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Original Research Article

Regulation of CXCL12 expression by canonical Wnt signaling in bone marrow stromal cells

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ABSTRACT

CXCL12 (stromal cell-derived factor-1, SDF-1), produced by stromal and endothelial cells including cells of the bone marrow, binds to its receptor CXCR4 and this axis regulates hematopoietic cell trafficking. Recently, osteoclast precursor cells were found to express CXCR4 and a potential role for the CXCL12-CXCR4 axis during osteoclast precursor cell recruitment/retention and development was proposed as a regulator of bone resorption. We examined the role of canonical Wnt signaling in regulating the expression of CXCL12 in bone marrow stromal cells. In mouse stromal ST2 cells, CXCL12 mRNA was expressed, while its expression was reduced in Wnt3a over-expressing ST2 (Wnt3a-ST2) cells or by treatment with lithium chloride (LiCl). Wnt3a decreased CXCL12 levels in culture supernatants from mouse bone marrow stromal cells. The culture supernatant from Wnt3a-ST2 cells also reduced migratory activity of bone marrow-derived cells in a transwell migration assay. Silencing of glycogen synthase kinase-3 β decreased CXCL12 expression, suggesting that the canonical Wnt signaling pathway regulates CXCL12 expression. In a transfection assay, LiCl down-regulated the activity of a reporter gene, a 1.8 kb

fragment of the 5'-flanking region of the *CXCL12* gene. These results show that canonical Wnt signaling regulates *CXCL12* gene expression at the transcriptional level, and this is the first study linking chemokine expression to canonical Wnt signaling.

Introduction

The Wnt secreted proteins of molecular weight ~40kd are a family of glycosylated-lipid-modified proteins which are powerful regulators of embryonic development, cell differentiation, and proliferation (Logan and Nusse, 2004, Sethi and Vidal-Puig, 2010). Two distinct Wnt signaling pathways, the β -catenin-dependent canonical pathway and the β -catenin-independent so-called “noncanonical” pathway, including the Wnt/planar cell polarity pathway and the Wnt/ Ca^{2+} pathway, have been described. Two types of Wnt proteins have also been identified, one class of which comprises the canonical Wnts such as Wnt1 and Wnt3a. The other class is the “noncanonical” Wnts such as Wnt5a and Wnt11 which act independently of or inhibit β -catenin signaling (Logan and Nusse, 2004). According to the model of canonical Wnt action, in cells lacking a Wnt signal, glycogen synthase kinase (GSK) -3 β phosphorylates β -catenin, inducing rapid degradation of β -catenin via the ubiquitin-proteasome pathway (MacDonald et al., 2009, Sethi and Vidal-Puig, 2010). Canonical Wnt signaling causes stabilization of β -catenin which then translocates to the nucleus, where it interacts with the transcription factors that regulate expression of several target genes including *c-myc* and osteoprotegerin (*OPG*) (MacDonald et al., 2009,

Sato et al., 2009).

The Wnt signaling pathway has also been reported to be involved in regulation of bone formation (Krishnan et al., 2006, Liu et al., 2008, Tamura et al., 2010). Loss of function of β -catenin in osteoblasts leads to low bone mass caused by increased numbers of osteoclasts resulting in increased bone resorption, indicating enhanced osteoclastogenesis from hematopoietic progenitor cells (Glass et al., 2005). Previously, we have shown that canonical Wnt signaling induces *OPG* expression and analysis of the murine *OPG* gene promoter revealed that constitutively active forms of β -catenin regulate transcription of *OPG* via a promoter region containing two responsive sites (Sato et al., 2009). In addition, canonical Wnt signaling reduces receptor activator of NF κ B ligand (*RANKL*) expression (Spencer et al., 2006, Sato et al., 2009). These observations suggest that canonical Wnt signaling regulates bone turnover via osteoclast formation from hematopoietic progenitor cells.

The Wnt signaling pathway plays a role in the regulation of hematopoiesis in bone marrow. Activation of canonical Wnt signaling enhances hematopoietic stem cell (HSC) self-renewal (Reya and Clevers, 2005, Staal and

Luis, 2010). In contrast, mice with conditional inactivation of β -catenin show normal hematopoietic cell development and repopulating activity of HSC (Cobas et al., 2004, Staal and Luis, 2010). These controversial results indicate that the precise role of canonical Wnt signaling in bone marrow remains unclear. Recently Kim *et al.* (2009) reported that the population of canonical Wnt receptor molecules and β -catenin accumulation were predominantly enriched in the stromal rather than the hematopoietic compartment of bone marrow (Kim et al., 2009). Therefore, we investigated the role of canonical Wnt function in bone marrow stromal cells.

Hematopoiesis and mobilization of mature blood cells into the bloodstream are dependent on various types of chemokines present in the bone marrow. Chemokines are small molecular weight molecules that function as chemoattractants and serve as regulators of blood cell maturation, trafficking and homing (Schall and Bacon, 1994, Howard et al., 1996). CXCL12 (stromal derived factor: SDF-1 α) and SDF-1 β belong to the C-X-C chemokine family and were originally isolated from a murine bone marrow stromal cell line (Broxmeyer, 2008). Both molecules are derived by alternative splicing of the *CXCL12* gene and share similar biological activities. CXCL12 is widely produced by stromal

and endothelial cells including bone marrow, skeletal muscle, liver, brain and heart, and CXCL12 binds predominantly to a G-protein coupled transmembrane glycoprotein cell surface chemokine receptor, CXCR4, widely expressed by several types of tissue-committed stem cells including HSC or hematopoietic progenitor cells (Ratajczak et al., 2006). It has been reported that CXCL12-CXCR4 interactions mediate to maintain HSC niches on the endosteal surface of bone; the CXCL12-CXCR4 axis is involved in the homeostatic release of HSC from the bone marrow into the circulation, and also maintains the HSC pool in the bone marrow microenvironment, the so-called stem cell niche (Petit et al., 2002). In vitro, a variety of cytokines and signaling molecules such as interleukin-1 β , platelet-derived growth factor-BB, transforming growth factor (TGF) β 1 and tumor necrosis factor- α stimulate CXCL12 expression and protein secretion (Jung et al., 2006). However, the regulation of CXCL12 production by Wnt signaling remains poorly understood.

In this report, we demonstrate that canonical Wnt signaling regulates CXCL12 production via the *CXCL12* gene promoter in bone marrow stromal cells.

Materials and Methods

Cell cultures

Cells of the mouse stromal cell line ST2 (RIKEN Cell Bank, Tsukuba, Japan) were cultured in α -MEM containing 100 μ g/ml of kanamycin (Meiji, Tokyo, Japan) and supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Inc. Lenexa, KS) at 37°C in 100-mm cell culture dishes (Corning, Corning, NY) in a humidified atmosphere of 5% CO₂ in air. For osteoblastic differentiation of ST2 cells, the cells were cultured in α -MEM containing both 5 mM β -glycerophosphate and 100 μ g/ml of ascorbic acid for 2 weeks. During culture, the medium was replaced every 3 days.

Bone marrow stromal cell (BMSC) cultures

Isolation of mouse bone marrow stromal cells (BMSCs) was as described previously (Matsumoto et al., 1995). Briefly, marrow was flushed from the bones with Dulbecco's modified Eagle's medium containing 20% FBS, 100 μ g/ml of kanamycin, and 10⁻⁸ M dexamethasone (Sigma-Aldrich). After 7-10 days, the

cells reached confluence and were ready for use in experiments.

Expression plasmids

The plasmid constructs Wnt3a-pUSEamp and Wnt5a-pUSEamp were purchased from Upstate Biotechnology (Charlottesville, VA). The GSK-3 β hairpin siRNA expression plasmid, pU6-GSK-3 β HP1, was kindly provided by Dr. D.L. Turner (Univ. Michigan, Ann Arbor, MI) (Yu et al., 2003a). The sgRNA expression plasmids for silencing GSK-3 β genes (pRNA Tin-H1.2/Neo-sgGSKL) were constructed as described previously (Nakashima et al., 2007). pU6pro (Yu et al., 2003a) or pRNATin-H1.2/Neo-Luc (Nakashima et al., 2007) was used as a non-targeting control. The plasmid DNA was transfected into cells using the cationic lipid reagent Lipofectamine PLUS (Invitrogen, Carlsbad, CA) as described previously (Nakashima et al., 2005).

Generation of stable transfected cells and conditioned medium (CM)

ST2 cells were transfected with 1.0 μ g of Wnt3a-pUSEamp or Wnt5a-pUSEamp

as described previously (Nakashima et al., 2005). We denoted these cell lines Wnt3a-ST2 cells and Wnt5a-ST2 cells, respectively. The mouse Wnt3a-expressing cell line (L-Wnt3a cells) was cultured as described previously (Nemoto et al., 2009). Conditioned medium (CM) was collected from each cell line and replaced with 8 ml fresh medium; cells were then cultured for a further 3 days.

Reverse transcription-polymerase chain reaction (RT-PCR)

PCR was used to analyze the transcript levels of CXCL12, RANKL, macrophage colony stimulating factor (M-CSF), alkaline phosphatase (ALP), Wnt3a and Wnt5a. Total RNA was extracted from the cells at the indicated time points using Isogen (Nippongene, Toyama, Japan) and treated with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA. RT-PCR was performed as previously described (Nakashima et al., 2005). The primer sequences for each gene were as follows: CXCL12, 5'- AGAGTCCGAGGAACGCTG C -3' (forward), 5'- CCCTGGCACTGAACTGGA-3' (reverse); RANKL, 5'- GTCACCTCTGTCCTCTTGGTAC-3' (forward), 5'- TGAAACCCCAAAGTACGTCG

-3' (reverse); M-CSF, 5'- TTGCCAAGGAGGTGTCAGAA-3' (forward),
5'-TATTGGAGAGTTCCTGGAGC -3' (reverse) ; Wnt3a, 5'-
ATTGAATTTGGAGGAATGGT -3' (forward), 5'- CTT
GAAGTACGTGTAACGTG-3' (reverse) ; ALP, 5'-
ATTGCCCTGAAACTCCAAAACC-3' (forward), 5'-
CCTCTGGTGGCATCTCGTTATC-3' (reverse) ; Wnt5a, 5'-
TCCTAGAGAGCGCACGCAT -3' (forward), 5'- CAGCTTGCCCCGGCTGTTGA
-3' (reverse) ; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-
TCCACCACCCTGTTGCTGTA -3' (forward), 5'-
ACCACAGTCCATGCCATCAC-3' (reverse). To account for any difference in the
amount of RNA, *GAPDH* was chosen as our endogenous control and amplified
using the primers described above. The amplification products were
electrophoresed on 2% agarose gels.

Quantification of gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) was performed using assay-on-demand

TaqMan probes (Applied Biosystems, Foster city, CA) and StepOne[®] real time PCR system. The relative level of gene expression was quantified using the comparative C_T method with *GAPDH* as the endogenous control.

Quantification of CXCL12 by enzyme-linked immunosorbent assay (ELISA)

The level of CXCL12 protein was quantified using the mouse CXCL12 Immunoassay (Quantikine[®] HS; R&D Systems, Minneapolis, MN).

Western blot analysis

Whole-cell extracts, dissolved in a-buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM dithiothreitol), were separated by SDS/ 10% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with anti-GSK-3 β antibodies (BD biosciences, Palo Alto, CA) using the ECL Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

In vitro cell migration assay

Mouse bone marrow cells (1.5×10^5 in 100 μ l) were loaded into Transwell chambers (5- μ m pore filter Transwell, 24-well cell clusters; Corning, Corning, NY). Chambers were then placed into separate wells containing 600 μ l of medium, either CM from ST2 cells, Wnt3a-ST2 cells or Wnt5a-ST2 cells, or control medium with or without 80 ng/ml of CXCL12 (R&D Systems). After overnight incubation at 37°C in 5% CO₂ in air, the upper chambers were removed and cells on the bottom surface of the filter were fixed with 100% methanol and stained with 1% toluidine blue. Cells were counted under a microscope at X40 magnification.

Osteoclast cultures

Murine bone marrow macrophages were obtained from the Primary Cell Co., Ltd. (Sapporo, Japan). Cells (2×10^5 in 100 μ l) were incubated in 48-well culture plates in 100 μ l of DMEM with 50 ng/ml of M-CSF and 25 ng/ml of RANKL. In

order to investigate the effect on osteoclast formation, 50 or 100 μ l of CM from Wnt3a-ST2 cells was added to the wells. The same amount of ST2 CM was added to separate wells as a control. After being cultured for 5 days, cells were fixed in 4% paraformaldehyde, and then tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts) activity was evaluated histochemically using the Sigma-Aldrich kit according to the manufacturer's instructions. Numbers of TRAP-positive osteoclasts were counted. Each experiment was performed in triplicate.

Reporter constructs and assay for luciferase activity

Topflash is a Tcf reporter plasmid containing three copies of the Lef1/Tcf binding site (wild type) upstream of the thymidine kinase minimum promoter and luciferase open reading frame (Upstate Biotechnology, Lake Placid, NY). Luciferase reporter plasmids for the *CXCL12* promoter were generated as follows. The 1884-bp *CXCL12* promoter fragment (-1834 to +50) was isolated from mouse genomic DNA by PCR and subcloned into the pGL4.12 vector (Promega) to generate the luciferase reporter plasmid (pCXCL12-1834Luc). The

deletion reporter constructs (pCXCL12-420Luc) (-420 to +50 CXCL12 promoter fragment) were made by PCR amplification. The nucleotide sequences of each promoter region were verified by sequencing. The reporter assay was performed as described previously (Nakashima et al., 2005).

Reagents

Lithium chloride was purchased from Sigma-Aldrich.

Statistical analysis

All experiments were repeated three to four times and representative results are shown. The data are reported as the mean \pm standard deviation, and were analyzed by Student's t-test, where values of $p < 0.05$ were considered significant.

Results

Wnt3a down-regulates CXCL12 expression in ST2 cells

To evaluate a potential role for canonical Wnt signaling in regulating *CXCL12* expression of bone marrow stromal cells, we generated ST2 cell lines that expressed either *Wnt3a* (Wnt3a-ST2 cells), which stimulates canonical Wnt signaling, or non-canonical *Wnt5a* (Wnt5a-ST2 cells). As shown in Fig. 1A, while untransfected ST2 cells did not produce any detectable expression of *Wnt3a* or *Wnt5a*, Wnt3a-ST2 cells and Wnt5a-ST2 cells expressed high levels of *Wnt3a* and *Wnt5a* mRNA, respectively, as detected by RT-PCR (Fig. 1A). To confirm activation or suppression of canonical Wnt signaling in these cells, we transfected with Topflash, the reporter plasmid that carries six tandem repeats of the Lef1/Tcf binding site. The promoter activity of Topflash is enhanced in Wnt3a-ST2 cells but suppressed in Wnt5a-ST2 cells (Fig. 1B). Using these cultures, we examined whether Wnt signaling specifically modulates *CXCL12* gene expression. We found that *CXCL12* mRNA was highly expressed in ST2 cells, whereas its expression level in Wnt3a-ST2 cells was reduced (Fig.1A). ST2 cells express at least two cytokines essential for osteoclast differentiation:

RANKL and M-CSF. *RANKL* expression was suppressed in Wnt3a-transfected cells (Fig. 1A and 1E), whereas expression of *M-CSF* was not changed after stable transfection with either Wnt3a or Wnt5a (Fig. 1A).

The expression level of *CXCL12* was also determined by real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay. The level of *CXCL12* mRNA was reduced by 76% in response to Wnt3a transfection (Wnt3a-ST2 cells) compared to untransfected ST2 cells (Fig. 1C left). The amount of *CXCL12* protein released from Wnt3a-ST2 cells also decreased by 47% compared with control ST2 cells (Fig. 1D), indicating that Wnt3a specifically down-regulates *CXCL12* expression in ST2 cells. To confirm that regulation by Wnt3a also occurs in BMSC, we examined *CXCL12* expression in mouse BMSC following addition of Wnt3a-CM, using qRT-PCR. As shown in Fig. 1C right, while control-CM did not reduce *CXCL12* expression, the addition of 20% (v/v) of Wnt3a-CM down-regulated *CXCL12* expression.

To investigate whether regulation of *CXCL12* expression depends upon differentiation stage, ST2 cells were cultured in α -MEM containing ascorbic acid and β -glycerophosphate for 2 weeks. These cells have been shown to exhibit

characteristics typical of osteoblasts, with induction of expression of mRNAs for marker proteins of osteoblastic differentiation and formation of mineralized nodules (Otsuka et al., 1999). In these cultures, following addition of Wnt3a-CM or control-CM, then culture for 24 h, *CXCL12* expression was determined using qRT-PCR. As shown in Fig. 1F, *CXCL12* expression in differentiated ST2 cells (2 weeks) was lower (~21%) than in undifferentiated ST2 cells (1 day). While control-CM did not reduce *CXCL12* expression, the addition of 20% (v/v) of Wnt3a-CM also did not regulate *CXCL12* expression in differentiated ST2 cells (Fig. 1F).

LiCl down-regulates CXCL12 expression in ST2 cells

Since LiCl is known to enhance canonical Wnt signaling, we analyzed the effects on *CXCL12* expression of culturing cells with LiCl. When we analyzed the time course of *CXCL12* mRNA expression in ST2 cells, we found that the level of *CXCL12* mRNA began to decrease after 3 h and a time-dependent decrease in *CXCL12* mRNA levels was observed up to 48 h (Fig. 2A). The level of *CXCL12* mRNA decreased significantly after addition of 2 mM LiCl and further decreased

with doses up to 20 mM (Fig. 2B). The level of CXCL12 protein in ST2 cells declined to 25% or 17% upon treatment with 10 or 20 mM LiCl, respectively, for 24 h (Fig. 2C).

Knock-down of GSK-3 β Decreases CXCL12 Production

Since GSK-3 β is known to mediate canonical Wnt signaling, we examined the effect of knockdown of GSK-3 β using either RNA interference or tRNase Z^L-utilizing efficacious gene silencing (TRUE gene silencing) (Tamura et al., 2003, Nakashima et al., 2007, Elbarbary et al., 2009) upon CXCL12 induction. Knock-down of GSK-3 β was confirmed by Western blot analysis. We found that the level of GSK-3 β protein decreased to approximately 10% (GSK-3 β HP1, lane 2) or 23% (sgGSKL, lane 3) of the nontargeted control level (lane 1), confirming that both the siRNA and the sgRNA were effective in silencing the endogenous GSK-3 β gene (Fig. 2D). Since suppression of the GSK-3 β protein might lead to down-regulation of canonical Wnt signaling, levels of CXCL12 expression were examined. Both CXCL12 mRNA expression and CXCL12 protein in the culture supernatant of ST2 cells significantly decreased after transfection with

GSK-3 β HP1 or sgGSKL (Fig. 2E and 2F). These results indicate that *CXCL12* induction is GSK-3 β dependent, and that the canonical Wnt signaling pathway may be involved in regulating *CXCL12* induction.

Repression of the migration of bone marrow cells by Wnt3a in ST2 cells

To evaluate the potential biological relevance of the regulation of *CXCL12* by canonical Wnt signaling, we examined whether it has any effect on the directed migration of cells. Migration of mouse bone marrow cells was measured in a transwell migration assay. As shown in Fig. 3, this assay revealed extensive migration of cells towards recombinant *CXCL12*. These cells also exhibited substantial migration towards CM from ST2 cells. The migration of cells towards CM from Wnt3a-ST2 cells, not but from Wnt5a-ST2 cells, was reduced compared with ST2 cell CM. These findings indicate that canonical Wnt signaling can suppress *CXCL12* production by ST2 cells, thereby restricting their ability to promote bone marrow cell migration.

Canonical Wnt signaling-mediated inhibition of osteoclast formation in vitro

To investigate the effects of canonical Wnt signaling on ST-2 cells in osteoclastogenesis, we performed an in vitro assay for osteoclast formation using primary bone marrow macrophages. Addition of CM from Wnt3a-ST2 cells caused a dose-dependent decrease in the formation of TRAP-positive multinucleated osteoclasts and mononuclear cells remained predominant (Fig. 4). By contrast, addition of CM from ST2 cells resulted in typical cell fusion and osteoclastogenesis (Fig. 4). These results indicate that canonical Wnt signaling in osteoblasts or stromal cells may act indirectly on the down-regulation of osteoclast differentiation and formation, not only by regulating RANKL but also by regulation of CXCL12.

Negative regulation of CXCL12 promoter activity by canonical Wnt signaling

To investigate the mechanism by which canonical Wnt signaling inactivates *CXCL12* transcription, we cloned an approximately 1.8 kilobase pair

mouse genomic DNA fragment corresponding to the 5'-flanking promoter region (-1834/+50) of the *CXCL12* gene. The *CXCL12* promoter region was ligated into a luciferase reporter expression vector (pCXCL12-1834luc) to examine its responsiveness to canonical Wnt stimulation. Although LiCl did not alter luciferase activity on a control luciferase plasmid (pGV-C), transient transfection of pCXCL12-1834luc into ST2 cells together with LiCl treatment resulted in a significant decrease in luciferase activity (Fig. 5). The luciferase activity of the construct in which the 5'-end was deleted up to nucleotide position -420 (pCXCL12-420luc) was also suppressed by LiCl treatment (Fig. 5). This analysis implies the presence of transcriptional machinery that is sensitive to interference by canonical Wnt signaling and that regulates transcriptional activity through interaction with the *CXCL12* gene promoter downstream from nucleotide position -420.

Discussion

In bone marrow, CXCL12 appears to be constitutively expressed by stromal and endothelial cells. In the present study, we used the stromal cell line ST2, derived from mouse bone marrow, to examine CXCL12 expression. In normal culture, ST2 cells have the characteristics of preadipocytes and none of the features typical of the osteoblastic phenotype (Yamaguchi et al., 1996). However ST2 cells cultured with ascorbic acid exhibited characteristics typical of osteoblasts with the formation of mineralized nodules, indicating that ST2 cells are pre-osteoblastic stromal cells (Otsuka et al., 1999). Our study shows that ST2 cells express high levels of CXCL12 and that this expression is regulated by canonical Wnt signaling. Further, the ability of bone marrow cells to migrate in a Transwell assay was evaluated in response to CM collected from ST2 cells. Migration activity was found to be significantly inhibited by Wnt3a transfection into ST2 cells. Our observations suggest that CXCL12 is in part responsible for the activity found in CM. We also show that Wnt3a decreases CXCL12 expression in primary BMSCs, suggesting that canonical Wnt signaling might regulate CXCL12 expression in the bone marrow microenvironment.

Two fundamental processes are supported by the bone marrow: The production of mature blood cells from HSCs and the formation of mineralized bone tissue by osteoblasts. Several groups have demonstrated that events centered at the endosteal surfaces are critical for hematopoietic regulation, particularly by cells which share an osteoblastic phenotype (Calvi et al., 2003, Zhang et al., 2003, Arai et al., 2004). CXCL12 is a powerful chemoattractant for mature and early hematopoietic cells that express the CD34 antigen (Lataillade et al., 2000). Canonical Wnts are present in the bone marrow microenvironment and constitute a pivotal group of molecules controlling HSC cell proliferation and differentiation. Recently, Kim *et al.* reported activation of canonical Wnt signaling in the endosteal stroma of bone marrow by systemic injection of Wnt3a (Kim et al., 2009, Oh, 2010). Down-regulation of *CXCL12* by canonical Wnt signaling in stroma may therefore play a role in regulating HSC and progenitor mobilization. Proteolytic degradation of CXCL12 in the bone marrow has also been shown to play a role in regulating HSC and progenitor mobilization into the peripheral circulation (Lévesque et al., 2003). However, the mechanism underlying this effect is not yet known.

Regulation of CXCL12 production by various growth factors and

inflammatory cytokines has been reported. Wright *et al.* (Hatakeyama *et al.*, 2003) reported that TGF- β 1 down-regulates *CXCL12* expression in the stromal cell line MS-5. Similar to our studies, this decrease in *CXCL12* production by MS-5 cells correlated with decreased *CXCL12*-dependent migratory activity (Hatakeyama *et al.*, 2003). They indicate that “primitive” mesenchymal “fibroblast-like” precursors within the bone marrow stroma produce *CXCL12* at an early stage of their maturation. Evidence from several *in vitro* and *in vivo* studies support the hypothesis that canonical Wnt signaling stimulates the differentiation of osteoblasts. For example, stimulation of canonical Wnt signaling using constitutively active forms of β -catenin induced ALP activity and also induced mineralization of osteoblastic cells (Bain *et al.*, 2003, Nakashima *et al.*, 2005, Tamura *et al.*, 2010). Our present results, showing that canonical Wnt signaling down-regulates *CXCL12* expression supports the idea that *CXCL12* is predominantly expressed by osteoblastic precursor cells at a primitive stage. Thus, it appears that expression declines once cells commit to a differentiation pathway by activation of canonical Wnt signaling. The decrease in *CXCL12* expression may therefore represent a useful marker of osteoblast commitment or differentiation.

In this study we have shown that canonical Wnt signaling inactivates the CXCL12 gene promoter. Upon activation by a canonical Wnt signal, the HMG-box protein Lef1/Tcf can form a complex with β -catenin. The complex converts into a transcriptional regulator of target genes. A number of target genes have been identified that respond to canonical Wnt signaling and that have a Lef1/Tcf binding motif (Yochum et al. 2007). We performed a search of CXCL12 gene promoter sequences downstream from nucleotide position -420 for potential Lef1/Tcf binding consensus sequences. This search identified **the** putative Lef1/Tcf binding site (TACAAAG) in the murine CXCL12 promoter located at positions -185 to -178 relative to the translational start codon. This site is a perfect match to the consensus core Lef1/Tcf binding sequence 5'-CTTTGA/TAT-3'. Previously, Spencer *et al.* (Spencer *et al.* 2006) reported that activation of canonical Wnt signaling down-regulated RANKL expression in osteoblasts. Consistent with our studies, they have also shown that canonical Wnt signaling down-regulates RANKL promoter activity, and identified five Lef1/Tcf binding sites in the 5' proximal region of the human RANKL promoter, implicating RANKL as a potential transcriptional target for Wnt signaling. Our present results suggest that the Lef1/Tcf binding site in the CXCL12 promoter

located at positions -185 to -178 functionally interacts with β -catenin/Tcf-1, and that these complexes then down-regulate expression of CXCL12. However, the precise mechanism responsible for this suppression is unclear. Further investigation is required to understand the mechanisms by which Wnt signaling mediates suppression of gene promoter transcriptional activities.

Bone mass is dependent on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. Osteoclasts are derived from granulocyte-macrophage colony forming units (CFU-GM) in bone marrow; osteoclast precursor cells then fuse and differentiate into mature osteoclasts. This differentiation and maturation is mediated by RANKL, which is expressed on osteoblasts, and by receptor activator of NF κ B (RANK), a cognate receptor expressed on osteoclast precursor cells (Theoleyre et al., 2004). We have previously shown that canonical Wnt signaling regulates expression of *OPG* and *RANKL*, a finding which supports a role for osteoblastic canonical Wnt signaling in the down-regulation of osteoclast differentiation and formation via regulation of *OPG* and *RANKL* (Sato et al., 2009, Tamura et al., 2010). During osteoclastogenesis, CXCL12 has been reported to recruit osteoclast precursors by inducing chemotaxis and collagen transmigration (Yu et al., 2003b, Gronthos

and Zannettino, 2007). This may serve as a mechanism to localize osteoclast precursors to the bone surface for subsequent activation into resorptive osteoclasts, indicating that CXCL12 is a crucial osteoclastogenesis factor that enhances the formation and activity of osteoclasts and increases bone resorption *in vivo*. Our studies suggest that canonical Wnt signaling may act indirectly on the down-regulation of osteoclast differentiation and formation, not only by regulating OPG and RANKL but also by regulation of CXCL12 in the bone marrow.

In conclusion, we have shown that canonical Wnt signaling regulates CXCL12 gene expression, and this is the first study linking chemokine expression to Wnt signaling in bone marrow. As such, these investigations may provide important new information pertaining to the molecular basis of the regulation of HSC or osteoclast precursors in the bone marrow microenvironment.

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FIGURE LEGENDS

Figure 1

Wnt3a down-regulates CXCL12 expression in ST2 cells

ST2, Wnt3a-ST2 and Wnt5a-ST2 cells were harvested at confluence and total RNA was extracted. The mRNA expression of CXCL12, receptor activator of NF κ B ligand (RANKL), macrophage colony stimulating factor (M-CSF), alkaline phosphatase (ALP), Wnt3a and Wnt5a was determined by RT-PCR (A). Transcriptional activity of Topflash reporter (B). Cells were transiently transfected in 24-well plates with 0.1 μ g of Topflash, after which cells were cultured for a further 24 h. Luciferase activity was determined as described in Materials and Methods. Normalized luciferase activity is represented as fold induction over ST-2 cells (B). CXCL12 mRNA expression was analyzed by quantitative RT-PCR (qRT-PCR) (C). Total cellular RNA was extracted from control or transfected ST2 cells and transcripts were analyzed by qRT-PCR (C, left). Confluent mouse bone marrow stromal cells (BMSC) were incubated in Dulbecco's modified Eagle's medium containing 5% FBS and 10^{-8} M dexamethasone in the presence of 20% (v/v) control-CM (cont.-CM) or

Wnt3a-CM for 24 h (C, right). CXCL12 protein levels in the culture supernatant were determined by ELISA (D). RANKL mRNA expression was analyzed by quantitative RT-PCR (qRT-PCR) (E). ST2 cells were cultured in α -MEM containing ascorbic acid and β -glycerophosphate for 2 weeks or 1 day after addition of Wnt3a-CM or control-CM, then cultured for a further 24 h and CXCL12 expression was determined using qRT-PCR.

Figure 2

Regulation of CXCL12 expression by canonical Wnt signaling

ST2 cells were plated at 1×10^5 cells/cm² and cultured as described. After 24h, 10mM (A) or indicated concentration (B and C) of LiCl was added, after which the cells were cultured for 3 -48 h (A) or 24 h (B and C). Total RNA was extracted from the cells and the mRNA levels of CXCL12 were determined by qRT-PCR (A and B). CXCL12 protein levels in the culture supernatant were determined by ELISA (C). ST2 cells were transiently transfected in 100 mm dishes with 6 μ g of vector. After the cells were cultured for a further 48 h, the levels of GSK3 β protein in the cells were determined by Western blot analysis using a GSK3 β

antibody (D): Lane 1 with pU6pro (empty vector) (control); lane 2 with pU6-GSK3 β HP1 (GSK3 β siRNA expression plasmid) (GSK3 β -HP1); lane 3 with Tin-H1.2/Neo-sgGSKL (GSK3 β sgRNA expression plasmid) (GSK3 β -sgRNA)

The levels of CXCL12 mRNA expression in the cells were determined by qRT-PCR (E). The level of CXCL12 protein in the culture supernatants was determined by ELISA (F). Data are means \pm S.D. Each assay represents a separate experiment performed in triplicate. Asterisks indicate significant differences ($p < 0.005$, t test for paired data). Each assay represents a separate experiment performed in triplicate.

Figure 3

Repression of the migration of bone marrow cells by Wnt3a in ST2 cells

Migration of mouse bone marrow cells was determined by the transwell assay. Chambers were placed into separate wells containing medium only (-), recombinant CXCL12 (80 ng/ml), or conditioned medium (Cond. Med.) from ST2 cells, Wnt3a-ST2 cells or Wnt5a-ST2 cells. After overnight culture, migrated cells were counted. Cell migration is represented as relative migration ratio

compared with that of recombinant CXCL12 (80 ng/ml) which is set at 100%.

Data are means \pm S.D. of at least three experiments, * $p < 0.05$.

Figure 4

Canonical Wnt signaling-mediated inhibition of osteoclast formation in vitro

Murine bone marrow macrophages (2×10^5 in 100 μ l) were incubated in 48-well culture plates in 100 μ l of DMEM with 50 ng/ml of M-CSF and 25 ng/ml of RANKL. Then, 100 or 50 μ l of CM from Wnt3a-ST2 cells was added to the wells.

The same amount of ST2-CM was added as a control. Each experiment was performed in triplicate. After culture for 5 days, cells were fixed, and then tartrate-resistant acid phosphatase (TRAP) activity was evaluated histochemically (A). ST2-CM (left), 100 μ l of CM from Wnt3a-ST2 cells (right).

Numbers of TRAP-positive multinuclear cells (MNCs) (%) are shown (B). Each experiment was performed in triplicate. Values are expressed as means \pm SE.

Figure 5

Down-regulation of CXCL12 promoter activity by LiCl

Transcriptional activity of the 5' region of the CXCL12 gene promoter in luciferase reporter constructs. ST2 cells were transiently transfected in 24-well plates with 0.1 µg of a reporter plasmid (pCVCL12-1834luc or pCXCL12-420luc), or control luciferase plasmid (pGV-C). After the cells were cultured with or without 10 mM LiCl for a further 48 h, luciferase activity was determined. Luciferase activity is represented as the relative reduction compared to pCXCL12-1834luc with vehicle. Data means \pm S.D. Each assay represents a separate experiment performed in triplicate. * $p < 0.05$.

Fig. 1

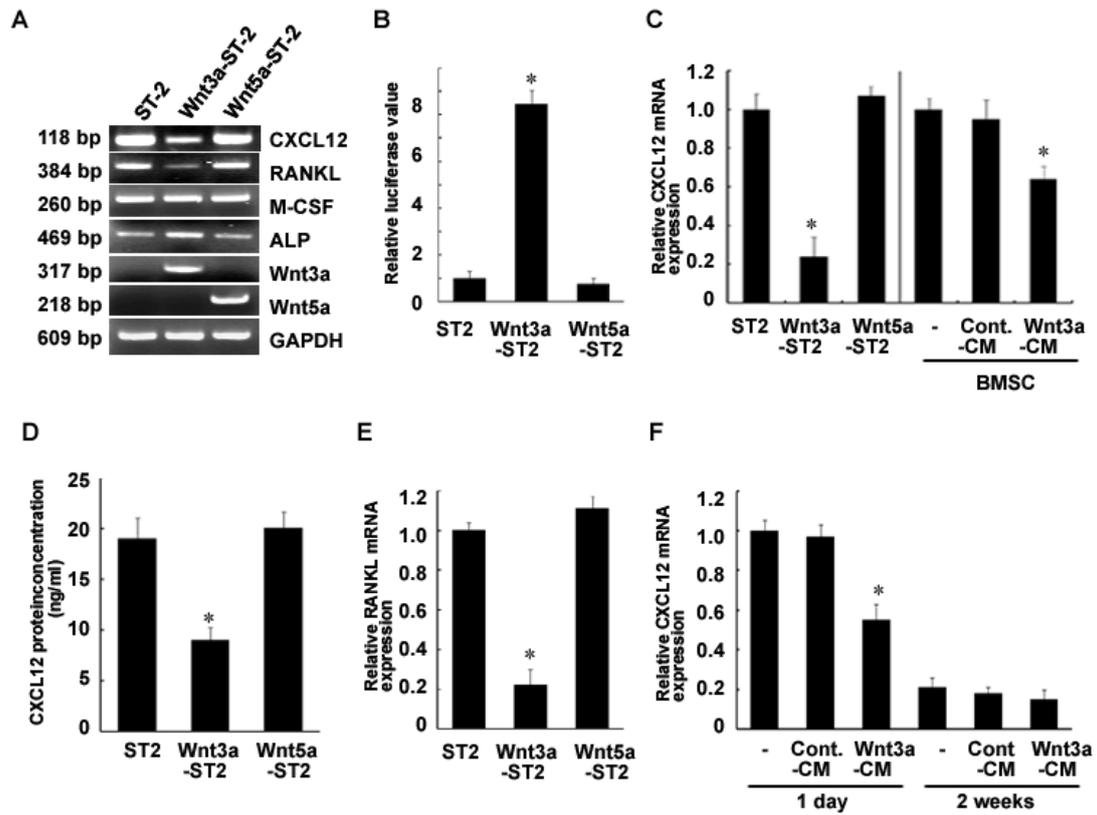


Fig. 2

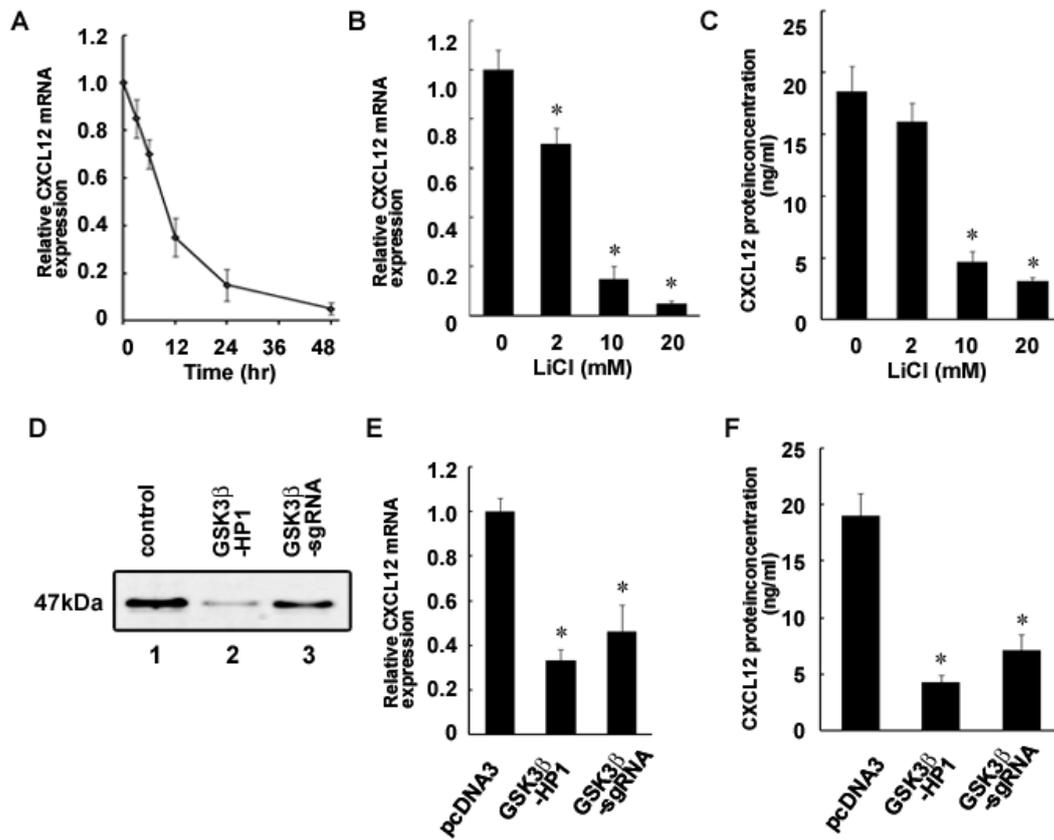


Fig. 3

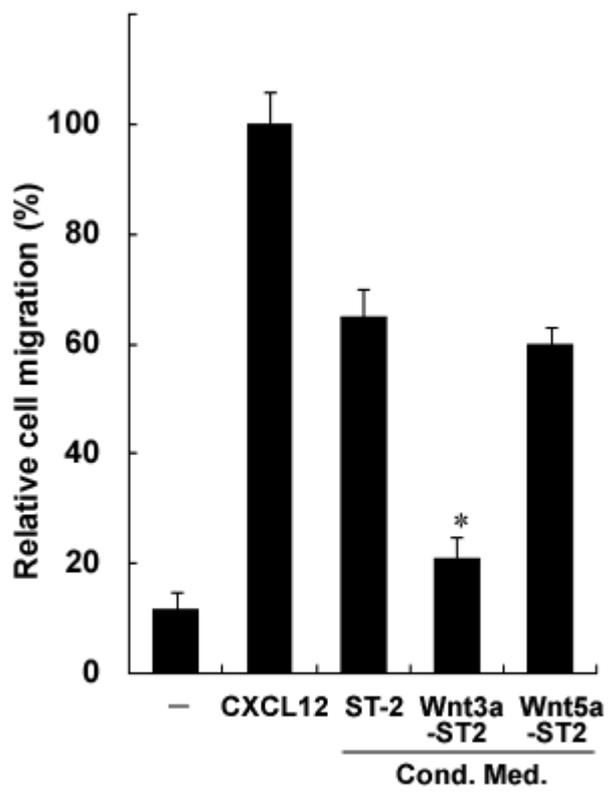


Fig. 4

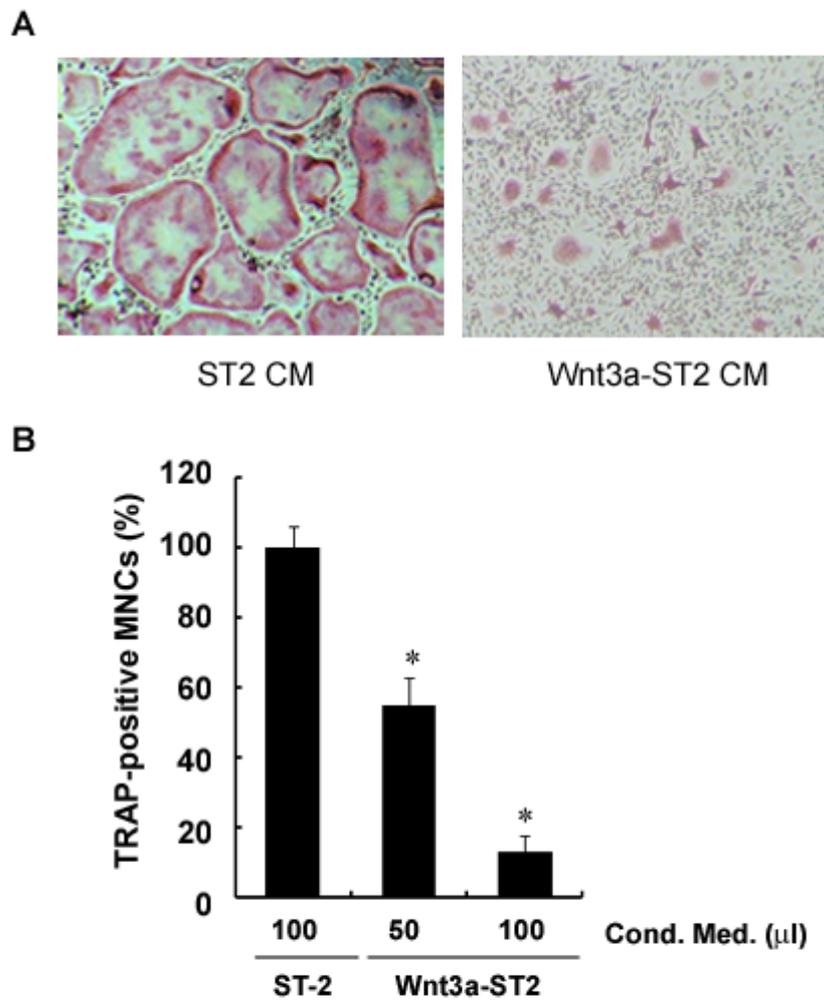


Fig. 5

