Up-regulation of BMP2/4 signaling increases both osteoblast-specific marker expression and bone marrow adipogenesis in Gja1Jrt/+ stromal cell cultures

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ABSTRACT Gja1Jrt/+ mice carry a mutation in one allele of the gap junction protein α1 gene (Gja1), resulting in a G60S connexin 43 (Cx43) mutant protein that is dominant negative for Cx43 protein production of <50% of wild-type (WT) levels and significantly reduced gap junction formation and function in osteoblasts and other Cx43-expressing cells. Previously we reported that Gja1Jrt/+ mice exhibited early-onset osteopenia caused by activation of osteoclasts secondary to activation of osteoblastic lineage cells, which expressed increased RANKL and produced an abnormal resorption-stimulating bone matrix high in BSP content. Gja1Jrt/+ mice also displayed early and progressive bone marrow atrophy, with a significant increase in bone marrow adiposity versus WT littermates but no increase in adipose tissues elsewhere in the body. BMP2/4 production and signaling were increased in Gja1Jrt/+ trabecular bone and osteogenic stromal cell cultures, which contributed to the up-regulated expression of osteoblast-specific markers (e.g., Bsp and Ocn) in Gja1Jrt/+ osteoblasts and increased Pparg2 expression in bone marrow-derived adipoprogenitors in vitro. The elevated levels of BMP2/4 signaling in G60S Cx43-containing cells resulted at least in part from elevated levels of cAMP. We conclude that up-regulation of BMP2/4 signaling in trabecular bone and/or stromal cells increases osteoblast-specific marker expression in hyperactive Gja1Jrt/+ osteoblasts and may also increase bone marrow adipogenesis by up-regulation of Pparg2 in the Cx43-deficient Gja1Jrt/+ mouse model.

INTRODUCTION Gap junctions and hemichannels mediate cellular communication by allowing the passage of small molecules and ions (e.g., ATP, Ca2+, IP3, cAMP) directly between cells and between cells and their extracellular environment, respectively (Loewenstein, 1981; Alexander and Goldberg, 2003). Connexin 43 (Cx43), one member of the large connexin protein family, is the major gap junctional protein found in bone and is expressed by osteoblasts, osteocytes (Civitelli et al., 1993; Donahue et al., 1995), and bone marrow stromal cells (including osteoblast and adipocyte precursors; Dorshkind et al., 1993). Other members of the connexin protein family expressed in bone are Cx45 (Steinberg et al., 1994), Cx46 (Koval et al., 1997; Sanches et al., 2009), and Cx37 (Yamada et al., 2007; Paic et al., 2009), although their expression is much lower than that of Cx43. In bone, Cx43 is important in mediating hormonal and molecular signals (Chung et al., 2006; Plotkin et al., 2008; Zhang et al., 2011), fracture
repair (Loiselle et al., 2012), mechanical loading (Grimston et al., 2008, 2012; Zhang et al., 2011), and control of the subpopulation makeup of the stroma (Gonzalez-Nieto et al., 2012).

Cx43 gap junction function is critical to the processes of osteoblast and osteocyte differentiation and activity and bone formation and maintenance and has been studied extensively through the generation of Cx43-knockout (Lecanda et al., 2000), conditionally deleted (Chung et al., 2006; Watkins et al., 2011; Zhang et al., 2011; Bivi et al., 2012; Gonzalez-Nieto et al., 2012), and point-mutation mutant mice (Flenniken et al., 2005; Dobrowski et al., 2008; Zappitelli et al., 2013) and by overexpression of mutant Cx43 proteins in cell lines (McLachlan et al., 2008). The role of Cx43 in adipocytes and adipogenesis is less well studied. However, it has been reported that functional Cx43 gap junctions are present and required for mitotic expansion and C/EBPβ expression in preadipocytes (Yanagya et al., 2007) and that levels of Cx43 protein and gap junction formation and function are down-regulated during adipocyte differentiation (Azarnia and Russell, 1985; Umezawa and Hata, 1992; Yeganeh et al., 2012).

In addition to the important role of Cx43 channels in transport of signaling molecules, Cx43 has been shown to interact with intracellular structural and signaling molecules to modulate cellular signaling activities. For instance, Cx43 proteins have been proposed and/or shown to interact with Src kinase to activate ERKs in response to bisphosphonate-mediated cell survival signaling (Plotkin et al., 2002), with β-arrestin in response to PTH survival signaling (Bivi et al., 2011), and with protein kinase C during FGF2 signaling (Niger et al.). Cx43 has also been proposed to physically interact with β-catenin (Ai et al., 2000), although its involvement in Wnt and bone morphogenetic protein (BMP) signaling pathways is unknown.

Loss or disruption of Cx43 gap junctions and hemichannels in cells early in the osteogenic lineage has been reported to impair osteoblast differentiation, bone formation, and mineralization activities in various mouse models (Lecanda et al., 2000; Chung et al., 2006; McLachlan et al., 2008; Watkins et al., 2011). However, in Gja1+/− mice, in which a dominant-negative G60S Cx43 mutation results in a significant (>50%) reduction of Cx43 protein production, phosphorylation, and gap junction formation and function in osteoblasts (McLachlan et al., 2008) and other cell types (Flenniken et al., 2005), we recently showed that osteoblast differentiation and function are not decreased but are instead activated (Zappitelli et al., 2013). In particular, we found that Gja1+/− osteoblasts overexpress many osteoblast-associated genes, including Bsp, and deposit an abnormal resorption-stimulating bone matrix high in bone sialoprotein (BSP) content. In addition to its novel osteoblast phenotype, Gja1+/− is the only Cx43 mutant mouse model with a reported change in bone marrow adipogenesis, leading to progressive bone marrow atrophy beginning at 17 wk of age (Flenniken et al., 2005).

We now report that the G60S Cx43 mutation increases the expression level of osteoblast-specific markers in the osteoblasts by up-regulation of BMP2/4 production and signaling and that the increased BMP production by activated osteoblasts and/or stromal cells may also up-regulate Pparg2 (for peroxisome proliferator-activated receptor γ [Pparg2]) expression, leading to increased bone marrow adipogenesis.

RESULTS

The G60S Cx43 mutation concomitantly activates the osteoblast lineage and increases bone marrow adipogenesis in early-onset osteopenic Gja1+/− mice

As we previously reported, Gja1+/− mice, which carry a G60S Cx43 mutation resulting in reduced gap junction formation (Figure 1A) and function, exhibited early-onset osteopenia and changes in the structure and biomechanical properties of bone (Zappitelli et al., 2013). The osteopenic phenotype results from activation of osteoclasts secondary to activation of the osteoblast lineage both in trabecular bone in vivo and bone marrow stromal cultures. We confirmed here that activation of Gja1+/− osteoblastic cells resulted in increased bone nodule formation, increased osteoblast marker expression (with Bsp being the most highly and significantly up-regulated), and production of an abnormal bone matrix high in BSP content (Figure 1, B and C), which we previously showed stimulated resorption (Zappitelli et al., 2013).

As early as 7 wk of age, Gja1+/− mice exhibited increased bone marrow atrophy versus wild-type (WT) mice (Figure 2A; Flenniken et al., 2005). Histomorphometry confirmed that whereas adipocyte size (adipocyte volume/adipocyte number) and percentage marrow fat (adipocyte volume/marrow volume) were not significantly different between genotypes, adipocyte density (adipocyte number/tissue volume, mm−2) was significantly increased at 2 and 4 mo of age in Gja1+/− versus WT bone marrow (Figure 2B). Expression of all adipocyte markers tested, including Pparg2, the master adipogenic transcription factor, and downstream adipogenic markers aP2, LPL, and Adipsin, were significantly increased in Gja1+/− versus WT bone marrow (Figure 2C).

The increase in adipogenesis in Gja1+/− mice was restricted to the bone marrow, as evidenced by the fact that neither body mass composition (Supplemental Figure S1A) nor expression of adipocyte markers in the epididymal fat pads (Supplemental Figure S1B) was significantly different between Gja1+/− and WT mice at any age tested. Of note, Gja1+/− stromal cells cultured under adipogenic conditions displayed significantly increased expression of adipocyte markers at day 1 compared with WT cells but not thereafter (Figure 3); consistent with this, no difference was seen in oil red O staining at endpoint between genotypes (Supplemental Figure S2).

The BMP2/4 and Wnt/β-catenin signaling pathways are up-regulated in Gja1+/− trabecular bone and osteogenic stromal cell cultures, but only BMP2/4 is responsible for the increase in osteoblast-specific gene expression

We next sought to identify which signaling pathway(s) downstream of Cx43 were altered by the G60S Cx43 mutation and might account for the increased expression of markers in the Gja1+/− osteoblasts. Using a Pathway Finder QPCR array and RNA isolated from Gja1+/− and WT trabecular bone samples, we identified and selected two candidate pathways for further analysis, the BMP2/BMP4 pathway and the Wnt/β-catenin pathway (based on expression differences of 1.5-fold or greater between genotypes; Table 1). Given the reported ability of β-catenin to interact physically with Cx43 (Ai et al., 2000), we first examined the Wnt/β-catenin signaling pathway. Whereas total β-catenin protein was significantly increased in Gja1+/− versus WT stromal cells, the level of transcriptionally active β-catenin was not (Supplemental Figure S3A). When expression of Axin2 and naked cuticle 1 (Nkd1), two direct targets of β-catenin signaling, was assessed via quantitative PCR, we found that Axin2 was unaffected, but expression of Nkd1 was significantly up-regulated in Gja1+/− versus WT stromal cells (Supplemental Figure S3B). The inconsistent changes in Wnt/β-catenin target genes suggested that this pathway was not involved in the increased marker expression by Gja1+/− osteoblasts. This was confirmed by treating WT and Gja1+/− stromal cells with a Wnt signaling inhibitor, IWP-2. Treatment with 1 μM IWP-2 significantly reduced Wnt/β-catenin signaling in both WT and Gja1+/− stromal cells, as evidenced by down-regulation of expression of Axin2 (Supplemental Figure S3C);
increased in the bone marrow but not elsewhere in the body.

As summarized so far, adipocyte density and volume were increased in adipogenic stromal cultures and CFU-O were higher in the presence of conditioned medium from Jrt/+ osteogenic stromal cells, and Bmp2 expression remained higher in Jrt/+ than in WT stromal cells, regardless of the IWP-2 concentration used (Supplemental Figure S3D).

Therefore we next examined the BMP2/4 signaling pathway and confirmed that expression of Bmp2, Bmp4, and Tcf7 for transcription factor 7 (Tcf7) was significantly increased in RNA isolated from both Jrt/+ and WT stromal cell cultures (Figure 4A) and osteogenic stromal cell cultures (Figure 4B) versus WT specimens. BMP2/4 signaling, determined by immunoblotting for phosphorylated SMAD1/5/8 (pSMAD1/5/8) proteins, was also significantly increased in Jrt/+ versus WT stromal cell cultures. Levels of SMAD1 were unaffected between genotypes (Figure 4C).

To determine whether the up-regulated marker expression in Jrt/+ osteoblasts resulted directly from up-regulated BMP2/4 signaling, we treated osteogenic stromal cell cultures with Noggin, a BMP2/4-signaling inhibitor. The dose of Noggin required for either half (ID50) or maximal knockdown of both Bsp and Ocn (for osteocalcin [Ocn]) expression was higher in Jrt/+ than WT cells (e.g., ID50 = 12.5–25 ng/ml Noggin in WT cells vs. ID50 = 100–200 ng/ml Noggin in Jrt/+ cells; Figure 5A). The significant reduction in pSMAD1/5/8 levels confirmed that Noggin treatment knocked down BMP2/4 signaling in cells of both genotypes (Supplemental Figure S4).

**Up-regulated BMP2/4 production by Jrt/+ osteogenic stromal cell cultures increases adipocyte gene expression in adipogenic stromal cultures**

As summarized so far, adipocyte density and volume were increased in the bone marrow but not elsewhere in the body (e.g., fat pads, trunk) of Jrt/+ mice, suggesting that the increase was dependent on factors in the marrow microenvironment. The fact that increased expression of adipocyte-associated genes was seen only at early times, that is, day 1, but not thereafter in Jrt/+ versus WT stromal cell cultures also supported the possibility that cells other than adipocytes or their endogenous factors in the marrow microenvironment were responsible for the increased adipogenesis. Previously, we showed that mature endosteal osteoblasts are dislodged from bone surfaces when bone marrow is flushed from long bones, and they remain viable for only short periods of time in culture (Malaval et al., 1994). We therefore hypothesized that it is the hyperactive Jrt/+ osteoblasts that are responsible, potentially through their increased production of BMP2/4, for increased marrow adipogenesis in Jrt/+ mice. The increased Bmp2/4 expression and signaling in Jrt/+ versus WT osteogenic stromal cell cultures at confluence (Figure 4, B and C) supports this possibility. To test this hypothesis further, we cultured stromal cells under adipogenic conditions but further supplemented with addition of either WT or Jrt/+ osteogenic stromal cell–conditioned medium. The expression of Pparg2 was significantly increased when cells of either genotype were grown in the presence of Jrt/+ or Jrt/+ osteogenic stromal cell–conditioned medium. The expression of Pparg2 was significantly increased when cells of either genotype were grown in the presence of Jrt/+ conditioned medium versus those supplemented with WT conditioned medium (Figure SB). To determine whether this increase was due to increased BMP2/4 in the Jrt/+ conditioned medium, we next treated adipogenic stromal cells supplemented with Jrt/+ conditioned medium with Noggin for 48 h. In cultures supplemented with Jrt/+ conditioned medium, treatment with 6.25 ng/ml Noggin significantly reduced the expression of Pparg2 in both WT and Jrt/+ adipogenic cells (Figure SC).
Increased levels of cAMP contribute to the up-regulation of BMP2/4 signaling in Gja1<sup>−/+</sup> versus WT osteogenic stromal cell cultures

To establish the link between decreased G60S Cx43 channel function and the increased activity of Gja1<sup>−/+</sup> osteoblasts including increased BMP2/4 production, we next compared the intracellular concentrations of several molecules and ions known to be transported through Cx43 channels in WT and Gja1<sup>−/+</sup> stromal cells. Whereas intracellular levels of ATP and Ca<sup>2+</sup> were not different between genotypes, cAMP levels were significantly increased in Gja1<sup>−/+</sup> versus WT stromal cells cultured under osteogenic conditions (Figure 6A). To determine whether cAMP signaling was up-regulated, we quantified phosphorylation of cAMP-responsive element-binding protein (CREB), a cAMP-responsive transcription factor, given the relatively rapid decay of cAMP and low basal levels of pCREB/CREB, we performed the assay in the presence of 3-isobutyl-1-methylxanthine (IBMX), a nonselective phosphodiesterase inhibitor, which inhibits cAMP breakdown, thereby amplifying any differences in levels of cAMP and its targets between genotypes. Levels of pCREB/CREB increased significantly in Gja1<sup>−/+</sup> cells after 2 h of IBMX treatment, whereas no change in cAMP levels was detectable in WT cells; of note, pCREB/CREB levels were also significantly increased in Gja1<sup>−/+</sup> versus WT stromal cells at 2 h of IBMX treatment (Figure 6B). To determine whether increased cAMP signaling in Gja1<sup>−/+</sup> cells was the mechanism behind the increased BMP2/4 production, we next treated WT and Gja1<sup>−/+</sup> stromal cells with an inhibitor of cAMP signaling and assessed Bmp2 expression. Myristoylated cAMP-dependent protein kinase inhibitor (mPKI) knocks down cAMP signaling by interfering with the activation of protein kinase A (Ashby and Walsh, 1972, 1973). Treatment with mPKI had no significant effect on Bmp2 expression in WT stromal cells but knocked down Bmp2 expression in Gja1<sup>−/+</sup> cells to WT levels (Figure 6C).

DISCUSSION

G60S Cx43-containing cells exhibit significantly reduced levels of total CX43 protein, gap junction formation, and gap junction coupling between cells (Flenniken et al., 2005; McLachlan et al., 2008), which result in an early-onset osteopenic phenotype in Gja1<sup>−/+</sup> mice due to activation of osteoclast bone resorption by production of an abnormal resorption stimulating bone matrix, high in BSP, as well as changes in receptor activator of nuclear factor-κB ligand (RANKL)–osteoprotegerin (OPG) signaling, both of which arise from activation of osteoblast lineage cells (Zappitelli et al., 2013). We now report that the activation (i.e., up-regulated Bsp and Ocn expression) of the osteoblast lineage results from increased BMP2/4...
production and signaling. Increased BMP2/4 also increases Pparg2 expression to promote bone marrow adipogenesis in Gja1/Jrt/+ versus WT mice. Our data also suggest that the increased production of BMP2/4 is due to reduced gap junction intercellular communication and consequent build-up of intracellular cAMP and its downstream signaling in Gja1/Jrt/+ osteogenic stromal cells.

**Increased BMP2/4 signaling results in up-regulated osteoblast-specific marker expression in Gja1/Jrt/+ osteoblasts**

Gja1/Jrt/+ mice, like other Cx43 mutant mice exhibiting reduced gap junction formation and function, are osteopenic, but the osteopenia results not from decreased osteogenesis and osteoblast activity, but from osteoblast hyperactivity, which activates osteoclasts (Zappitelli et al., 2013). We show here that BMP2/4 production and signaling are significantly increased in Gja1/Jrt/+ versus WT trabecular bone and/or osteogenic stromal cells both in vivo and in vitro. The knockdown of expression of up-regulated genes such as Bsp and Ocn in Gja1/Jrt/+ stromal cell cultures to WT levels by Noggin treatment confirmed that the up-regulation of gene expression in Gja1/Jrt/+ osteoblasts is at least partly, if not entirely, due to up-regulated levels of BMP2/4. Whether the increase in BMP2/4 production is directly responsible for all of the up-regulated osteoblast activities (i.e., not only increased osteoblast-specific gene expression, but also production of an abnormal bone matrix due to increased BSP incorporation and changes in RANKL-OPG production), which result in activation of osteoclasts and therefore the early-onset osteopenic phenotype of Gja1/Jrt/+ mice, remains to be determined, for example, by knocking down BMP2/4 signaling in Gja1/Jrt/+ mice. We also cannot exclude the possibility that other intrinsic defects or disrupted signaling molecules or pathways may be responsible for or contribute to the activation of the Gja1/Jrt/+ osteoblasts.

To date, no other studies have attributed the osteopenic bone phenotypes of Cx43 mutants to increased BMP2/4 signaling, although osteoblast hyperactivity has also not been reported in other mouse models with decreased Cx43 gap junction formation and function. However, Gja1/Jrt/+ mice also exhibit all the classical features of oculodentodigital dysplasia (ODDD), including syndactyly (Flenniken et al., 2005). Alterations in BMP2 levels have been reported to underlie common phenotypic features of ODDD, although in contrast to the activation we report in Gja1/Jrt/+ cells, others have shown that disruption of Cx43 gap junction function causes a reduction in BMP2 signaling. For instance, Dobrowolski et al. (2009) showed that the syndactyly phenotype of the Cx43+/G133R and Cx43−/− mice arises from decreased interdigital apoptosis due to decreased SHH and BMP2, along with subsequent increase in FGF signaling due to increased FGF4 and FGF8. Kim et al. (2005) showed that disruption of Cx43 by antisense-oligonucleotides caused
by hemichannels may play a role in the up-regulation of BMP production and the hyperactive phenotype of the Gja1αβ+/+ osteoblasts.

Whereas the increase in BMP2/4 signaling is directly responsible for the increased expression of osteoblast-specific markers in hyperactive Gja1αβ+/+ osteoblasts, our data for the Wnt/β-catenin inhibitor IWP-2 indicate that the up-regulation of Wnt/β-catenin signaling is not. However, the inconsistent and variable changes in the Wnt/β-catenin pathway that we report in the Gja1αβ+/+ mice is consistent with recently published data by Bivi et al. (2013), who reported increased expression of total β-catenin protein and some Wnt target genes (e.g., Axin2) when Cx43 is disrupted in osteocytes (in bones of DMP1Cre;Cx43fl/fl mice and in the MLO-Y4 osteocytic cell line). At the same time, however, the expression of other Wnt/β-catenin target genes (e.g., cyclin D1 and Smad6) and Wnt-mediated transcription (assessed via a TCF/LEF1-luciferase reporter assay) were unaffected in Cx43-silenced MLO-Y4 cells (Bivi et al., 2013). These results, along with the data we present here, suggest that the accumulation of β-catenin protein in Cx43-deficient cells may not lead to increased Wnt/β-catenin-mediated transcription; instead, elevated levels of total β-catenin in Cx43 mutant mice may be involved in other cellular processes, such as mediating the responsiveness of cells to mechanical stimulation (i.e., enhancing the responsiveness of “primed” Cx43-deficient cells to mechanical stimulation) or to other signals/factors, independently of classical Wnt/β-catenin transcription. As such, it will be interesting to determine whether sensitivity to mechanical loading or unloading in the Gja1αβ+/+ mice is altered as a result of the increased β-catenin protein levels.

### Increased bone marrow adipogenesis in Gja1αβ+/+ mice

Whether the Gja1αβ+/+ mouse bone marrow adipocyte phenotype is a direct consequence of altered Cx43 function in adipogenic cells and/or is indirect through other cell types is not yet clear. Adipogenesis and expression of adipocyte markers are increased in Gja1αβ+/+ bone marrow but not at other anatomical sites in Gja1αβ+/+ mice. Similarly, several adipocyte markers and BMP2 were increased at very early times in cultures of Gja1αβ+/+ versus WT stromal cells but not later, and no increase in adipogenesis was detectable in these cultures. We therefore hypothesized that the up-regulated production of BMP2 and BMP4 in Gja1αβ+/+ trabecular bone and osteogenic stromal cell cultures is responsible for the increased marrow adipogenesis in vivo and in stromal cultures, respectively. The fact that Noggin abrogated the increased expression of Pparg2 in adipogenic stromal cells treated with Gja1αβ+/+ osteogenic stromal cell-conditioned medium is consistent with such a hypothesis. Similarly, no other Cx43 mutant mouse models have been described to have an adipocyte phenotype or an increase in bone marrow adipogenesis, either because the marrow has not been screened in other models or, more likely, reflecting the unique activated osteoblast phenotype seen in the Gja1αβ+/+ model with its associated up-regulated BMP production. Thus no increase in adipogenesis was described in the global G138R mutant, Cx43+/-G138R, or the conditional deletion and conditional G138R mutant, DM1Cre;Cx43fl/fl and DM1Cre;Cx43fl>Gja1αβ+/fl mice, whose osteoblasts are dysfunctional but not hyperactive as in the Gja1αβ+/+ mice (Dobrowolski et al., 2008; Watkins et al., 2011).

It is also worth noting that early in adipogenesis in vitro, Cx43 is highly phosphorylated and localized in the plasma membrane of 3T3-L1 cells (Azarnia and Russell, 1985) and H-1/A stromal cells (Umezawa and Hata, 1992), and functional gap junctions are required in these early stages (Yanagiya et al., 2007). However, as adipocytes differentiate into mature adipocytes, the level of

### Table 1: Results of the Mouse Signal Transduction Pathway Finder RT2 Profiler PCR Array.

| Gene symbol | Gene name                        | Expression, fold change vs. WT |
|-------------|----------------------------------|-------------------------------|
| Bmp2        | Bone morphogenetic protein 2     | 2.33                          |
| Bmp4        | Bone morphogenetic protein 4     | 3.00                          |
| Ccl2        | Chemokine (C-C motif) ligand 2    | −1.56                         |
| Cdkn2a      | Cyclin-dependent kinase inhibitor 2A | −1.58                        |
| Cxcl1       | Chemokine (C-X-C motif) ligand 1  | −2.53                         |
| Egr1        | Early growth response 1           | 1.79                          |
| Fn1         | Fibronectin 1                     | −2.02                         |
| Hhip        | Hedgehog-interacting protein      | 2.79                          |
| Il1a        | Interleukin 1α                    | 1.65                          |
| Il4ra       | Interleukin 4 receptor α          | −1.75                         |
| Mmp10       | Matrix metallopeptidase 10        | −1.91                         |
| Pparg       | Peroxisome proliferator-activated receptor γ | 2.53                      |
| Selp        | Selectin, platelet                | −3.82                         |
| Tcf7        | Transcription factor 7, T-cell specific | 3.21                      |
| Tfrc        | Transferrin receptor              | 1.69                          |
| Pme-pa1     | Prostate transmembrane protein, androgen induced 1 | −1.56 |
| Vcam1       | Vascular cell adhesion molecule 1 | 1.54                          |
| Vegfa       | Vascular endothelial growth factor A | 1.52                      |
| Wisp1       | WNT1-inducible signaling pathway protein 1 | 1.89                      |

Genes of interest were identified as those whose expression was changed 1.5-fold or greater in Gja1αβ+/+ vs. WT samples. RNA was isolated from trabecular bone of 2-mo-old WT and Gja1αβ+/+ mice; n = 2, and each sample was the combination of n ≥ 2 independent biological samples.
Cx43 protein declines via proteasomal degradation, an effect reported to be essential for the development of mature adipocytes (Yeganeh et al., 2012). In Gja1<sup>+/−/+</sup> mice, however, the reduction in Cx43 expression and gap junction function appears to have no effect on adipogenesis in epididymal fat pads or elsewhere, except in the bone marrow, where our data suggest that the adipogenic consequences of Cx43 deficiency are indirect and via osteoblasts and possibly other BMP2/4-producing cells. The data suggest that only a low level of gap junctional communication is necessary for the early differentiation stages of adipogenesis, levels commensurate with the low residual gap junction communication seen in Gja1<sup>+/−/+</sup> cells, or that other pathways, such as an enhanced BMP2/4 pathway, can compensate for low Cx43. However, we cannot entirely rule out a direct action of the G60S Cx43 mutation on bone marrow adipocyte precursors, which may behave phenotypically differently than adipocytes in other tissues. Future experiments will be aimed at assessing the direct effects of the germ line G60S Cx43 mutation in bone marrow–derived adipocyte precursors and testing more rigorously the link between increased BMP2/4 production and increased bone marrow adipogenesis in Gja1<sup>+/−/+</sup> mice.

Increased intracellular cAMP in G60S Cx43 osteogenic stromal cells plays a role in increased BMP2/4 production

To uncover a link between the G60S Cx43 mutant channels and the up-regulation of BMP2/4 production by Gja1<sup>+/−/+</sup> cells, we investigated intracellular levels of several signaling molecules and ions known to be released via Cx43 channels. Although our results revealed only a small increase in levels of intracellular cAMP, a second messenger signaling molecule known to pass through Cx43 gap junctions (Lawrence et al., 1978), such small changes can have profound impacts on cell activities. Consistent with this, treatment with the cAMP-dependent protein kinase inhibitor mPKI reduced the elevated levels of Bmp2 expression in Gja1<sup>+/−/+</sup> cells to WT levels, suggesting that increased cAMP signaling was at least partly responsible for the up-regulation of Bmp2 expression. We cannot exclude the possibility that the transport of other small second messenger molecules, which we did not test, were also affected by the G60S Cx43 mutation and involved in the up-regulation of BMP2/4 production and/or the expression of downstream osteoblast markers in Gja1<sup>+/−/+</sup> cells. Further analysis of the G60S Cx43 mutation on hemichannel and gap junction channel conductance, permeability, pore size, and specificity are underway.

Of importance, we and others have found no evidence of changes in or compensation by Cx45 (gene or protein expression levels; unpublished data) in Cx43-mutant ODDD mouse models to account for alteration in levels of BMP2/4 molecules (i.e., up-regulated BMP2/4 levels in Gja1<sup>+/−/+</sup> mice or down-regulated BMP2 levels in Cx43<sup>+/−</sup>/Cx138R mice; Dobrowolski et al., 2008). This likely reflects the inability of connexin family members to adequately compensate for one another, since the channels have different functions.

**FIGURE 4:** BMP2/4 signaling is increased in Gja1<sup>+/−/+</sup> in vivo and in vitro. Expression of Bmp2, Bmp4, and Tcf7 was increased in Gja1<sup>+/−/+</sup> vs. WT (A) trabecular bone at 4 mo of age (n ≥ 8) and in (B) osteogenic stromal cultures at confluence (n ≥ 3). (C) Levels of pSMAD1/5/8 were significantly increased in Gja1<sup>+/−/+</sup> vs. WT confluent osteogenic stromal cultures. Levels of SMAD1 were unchanged. One representative blot is shown; n ≥ 4. *p < 0.1, **p < 0.05, ***p < 0.01.
molecular and ionic permeabilities (Steinberg et al., 1994; Veenstra et al., 1994; Koval et al., 1995), serving different functions as cells differentiate (Lecanda et al., 2000). Alternatively, however, elevated levels of Cx43 protein have been proposed to partially compensate for loss of Cx43 in Cx43−/− neonatal calvaria osteoblasts (Lecanda et al., 2000); it is possible that compensatory mechanisms by other connexins can be influenced on the basis of whether Cx43 is entirely deleted, as in the knockout, or simply mutated, as in the G60S or G138R mutants.

In summary, we report the novel findings that up-regulation of BMP2/4 signaling in trabecular bone and/or stromal cells increases osteoblast-specific marker expression in hyperactive Gja1−/−+ osteoblasts and may also increase bone marrow adipogenesis by up-regulation of Pparg2 in the Cx43-deficient Gja1−/−+ mouse model. We also report that increased cAMP signaling may promote the up-regulated production of BMP2 and BMP4 signaling molecules by Gja1−/−+ cells.

**FIGURE 5:** Up-regulated BMP2/4 signaling is responsible for the increased osteoblast marker expression and the increased Pparg2 expression in bone marrow–derived adipocytes and adipogenic precursors in Gja1−/−+ vs. WT mice. (A) The dose of Noggin required for either half (ID₅₀) or maximal knockdown of both Bsp and Ocn expression was higher in Gja1−/−+ vs. WT osteogenic stromal cells. One representative experiment is shown, and samples were run in triplicate; n = 3. (B) Both WT and Gja1−/−+ adipogenic stromal cells grown in the presence of Gja1−/−+ conditioned medium expressed higher levels of Pparg2 vs. those grown with the addition of WT conditioned medium; n ≥ 3. Solid and dashed lines indicate significant differences between cells cultured in either conditioned medium situation in WT and Gja1−/−+ cells, respectively. (C) Expression of Pparg2 declined significantly when cells of either genotype (cultured under adipogenic conditions and with the addition of Gja1−/−+ conditioned medium) were treated with Noggin; n = 5. Asterisks indicate significance between genotypes at that dosage concentration; *p < 0.05, **p < 0.01, and ***p < 0.001. Capital letters indicate significance between WT samples; lowercase letters indicate significance between Gja1−/−+ samples; letters are assigned in alphabetical order according to the dosages (e.g., significant difference vs. dose 0 is denoted A in WT and a in Gja1−/−+). p < 0.05 was considered statistically significant.

**MATERIALS AND METHODS**

**Animals studies**

Gja1−/−+ mice were generated as previously described (Zappitelli et al., 2013). The studies reported here were done on male Gja1−/−+ and WT littermates between 2 and 4 mo of age. All experimental procedures were performed in accordance with protocols approved by the Canadian Council on Animal Care and the University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee.

**Body composition**

Dual energy x-ray absorptiometry (PIXImus; Lunar, Madison, WI) was used to measure body composition (percentage fat, lean tissue mass) on the whole body (excluding the head).

**Immunocytochemistry**

Mouse embryonic fibroblasts cultured on glass coverslips were permeabilized in 1% Triton X-100 in phosphate-buffered saline.
inactivated FBS and antibiotics (100 μg/ml penicillin, 1 μg/ml streptomycin, 50 μg/ml gentamicin, 250 ng/ml Fungizone; standard medium) at 1 × 10^6 nucleated cells/35-mm dish. 

**Osteogenic assay.** After 3 d, the medium was changed to differentiation medium (standard medium supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate). 

**Adipogenic assay.** After 3 d, the medium was changed to differentiation medium (standard medium with 50 μg/ml ascorbic acid and 10^−5 M thiazolidinedione [BRL 49653]).

**Conditioned medium.** Conditioned medium was collected from osteogenic stromal cell cultures at confluence after 24–48 h of conditioning. The conditioned medium was then used at 50:50 with fresh adipogenic medium. 

**Inhibitor studies in stromal cells**

Stromal cells were cultured in standard medium with 50 μg/ml ascorbic acid until day 6 (matrix-forming time point). Cells were then treated for 24 h with 0.1 or 1 μM IWP-2 (I0536; Sigma-Aldrich, St. Louis, MO) in dimethyl sulfoxide (DMSO) or vehicle (DMSO) alone. RNA was isolated as described later. 

**Histochemistry**

The right femur fixed in 4% paraformaldehyde was embedded in a mixture of methyl methacrylate and glycolmethacrylate, and 5-μm sections were stained with hematoxylin and eosin (Carson, 1990). Images were captured and analyzed using a Bioquant Osteomager and Bioquant Osteo 2012 (Bioquant Image Analysis, Nashville, TN).

**Isolation of bone marrow cells**

Bone marrow cells were isolated from resected tibia and femora using a modification of a previously published method (Scutt et al., 2004). Cells were plated in α-MEM supplemented with 10% heat-inactivated FBS and antibiotics (100 μg/ml penicillin, 1 μg/ml streptomycin, 50 μg/ml gentamicin, 250 ng/ml Fungizone; standard medium) at 1 × 10^6 nucleated cells/35-mm dish.

**FIGURE 6:** Intracellular levels of cAMP and cAMP signaling are increased in Gja1^+/− vs. WT osteogenic stromal cells. (A) Intracellular levels of cAMP were significantly increased in confluent Gja1^+/− vs. WT osteogenic stromal cells. Intracellular levels of ATP and Ca^{2+} were unchanged; n ≥ 6. Levels of the signaling molecules and ions were normalized to WT levels. (B) Basal levels of pCREB/CREB were unaffected in osteogenic stromal cells isolated from 4-mo- old mice. However, treatment of cells with 1 mM IBMX for at least 2 h resulted in increased levels of pCREB/CREB in Gja1^+/− vs. WT cells. Two representative blots; n = 3. (C) Bmp2 expression decreased when Gja1^+/− osteogenic stromal cells were treated with 20 μM mPKI, a cAMP signaling inhibitor, for 15 min. Treatment of WT cells with mPKI had no effect on Bmp2 expression. One representative experiment; n ≥ 4. Solid and dashed lines indicate significant differences over time in WT and Gja1^+/− mice, respectively. *p < 0.05, **p < 0.01.
Noggin

Osteogenic cells. Stromal cells were cultured in standard osteogenic differentiation medium with vehicle (20 μg/ml acetic acid in 0.1% BSA-PBS) or 25, 50, 100, 200, or 500 ng/ml Noggin (ab50156; Abcam, Cambridge, MA). Cells were then treated with vehicle-treated wells contained mineralized nodules, and RNA was then isolated as described later.

Adipogenic cells. Stromal cells were cultured in standard adipogenic differentiation medium for 48 h and then treated with vehicle (20 μg/ml acetic acid in 0.1% BSA-PBS) or 25, 50, or 200 ng/ml Noggin for 48 h. RNA was isolated as described later.

3-Isobutyl-1-methylxanthine

Stromal cells were cultured in standard medium with 50 μg/ml ascorbic acid to confluence, serum starved (κ-MEM with 0.5% heat-inactivated FBS and antibiotics) overnight, and treated with 1 μM IBMX (I5879; Sigma-Aldrich) for 40 min, 2 h or 4 h. RNA and protein were isolated as described later.

mPKI (14–22)

Osteogenic cells. Stromal cells were cultured in standard medium with 50 μg/ml ascorbic acid until confluence. Cells were then treated with vehicle (water) or 5, 10, or 20 μM mPKI (PHZ1202; Life Technologies, Carlsbad, CA) for 24 h, and RNA was collected.

Adipogenic cells. Stromal cells were cultured in standard adipogenic differentiation medium for 48 h and then treated with vehicle (water) or 5, 10, or 20 μM mPKI for 24 h, and RNA was collected.

Quantitative reverse transcription PCR

Total RNA was isolated from bone, bone marrow, and cell cultures using TRIzol (Sigma-Aldrich) and reverse transcribed using SuperScript II (Invitrogen) and random hexamers. cDNA was combined with 0.5 μM each of the forward and reverse primers (Supplemental Table 2) and qPCR Green Supermix and run in the MyiQ Real-Time PCR system (Bio-Rad, Hercules, CA). Raw data were analyzed with PCR Miner (Zhao and Fernald, 2005) and normalized using the internal control transcript for ribosomal protein L32.

SA Biosciences Mouse Signal Transduction PathwayFinder PCR Array

RNA was isolated from trabecular bone samples. Sample preparation and RNA isolation were performed using TRIzol (Sigma-Aldrich) and SA Biosciences qPCR-Grade RNA isolation kit (Qiagen, Venlo, Netherlands) following manufacturer’s instructions. The Mouse Signal Transduction Pathway Finder RT² Profiler PCR Array (Qiagen) was used following the manufacturer’s instructions.

Protein isolation from bone and stromal culture and Western blotting

Long bones cleaned of surrounding tissue, epiphysis, and bone marrow were cut slightly below the growth plate to separate trabecular bone and washed in PBS. Stromal culture plates were washed with PBS. Proteins were extracted in cell lysis buffer as previously described (Thomas et al., 2004). Protein extracts (30 μg) underwent immunoblotting with antibodies of interest (Supplemental Table S2); actin was used as a loading control. Western blots were developed using chemiluminescence, imaged with Bio-Rad ChemiDoc-XRS+, and analyzed using Image Lab software (Bio-Rad).

Statistical analysis

Results are presented as mean ± SD. Experiments were repeated at least three times with independent biological samples. Statistical analysis was performed using Prism 4.0 software (GraphPad, La Jolla, CA). One-way analysis of variance was used to determine longitudinal significance in dosage experiments. Unpaired t test was used for direct comparisons between mutant and WT parameters; paired t test was used for comparisons within genotypes (e.g., changes over treatment time); n values presented are independent biological samples.

ACKNOWLEDGMENTS

We thank members of the Centre for Modeling Human Disease (www.cmrd.ca), particularly Celeste Owen, for their support; Ralph Zirngibl and Marco Cardelli from the Aubin lab for support and helpful discussions; the Center for Bone and Periodontal Research (www.bone.mcgill.ca) and Feryal Sarraf from the Faculty of Dentistry for expert technical assistance; and Liliana Attisano and Jane Mitchell for providing reagents and discussion. This work was supported by a Canadian Institutes of Health Research operating grant (FRN 69198 to J.E.A.), as well as scholarship support from the government of Ontario through the Ontario Graduate Scholarship (T.Z.), the Queen Elizabeth II-GSTT (T.Z.), and the Department of Medical Biophysics, University of Toronto (T.Z.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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