDS sample buffer, immunoblotted, and probed with anti-Runx2 or anti-phosphoserine antibodies, as indicated.

Scrape Loading

Assessment of gap junctional coupling was assessed using scrape loading induced dye transfer, as described by others, with several minor modifications (Le and Musil, 1998). MC3T3-E1 clone 4 cells were transfected with the indicated constructs, as described above. Two days after confluence, the fully confluent monolayer of cells was then rinsed three times in Ca²⁺- and Mg²⁺-free HBSS and then incubated in 0.5% solution of Lucifer yellow and 0.5% rhodamine dextran in Ca²⁺- and Mg²⁺-free HBSS. A scalpel was used to cut a single longitudinal line through the monolayer of cells. The cells were then incubated for 10 min in the dark to permit dye diffusion from the damaged cells at the scrape line to adjacent cells. Subsequently, the cells were rinsed in Ca²⁺- and Mg²⁺-containing HBSS, and fixed in 10% neutral buffered Formalin, and fluorescence was observed using an Eclipse 50i microscope and a 4 $\times$ Plan Apo objective (Nikon, Melville, NY). Images were captured using a Photometrics Coolsnap camera system (Tucson, AZ). Rhodamine dextran-positive cells indicate the damaged cells that initially take up the dye at the scrape line, because the molecular weight of rhodamine dextran (>10,000 Da) precludes passage through gap junction channels. Dye transfer was quantitated by measuring the distance from the rhodamine dextran-Lucifer yellow-positive cells at the scrape line to the most distal Lucifer yellow-positive cells perpendicular to the scrape line in the collected images. A minimum of 20 measurements were taken per field of view. Three fields of view were analyzed per experiment. The scrape loading was performed on triplicate cultures. All of the calculated measurements for dye diffusion were averaged for comparisons among groups.

Chromatin Immunoprecipitations

Chromatin immunoprecipitations (ChIPs) were performed as described previously, with the exception that the quantification of immunoprecipitated genomic DNA was assessed by real-time PCR (Stains *et al.*, 2003). The osteocalcin proximal promoter primers were as follows: mOG2 Promoter: forward, AGCCTGGCAGTCTCCGATT, and reverse, ATGCTGTGGTTGGTGATTGC; mOG2 open reading frames (ORF): forward, GTGCTGCCCTAAAGCCAAAC, and reverse, GGACAGGGAGGATCAAGTTCTG. Real-time PCR was performed on an Applied Biosystems 7300 Sequence Detection System (Foster City, CA) using the SYBR green method. Amplification of a single target was determined by examination of a dissociation curve. For traditional gels, the PCR products after 20 cycles of PCR were electrophoresed on 2% NuSieve 3:1 agarose gels (Cambrex, Walkersville, MD) and imaged using a UVP EpiChem gel documentation system and Labworks 4.0 software.

Immunostaining

Cells were grown to confluence and treated with 5 ng/ml FGF2, as described above, for the indicated time. Cells were then rinsed with HBSS and then fixed in 4% paraformaldehyde for 15 min. Cells were permeabilized in HBSS

+ 0.1% Triton X-100 for 10 min, blocked in HBSS + 10% normal goat serum, and then incubated with total or phospho-PKC δ or PKC ϵ antibodies in blocking buffer for 1 h at room temperature. After extensive rinsing the cells were incubated with anti-rabbit Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) for 1 h, rinsed in DAPI containing HBSS, and mounted onto slides using Fluoromount G. Fluorescence was monitored using a Nikon Eclipse 50i microscope, a 40 $\times$ Plan Apo objective and Photometrics Coolsnap camera system. Staining using nonimmune rabbit IgG and anti-rabbit Alexa Fluor 488 secondary antibodies were used as a negative control. Quantitation of nuclear/perinuclear fluorescence intensities was calculated by analyzing the collected microscopic images with Labworks 4.0 Image Acquisition and Analysis Software v4.0 (UVP Bioimaging Systems). Nuclear/perinuclear regions of interest were selected based on corresponding DAPI images for three fields of view from independent experiments per treatment group. Each field of view contained at least 35 nuclei. A background corrected integrated optical density (IOD) was then calculated for each region of interest in the pPKC δ images. Mean IODs were calculated from the IODs of all counted regions of interest for each group. The percentage of responding cells (% responding) was determined by counting the number of regions of interest in a field of view from FGF2-treated cells that had an IOD that was greater than two SDs above the mean IOD of the corresponding vehicle-treated samples. Averages and SDs were calculated from fields of view from three separate experiments.

Statistical Analysis

Data were assessed for statistical significance using a two-tailed *t* test (comparison of two data sets) or with analysis of variance (ANOVA, comparison of multiple data sets). A significant difference was determined as *p* < 0.05. All experiments were repeated in triplicate and have been repeated a minimum of three times (*n* $\geq$ 9).

RESULTS

Synergistic Activation of Osteocalcin Transcription by FGF2 and Cx43

The effect of the overexpression of Cx43 combined with FGF2 treatment on osteocalcin transcription was evaluated in confluent monolayers of MC3T3-E1 clone 4 cells that were transiently transfected with a -199OCN LUC reporter along with pSFFV-Cx43 or empty vector. After serum-starving overnight, the cells were treated with vehicle or 2.5–50 ng/ml FGF2 for 16 h. As illustrated in Figure 1A, FGF2 weakly stimulated osteocalcin transcription in empty vector (pcDNA)-transfected cells at all concentrations tested ($\square$). Osteocalcin transcription was synergistically increased when cells were treated with 5, 10, and 25 ng/ml FGF2 in the presence of overexpressed Cx43 ($\blacksquare$). Furthermore, the synergistic response to the addition of FGF2 was dependent on the concentration of Cx43 plasmid used in the transfection (data not shown). A maximal synergistic increase was observed in cells transfected with 1.6 μ g/well Cx43 and treated with 5 ng/ml FGF2, which exhibited a 4.8-fold increase in osteocalcin transcription relative to vehicle-treated, empty vector-transfected cells. The efficacy of Cx43 overexpression after transfection of the cells by immunoblotting is shown in Figure 1B.

Work from others has shown that the FGF2-responsiveness of the osteocalcin promoter is mediated primarily by regulating Runx2 binding to OSE2 elements, either through modulating Runx2 recruitment or through activity (Willis *et al.*, 2002; Xiao *et al.*, 2002; Kim *et al.*, 2003; Sierra *et al.*, 2004). We have previously shown that Cx43 gap junctions can regulate transcription from a Sp1/Sp3 binding "connexin-response element" in the osteocalcin proximal promoter (OCN CxRE; Stains *et al.*, 2003). To identify whether the FGF2-Cx43 synergistic effect on transcription is being mediated by Runx2, Sp1/Sp3, or both, we analyzed the effects of Cx43 and FGF2 on the Sp1/Sp3 binding OCN CxRE and the Runx2 cognate (p6xOSE2) luciferase reporters. As shown in Figure 2A, the overexpression of Cx43 markedly enhanced the transcriptional activation of the -199OCN LUC reporter by FGF2. Likewise, the synergistic action of Cx43 overex-

Figure 1. Synergistic induction of osteocalcin transcription by FGF2 and Cx43. (A) MC3T3 osteoblasts were transiently transfected with the -199OCN LUC reporter plasmid, along with either empty pcDNA vector or with pSFFV-Cx43 expression vector. After serum starvation, the cells were treated for 16 h with the indicate concentration of FGF2 or with vehicle. The samples were then analyzed for luciferase activity. Statistically significant difference relative to the respective paired vehicle-treated control for a, pcDNA or b, Cx43; $p < 0.05$; c indicates a more than additive (synergistic) response to the combined treatment with FGF2 and Cx43 overexpression. (B) Western blots of whole cell extracts from MC3T3 osteoblasts transiently transfected with pcDNA or pSFFV-Cx43 were probed with anti-Cx43 or anti-GAPDH (load control) antibodies.

pression combined with FGF2 treatment on transcription can be recapitulated on the Runx2-binding p6xOSE2 LUC construct (Figure 2C). In contrast, transcriptional synergy was not observed on the OCN CxRE LUC reporter, which remains exquisitely sensitive to Cx43, but not to FGF2, nor on p6xOSE2mut LUC reporter, which is unable to bind Runx2 (Figure 2, B and D).

On the basis of these data, we hypothesized that Cx43 may propagate and amplify FGF2-initiated signals. Accordingly, disruption of Cx43-gap junctions should limit the capacity to propagate signal molecules among coupled cells, resulting in activation of FGF2-dependent gene activity. To test this hypothesis, we utilized a model of connexin45 (Cx45) overexpression. Previously, we and others have reported that the overexpression of exogenous Cx45 in an endogenous Cx43 background results in diminished gap junction communication, leading to repressed gene transcription (Koval *et al.*, 1995; Li *et al.*, 1999; Martinez *et al.*, 2002; Stains *et al.*, 2003). Cells were transiently transfected with pcDNA-cCx45, SVFF-Cx43, or pcDNA. As shown in Figure 3, overexpression of Cx45 alone or coexpression of both Cx43 and Cx45 results in statistically significant reduction of the Cx43-FGF2-induced synergistic activation of osteocalcin transcription from both the -199OCN LUC and the Runx2-binding p6xOSE2 LUC reporters. Thus, the type of communicative function provided by Cx43, but not by Cx45 or Cx43/45 heteromeric gap junctions, is necessary to promote an amplified response to FGF2 stimulation. Next, we

Figure 2. FGF2-Cx43-induced synergism occurs via a Runx2 binding cognate. MC3T3 cells were transiently transfected with (A) -199OCN LUC reporter, (B) OCN CxRE LUC reporter, (C) a p6xOSE2 LUC reporter or (D) a p6xOSE2mut LUC reporter. The cells were cotransfected with expression vector and treated with vehicle or FGF2 (5 ng/ml) as indicated in Figure 1. Statistically significant difference, $*p < 0.05$; #indicates a more than additive response to the combined treatment with FGF2 and Cx43 overexpression.

disrupted Cx43 expression in MC3T3-E1 clone 4 cells by transient transfection with Cx43-specific siRNA oligonucleotides (Cx43 siRNA). As shown in Figure 4, when transfected with a nonspecific scrambled siRNA, osteocalcin transcription from the -199OCN LUC reporter is nearly doubled in response to FGF2 administration. Conversely, when Cx43 expression is "knocked down" by transfection with Cx43-directed siRNA, the transcriptional response to FGF2 is attenuated.

In total, these data show that increased expression of Cx43 can enhance osteocalcin transcription in response to FGF2 treatment. This effect is mediated at least in part by an OSE2 containing transcriptional element in the osteocalcin proximal promoter. Additionally, loss of Cx43 protein (Cx43 siRNA) or function (Cx45 overexpression) can attenuate the transcriptional response of MC3T3 osteoblasts to FGF2.

To ensure that the overexpression of Cx43 and/or disruption of Cx43 expression/function does, in fact, modulate gap junctional communication among the MC3T3 cells, we performed scrape loading experiments to examine the "dye coupling" among osteoblasts. This assay takes advantage of the ~0.5-kDa Lucifer yellow fluorescent dye to be passed among cells by certain gap junctions. As shown in Figure 5, pcDNA-transfected MC3T3 cells are moderately well coupled by gap junctions as indicated by Lucifer yellow transfer among cells in the monolayer. Overexpression of Cx43 in

Figure 3. Cx45 overexpression can attenuate the FGF2-Cx43 synergistic activation of osteocalcin transcription. MC3T3 osteoblasts were transiently transfected with (A) -199OCN LUC reporter, (B) a p6xOSE2 LUC reporter, or (C) a p6xOSE2mut LUC reporter. The cells were cotransfected with expression vectors as follows: empty pcDNA, Cx43, Cx45, or both Cx43 and Cx45. The total amount of expression plasmid in each reaction was maintained constant via the addition of empty pcDNA vector. Statistically significant difference, * $p < 0.05$; #indicates a more than additive response to the combined treatment with FGF2 and Cx43 overexpression.

these cells enhances the dye transfer among cells, as demonstrated by the increased diffusion distance of the Lucifer yellow from the scrape line. Conversely, disruption of Cx43 function by overexpression of Cx45 or siRNA-mediated knockdown of Cx43 (Cx43 siRNA) expression reduces the Lucifer yellow diffusion significantly below the levels observed for pcDNA-transfected cells. Fluorescence imaging of 10-kDa rhodamine dextran, which due to its molecular weight cannot be passed via gap junctions to adjacent cells, reveals the cells at the scrape line.

The FGF2-Cx43-Induced Synergistic Effect on Osteocalcin Transcription Is Dependent on PKC δ

The serine/threonine kinase PKC δ , a member of the novel protein kinase C family, is nearly ubiquitously expressed and plays a role in a large number of cellular processes (Dempsey *et al.*, 2000; Kikkawa *et al.*, 2002; Parker and Murray-Rust, 2004). Unlike classic protein kinase C family members, the activation of the novel PKCs are Ca^{2+} independent,

Figure 4. Knockdown of Cx43 attenuates the FGF2 responsiveness of the osteocalcin promoter. Transient transfection with siRNA directed against the Cx43 mRNA (Cx43 siRNA) attenuates the FGF2 response of the -199OCN LUC reporter when compared with cells transiently transfected with a nonspecific, scrambled siRNA (SCR siRNA). Inset, a Western blot of whole cell extracts from siRNA-transfected cells probed with anti-Cx43 antibodies. GAPDH antibodies were used as a loading control.

but remain sensitive to diacylglycerol. Kim *et al.* (2003, 2006) have shown that PKC δ can mediate FGF2-dependent activation of Runx2 transcription and binding activity in osteoblasts. Accordingly, we examined if disruption of PKC δ signaling would impact the FGF2-Cx43 synergistic effect on osteocalcin transcription. Indeed, treatment of the cells with 5 μM rottlerin, a chemical inhibitor of PKC δ (Gschwendt *et al.*, 1994) or with siRNA construct targeting PKC δ (siPRKCD15) blocks the FGF2-Cx43 synergism from both the -199OCN LUC and pOSE2 reporters (Figure 6). Inhibition of ERK or JNK cascades with chemical inhibitors and/or siRNA were unable to attenuate the effect of Cx43 on FGF2 responsive osteocalcin transcription (data not shown).

Furthermore, by immunoblotting we show that PKC δ is phosphorylated on threonine 505 within minutes after treatment with FGF2 (Figure 7A). The degree of PKC δ phosphorylation in response to FGF2 is markedly enhanced by overexpression of Cx43, correlating well with the synergistic activation of gene transcription (Figure 7B). The level of Runx2 protein, as determined by immunoblotting, is not noticeably increased by FGF2 treatment, nor by Cx43 overexpression; however, using coimmunoprecipitation, we show that the serine phosphorylation of Runx2 is enhanced by both FGF2 and Cx43 overexpression and is maximally enhanced when both FGF2 is present and Cx43 is overexpressed (Figure 7C). To determine the impact of Cx43 and FGF2 on Runx2 DNA-binding activity, we performed ChIP assays on the osteocalcin proximal promoter, using antibodies against Runx2 and primers adjacent to the proximal OSE2-Runx2-binding sites (Figure 7, D and E). Treatment with FGF2 in pcDNA-transfected cells increases Runx2 occupancy of the osteocalcin proximal promoter by ~100%. Similarly, Runx2 association with the osteocalcin promoter is increased by Cx43 overexpression in the absence of FGF2 treatment. Consistent with the observed transcriptional synergy, when cells overexpressed Cx43 and received FGF2, Runx2 occupancy of the osteocalcin proximal promoter increases more than 10-fold.

Figure 5. Modulation of dye coupling by alteration of Cx43 expression or function. MC3T3 osteoblasts were transiently transfected with pcDNA, pSFFV-Cx43, pcDNA-Cx45, or Cx43 siRNA. Forty-eight hours after transfection, gap junctional communication was assessed by the scrape-loading, dye-coupling assay. Top, representative fluorescence images are shown for the Lucifer yellow (left), rhodamine dextran (middle), or merged images (right) after transmission of the Lucifer yellow among transfected cells. Scale bar, 50 μ m. Bottom, quantitation of the average distance of transmission of Lucifer yellow from the cells proximal to the scrape line to the most distally Lucifer yellow-positive cell. Data are set relative to the pcDNA-transfected controls. Statistically significant difference from the pcDNA control; * $p < 0.05$.

To gain insight into the point of intersection of Runx2-PKC δ signaling, we performed immunocytochemistry on MC3T3 cells and monitored the distribution of PKC δ after FGF2 treatment. Immunocytochemistry reveals that PKC δ translocates to nuclear regions of FGF2-stimulated cells, as determined by codetection of DAPI-labeled nuclei and PKC δ immunostaining (Figure 8, A and B). In contrast, another novel PKC, PKC ϵ remains localized to the cytoplasm (Figure 8, C and D). We hypothesize that the translocation of PKC δ to the nucleus is likely associated with the phosphorylation and alteration of Runx2 DNA-binding activity and the concomitant increase in osteocalcin transcription. Notably, not all of the cells have robust nuclear staining for PKC δ after treatment with FGF2.

We next sought to examine whether modulation of Cx43 function or expression could impact the activation of PKC δ to the nucleus after FGF2 administration. Figure 9 reveals immunostaining of MC3T3 clone 4 cells stained with anti-phospho-PKC δ (Thr505) antibodies. In vehicle-treated cells (Figure 9, left column), the staining for phospho-PKC δ is diffusely visible in the cytoplasm and has a granulated or punctate appearance in the nuclei (Figure 9, small arrows).

On treatment with FGF2, the pattern of staining of some, but not all cells, is dramatically different from that observed in vehicle-treated cells, with a distinct reduction of cytoplasmic staining and a less punctate and generally more intense nuclear fluorescence (Figure 9, large arrows). Intriguingly, it appears qualitatively that the number of cells that have an altered pattern of staining after FGF2 treatment is increased by overexpression of Cx43 and reduced by Cx45 overexpression or transfection with Cx43 siRNA relative to the pcDNA-transfected samples.

We next attempted to provide quantitative analysis to these observations of the immunofluorescence data (Table 1), as described in *Materials and Methods*. DAPI-stained nuclei were used as a guide to define the nuclear region of interest. Fluorescence intensities of nuclear regions of interest were expressed as IOD. Treatment with FGF2 enhanced the IOD in the nuclear regions of all groups (pcDNA, Cx43, Cx45, and Cx43 siRNA). In contrast, transfection of the cells treated with vehicle alone did not affect the mean IOD. Overexpression of Cx43 when combined with FGF2 treatment significantly enhanced the mean IOD levels, indicating an increase in phospho-PKC δ within the nuclear region of interest. Disruption of Cx43 expression (Cx43 siRNA) or function (Cx45 overexpression) reduced the mean IOD relative to pcDNA-transfected FGF2 treated samples, but did not reach statistical significance. However, when we examine the number of nuclear regions of interest that have IOD values greater than two SDs above the mean IODs of their vehicle-treated samples, we observe that Cx43 overexpression enhances the percentage of nuclear regions of interest with elevated IODs (67.8 vs. 34.9%) and disruption of Cx43 expression (Cx43 siRNA) or function (Cx45 overexpression) diminishes the percentage of nuclear regions of interest with an elevated IOD (17.0 and 14.0%, respectively vs. 34.9%) relative to the FGF2-treated, pcDNA-transfected samples.

DISCUSSION

During bone formation, new bone is deposited in a highly coordinated manner, in which hundreds of cuboidal osteoblasts are found clustered along the bone surface. It is widely supported that osteoblasts and osteocytes coordinate their function through the rapid and efficient communication through gap junctions, in response to extracellular cues. In this study, we modulate gap junctional communication in osteoblasts by altering the expression or function of Cx43 and examine the impact on an FGF2-mediated transcriptional response. There is both *in vivo* and *in vitro* evidence that manipulating the relative abundance of Cx43 can affect osteoblast function. A gene dosage effect is observed using a model of conditional knockout of Cx43 in osteoblasts, as the anabolic response to PTH in heterozygous Cx43^{+/-} mice is intermediate between wild-type and Cx43 conditionally deleted animals (Chung *et al.*, 2006). Similarly, heterozygous Cx43^{+/-} mice demonstrate an intermediate response to mechanical load (Grimston *et al.*, 2008). Gene dosage effects are also apparent in the Cx43 null mouse model. Although no overt skeletal phenotype in Cx43^{+/-} animals is noted, primary osteoblast isolated from Cx43^{+/-} mice exhibit decreased dye coupling, reduced osteoblastic gene expression, and diminished mineralizing capacity, which is intermediate between cells from wild-type and homozygous null animals (Lecanda *et al.*, 2000). Likewise, cells from Cx43^{+/-} mice have deficient calcium mobilization in response to endothelin-1. Additionally, several *in vitro* studies have up-regulated (Steinberg *et al.*, 1994; Gramsch *et al.*, 2001) or down-regulated Cx43 abundance or function (Van der

Figure 6. Inhibition of PKC δ reduces Cx43-dependent activation of osteocalcin transcription by FGF2. MC3T3 osteoblasts were transiently transfected with (A) a -199OCN LUC reporter or (B) a p6xOSE2 LUC reporter constructs. In addition, the cells were cotransfected with pcDNA or Cx43 expression constructs. Before treatment with FGF2 (5 ng/ml), the cells were pretreated with 5 μ M rottlerin. (C) Cells were transiently cotransfected with p6xOSE2 LUC, pcDNA, or Cx43 expression constructs and a scrambled siRNA expression construct (SCR siRNA) or siRNA construct to knockdown PKC δ expression construct (PRKCD15 siRNA), as indicated. (D) Immunoblot for PKC δ showing the efficacy of the siRNA construct for PKC δ . SCR siRNA represents a scrambled siRNA construct. Cx43 66 siRNA is a construct designed to knock down Cx43 expression and is used as a negative control in this experiment. GAPDH antibodies were used as a loading control. Statistically significant difference, * $p < 0.05$; #indicates a more than additive response to the combined treatment with FGF2 and Cx43 overexpression.

Molen *et al.*, 1996; Lecanda *et al.*, 1998; Li *et al.*, 1999, 2006; Upham *et al.*, 2003; Plotkin *et al.*, 2008) with profound effects on osteoblast function.

Our ultimate goals are to gain insight into how osteoblasts utilize intercellular communication in order to regulate function and to identify biologically relevant molecules being passed through Cx43 gap junction channels in osteoblasts. Accordingly, our approach is to work our way from a defined transcriptional response affected by gap junctions back through the signaling cascade—systematically examining a path from the nucleus to membrane.

This is the first study to demonstrate that modulation of Cx43 can markedly impact the response of osteoblasts to

FGF2 treatment. Overexpression of Cx43 dramatically augments the effect of FGF2 on osteocalcin transcription in MC3T3 cells, whereas siRNA-mediated knockdown of Cx43 can attenuate the response of osteoblasts to FGF2. Additionally, we show that the Cx43-dependent regulation of the FGF2 response is mediated, at least in major part, by the novel protein kinase C family member, PKC δ . The fact that the chemical inhibitor rottlerin is unable to fully abrogate both the Cx43 overexpression response and the response to FGF2 alone, may indicate the involvement of other pathways, may be a result of only partial inhibition of function by the inhibitor or may be complicated by the known lack of specificity of rottlerin. It should be noted that the results

Figure 7. Activation of PKC δ by Cx43 and FGF2 synergistically affects Runx2 phosphorylation and DNA-binding activity. (A) Immunoblots of whole cell extracts from MC3T3 cells treated with FGF2 for the indicated number of minutes probed with phospho-PKC δ or total PKC δ antibodies. (B) Immunoblots of whole cell extracts from MC3T3 pcDNA- or Cx43-transfected cells treated with FGF2 for 16 h. The blots were probed with anti-phospho-PKC δ or anti-Runx2 antibodies. Anti-GAPDH antibodies are used as a loading control. (C) Whole cell extracts from pcDNA- or Cx43-transfected cells treated with either vehicle or FGF2 were immunoprecipitated with anti-Runx2 antibodies and subsequently immunoblotted using anti-phospho-serine antibodies. (D) ChIP was performed on cells that were transfected with pcDNA or Cx43 and treated with vehicle or FGF2. The immunoprecipitated material was analyzed by real-time PCR for the presence of the proximal osteocalcin promoter region, using a specific primer set. Data are shown as the abundance of PCR product associated with the bead fraction of the ChIP as a ratio of input DNA and represents the compiled data obtained from three separate experiments. The ChIP data are normalized to the pcDNA vehicle control. (E) A representative agarose gel of the ChIPs showing the PCR product obtained from the immunoprecipitated nucleic acid pulled down with anti-Runx2 antibodies after treatment with or without FGF2 in transfected cells. The same PCR primers to the osteocalcin proximal promoter were used in this experiment. Statistically significant difference, * $p < 0.05$.

Figure 8. Treatment with FGF2 causes PKC δ , but not PKC ϵ , to translocate to the nucleus. Immunofluorescence of MC3T3 osteoblasts treated with vehicle (A or C) or 5 ng/ml FGF2 (B or D) for 16 h. PKC δ (A and B) or PKC ϵ (C and D) are shown in green. Nuclear staining by DAPI is shown in blue. The bottom panels are merged images. Arrows indicate robust detection of PKC δ in the nucleus. White scale bar, 10 μ m.

with siRNA against PKC δ produce similar but not identical results, more effectively reducing the impact of Cx43 overexpression on osteocalcin transcription, but only partially blocking the FGF2 response. Still, the Cx43-FGF2-mediated synergy is fully abrogated by both methods, strongly implicating PKC δ in the Cx43-dependent transcriptional effects. This data also supports that pathways other than PKC δ also contribute to the ligand-receptor-mediated response. This is not unexpected as many pathways are activated immediately downstream of the activation of the FGF receptors (see below). Accordingly, we propose that Cx43-mediated enhancement of FGF2 signaling is because gap junctions are amplifying the cellular response to FGF2 by passing a sec-

ond messenger that acts up stream of PKC δ to control the osteoblast response.

Consistent with the work of others, we demonstrate that the effects of FGF2 on osteocalcin transcription converge on Runx2 and regulates its recruitment to its cognate element (OSE2; Willis *et al.*, 2002; Xiao *et al.*, 2002; Kim *et al.*, 2003, 2006; Sierra *et al.*, 2004). In the presence of Cx43 gap junctions, we see a more robust activation of Runx2 recruitment to the osteocalcin promoter and increased transcriptional activity after FGF2 treatment. The involvement of Cx43 in regulating the cellular responses to osteogenic cues provides insight into the molecular mechanisms underlying the skeletal phenotypes observed in mouse models of Cx43 genetic

Figure 9. Alteration of Cx43 expression or function alters the nuclear accumulation of phospho-PKC δ in response to FGF2 treatment. Cells were transiently transfected with pcDNA, pSFFV-Cx43, pcDNA-Cx45, or Cx43 siRNA. The transfected cells were subsequently fixed and stained with anti-phospho-PKC δ antibodies, after 30-min treatment with vehicle or FGF2 (5 ng/ml). Phospho-PKC δ staining is shown in green. Nuclear staining by DAPI is shown in blue. Vehicle-treated cells under all conditions show diffuse punctate nuclear staining (small arrows). On treatment with FGF2, the pattern of nuclear staining in some, but not all, cells is altered (large arrows). White scale bar, 10 μ m.

Table 1. Quantitation of nuclear phospho-PKC δ fluorescence intensity

	pcDNA vehicle (n = 133)	pcDNA FGF2 (n = 156)	Cx43 vehicle (n = 150)	Cx43 FGF2 (n = 151)	Cx45 vehicle (n = 141)	Cx45 FGF2 (n = 150)	Cx43 siRNA vehicle (n = 162)	Cx43 siRNA FGF2 (n = 151)
Mean IOD	3765	9187 ^a	3315	14853 ^{a,b}	3570	6657 ^a	3813	6900 ^a
SD	2007	6181	1551	9364	1287	4551	1464	3580
Ratio FGF2/Veh		2.44		4.48		1.86		1.81
% Responding		34.9		67.8 ^b		17.0 ^c		14.0 ^c
SD		7.2		14.1		5.1		3.8

IOD, integrated optical density; Ratio FGF2/Veh, mean IOD of FGF2 treated cells divided by the mean IOD of FGF2 treated cells within a transfection group; % responding, percentage of nuclear regions of interest with nuclear IODs two SDs or more above the corresponding vehicle treated sample.

^a Statistically significant change relative to vehicle treated samples ($p < 0.05$).

^b Statistically significant change relative to all other groups ($p < 0.05$).

^c Statistically significant change relative to pcDNA FGF2 and Cx43 FGF2 groups ($p < 0.05$).

deficiency (Lecanda *et al.*, 2000; Flenniken *et al.*, 2005; Chung *et al.*, 2006). Furthermore, these data suggest the intriguing possibility that increasing gap junction communication may augment the response of bone to anabolic therapies.

These data have led us to hypothesize that gap junctions amplify the response of a population of cells to an extracellular cue by spreading the activation to adjacent cells that may not directly “sense” the cue. We propose that an osteoblast, sensing an extracellular cue, such as a growth factor–receptor interaction, mounts a gap junction–independent “primary” response. This primary response is the classic response ligand receptor–initiated signaling cascade. When cells are coupled by Cx43 gap junctions, we suggest that second messengers generated in one cell by the ligand–receptor–mediated primary response can be propagated via gap junctions into adjacent cells where a gap junction–dependent “secondary” response is initiated. Importantly, this gap junction–dependent secondary response may occur in a cell that otherwise would not have responded to the extracellular cue. For example, the cell may lack the cognate receptor because of heterogeneity in receptor expression or because the cell may be sequestered in such a way that it is not directly exposed to the activating cue. The net effect is that the secondary response represents an amplification and coordination of the response of a population of cells. This model of “more cells responding to a cue when coupled by gap junctions” appears to be supported by the immunodetection of phospho-PKC δ in the nuclear region of osteoblasts after FGF2 stimulation. The number of cells with nuclear phospho-PKC δ after FGF2 treatment is greater in populations that have increased Cx43 expression, whereas the number of cells with nuclear phospho-PKC δ is diminished in populations with reduced Cx43 expression or function.

A few of our results were somewhat unexpected. For example, Cx43 did not have a synergistic increase on the transcriptional response of the –199 OCN LUC reporter to FGF2 at the highest dose tested (50 ng/ml). On the basis of the work of others, we suspect that this may be due to the fact that in MC3T3 cells it has been shown that increasing doses of FGF2 can lead to a delayed decrease in gap junctional coupling (Shiokawa-Sawada *et al.*, 1997). The inhibition of gap junctional coupling reaches its peak at ~50 ng/ml FGF2, the highest dose we tested. It is possible that at 50 ng/ml FGF2 the transient closing of gap junctions by FGF2 may offset the increase in gap junctional coupling caused by Cx43 overexpression. The impact is difficult to assess as the reported inhibition of gap junctional commu-

nication is delayed, occurring only 8 h after treatment, and transient, lasting only 24 h. At 5 ng/ml FGF2, the concentration we used in this study, we do not observe any inhibition of gap junctional coupling or Cx43 expression at 8 or 16 h (data not shown). Perhaps not too surprisingly, alteration of Cx43 levels altered osteocalcin transcription independent of FGF2. We suspect that this effect is a result of some signaling caused by the presence of 0.1% serum in the starvation media. Indeed, we have previously shown that inhibition of Cx43 can reduce the transcriptional response of osteocalcin to serum stimulation, albeit from a separate promoter element (Stains *et al.*, 2003). Unfortunately, attempts to culture cells in media completely devoid of serum, results in greatly reduced cell viability, precluding definitive support of this hypothesis. The impact of Cx43 on transcription in the absence of FGF2 treatment is slightly more robust on p6xOSE2 LUC than the –199 OCN LUC reporters. We think this is due to the following: there are six tandem copies of the OSE2 element in the former but only one OSE2 element in the later reporter constructs and other pathways may be influencing the numerous response elements in the native –199OCN LUC reporter. Consistent with the transcriptional impact of Cx43 alone on osteocalcin, we see increased basal phosphorylation of PKC δ , increased serine phosphorylation of Runx2, and increased Runx2 promoter occupancy in Cx43-expressing cells treated with vehicle relative to the pcDNA-transfected controls.

The data presented in this study hint at viable candidates for biologically relevant signals that are propagated by Cx43. Signal transduction from the activated FGF receptor is mediated by several factors, including phospholipase C γ (PLC γ ; Mohammadi *et al.*, 1991; Dailey *et al.*, 2005), the Grb2-SOS complex that in turn activates Ras and the ERK MAPK cascade (Gotoh, 2008), cytosolic phospholipase A2 (cPLA2) and phospholipase D (Ahmed *et al.*, 1994; Sa and Fox, 1994; Bolego *et al.*, 1997; Munaron *et al.*, 1997; Sa and Das, 1999; Oh *et al.*, 2007). Known second messengers generated downstream of the activation of these mediators include inositol 1,4,5-trisphosphate (IP3) and other inositol phosphate derivatives, diacylglycerol (DAG), calcium, and arachidonic acid. Furthermore, of these second messengers generated in response to FGF2, DAG, arachidonic acid, and inositol phosphate derivatives, specifically the water-soluble inositol hexaphosphate (IP6), have been shown to regulate the activation of PKC δ (Gschwendt, 1999; Dempsey *et al.*, 2000; O’Flaherty *et al.*, 2001; Steinberg, 2004; Vucenik *et al.*, 2005).

Additional insight into the biologically relevant second messenger propagated via Cx43 in response to FGF2 may be gleaned from the permeability of gap junctions. Depending on the connexin subunits that make up a gap junction channel, gap junctions have varying size and charge permeability characteristics (Goldberg *et al.*, 2004; Weber *et al.*, 2004). Gap junctions formed by Cx43 have among the largest pore diameter, permitting the diffusion of molecules up to 1.2 kDa, with permeability preferences for molecules that are negatively charged (Weber *et al.*, 2004; Harris, 2007). The second messengers produced in response to FGFR activation, IP₃, DAG, calcium and arachidonic acid, are all small enough to theoretically pass through Cx43 channels. Importantly, Cx45 gap junctions form channels with markedly reduced pore size, permitting the diffusion of molecules of up to ~0.3 kDa and exhibiting a preference for positively charged molecules (Veenstra *et al.*, 1994, 1995; Weber *et al.*, 2004). Previous work has shown that overexpression of chicken Cx45 in Cx43-expressing osteoblasts, results in a dramatic decrease in the chemical and electrical coupling of the resultant gap junctions, indicating that in a mixed Cx43/Cx45 background the biophysical properties of Cx45 prevail (Koval *et al.*, 1995; Li *et al.*, 1999; Martinez *et al.*, 2002). The fact that Cx45 can attenuate the amplification of FGF2 signaling caused by Cx43 implies that the signal being transduced by the gap junctions is diffusible through Cx43 channels, but not Cx45 or Cx43/Cx45 heteromeric channels. These data suggest that the signaling molecule being communicated by Cx43 in response to FGF2 is probably between 1.2 and 0.3 kDa in mass and likely anionic. The possibility of the diffusion of lipophilic molecules, such as DAG or arachidonic acid, through gap junctions as signal molecules must be carefully considered, as recent data has suggested that prostaglandins are released from osteoblasts via Cx43 hemichannel activity in response to fluid flow (Cherian *et al.*, 2003, 2005). Another class of molecules that should not be ignored are the inositol polyphosphates, which extend beyond just IP₃ and calcium signaling to a diverse set of cellular functions (York *et al.*, 2001).

It is important to note that although we model that the effects of Cx43 function are mediating gap junction intercellular communication, a compelling and expanding body of research has demonstrated that Cx43 can also function as unopposed hemichannels to regulate responses to fluid flow (Cherian *et al.*, 2005; Genetos *et al.*, 2007; Siller-Jackson *et al.*, 2008) and the antiapoptotic actions of bisphosphonates (Plotkin *et al.*, 2002; Plotkin *et al.*, 2005) in osteoblast or osteocytes-like cells. None of the approaches we have utilized in this study can specifically rule out that the results we observe could be attributable to hemichannel function. Future studies will attempt to more specifically determine the specific contributions of gap junction and/or hemichannels to our model.

In total, these data provide critical insights into the molecular details of how osteoblasts utilize Cx43 to coordinate and amplify cellular responses and suggest potential second messengers being propagated through the Cx43 gap junction channel as well. Further, we establish that overexpression of Cx43 permit osteoblasts to respond more potently to extracellular cues. Thus, it is conceivable that enhancement of Cx43 may be a way to augment anabolic therapies to increase osteoblast function.

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