Osteoblastic heparan sulfate glycosaminoglycans control bone remodeling by regulating Wnt signaling and the crosstalk between bone surface and marrow cells

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Stimulating bone formation is an important challenge for bone anabolism in osteoporotic patients or to repair bone defects. The osteogenic properties of matrix glycosaminoglycans (GAGs) have been explored; however, the functions of GAGs at the surface of bone-forming cells are less documented. Syndecan-2 is a membrane heparan sulfate proteoglycan that is associated with osteoblastic differentiation. We used a transgenic mouse model with high syndecan-2 expression in osteoblasts to enrich the bone surface with cellular GAGs. Bone mass was increased in these transgenic mice. Syndecan-2 overexpression reduced the expression of receptor activator of NF-κB ligand (RANKL) in bone marrow cells and strongly inhibited bone resorption. Osteoblast activity was not modified in the transgenic mice, but bone formation was decreased in 4-month-old transgenic mice because of reduced osteoblast number. Increased proteoglycan expression at the bone surface resulted in decreased osteoblastic and osteoclastic precursors in bone marrow. Indeed, syndecan-2 overexpression increased apoptosis of mesenchymal precursors within the bone marrow. However, syndecan-2 specifically promoted the vasculature characterized by high expression of CD31 and Endomucin in 6-week-old transgenic mice, but this was reduced in 12-week-old transgenic mice. Finally, syndecan-2 functions as an inhibitor of Wnt-β-catenin–T-cell factor signaling pathway, activating glycogen synthase kinase 3 and then decreasing the Wnt-dependent production of Wnt ligands and R-spondin. In conclusion, our results show that GAG supply may improve osteogenesis, but also interfere with the crosstalk between the bone surface and marrow cells, altering the supporting function of osteoblasts. Cell Death and Disease (2017) 8, e2902; doi:10.1038/cddis.2017.287; published online 29 June 2017

Improving bone formation is an important issue to rescue bone loss in aging patients or to repair bone defects after fracture or tumor resection. Glycosaminoglycans (GAGs) are key component of the bone matrix and cell surface that modulate the bioavailability and activity of various osteoclastic and osteogenic factors. Synthetic sulfated GAGs showed osteogenic properties in vitro and were proposed to be useful for biomaterial coating; however, contrasting results were obtained and the effects of GAGs on bone formation and resorption are still unclear. Moreover, GAG accumulation was shown to have a role in the bone diseases associated with mucopolysaccharidoses or Leri pleonosteosis. In the various studies, only soluble or matrix-associated GAGs were considered. To design more optimal GAGs for clinical use in bone regeneration, we need to better understand the functions of endogenous cellular GAGs. Moreover, GAGs are unique to each cell type because they depend on the assembly machinery and modifying enzymes and the expression pattern of the proteoglycans. GAGs at the surface of osteoblastic cells could be major factors in the bone surface environment.

Recently, sulfated hyaluronan and chondroitin sulfate were reported to inhibit sclerostin and to enhance bone regeneration in diabetic rats. Thus, a key function of GAGs in fostering osteogenesis may involve modulating Wnt signaling. Indeed, in addition to sclerostin activity, that of Wnt proteins and many other Wnt modulators also depends on interactions with proteoglycans.

Syndecans are cell-surface heparan sulfate proteoglycans (HSPGs). They are low-affinity co-receptors with roles in docking, protection and concentration of their ligands. Syndecans also interact with high-affinity receptors and integrins to modulate intracellular signaling. Syndecans positively or negatively modulate Wnt signaling. Among the four syndecans, syndecan-2 was especially linked to osteoblastic differentiation during mouse development and in adult bone. In the embryo, syndecan-2 is expressed in the peristeum at the onset of osteogenesis, and its expression increases during osteoblast differentiation. Syndecan-2 is upregulated by osteogenic factors such as bone morphogenetic protein-2 and Runx2. Hence, the syndecan-2 level

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appears to be tightly controlled in osteoblastic cells. Overexpression of syndecan-2 in osteosarcoma cells alters multiple pathways involving PI3K, mitogen-activated protein kinases, nuclear factor kappa-B (NF-kB) and protein kinase C δ, and also canonical and non-canonical Wnt pathways. On another hand, syndecan-2 supports neoangiogenesis during development in zebrafish and in tumors, whereas shed syndecan-2 alters angiogenesis through the inhibition of endothelial cell migration. These data suggest that osteoblastic syndecan-2 could have a role in the relationship between angiogenesis and osteogenesis.

Here, we used a transgenic mouse model with syndecan-2 expression increased specifically in osteoblasts to identify the role of this proteoglycan and the associated GAGs during bone remodeling. Syndecan-2 overexpression enriched the bone surface with heparan sulfate and resulted in increased bone mass because of a strong inhibition of resorption. Syndecan-2 overexpression enhanced apoptosis of bone marrow cells and decreased osteoblast and osteoclast precursor populations. Syndecan-2 upregulated the osteogenic vasculature in young mice but did not prevent the loss of this specific endothelium with age. It downregulated the Wnt–β-catenin–T-cell factor (TCF) pathway, thereby changing the osteoblastic environment. Our data identify osteoblastic heparan sulfate GAGs as novel modulators of the crosstalk between osteoblasts and their microenvironment.

### Results

**Higher level of syndecan-2 and heparan sulfate GAGs at the bone surface increased bone mass.** Syndecan-2 was detected in bone marrow cells (BMCs), osteoblasts and osteocytes in wild-type (WT) mice (Figure 1a). We generated the C57BL/6-B6D2 Tg(Coll1(2.3)-SCD2) (Coll-Synd2) transgenic mouse model by using the syndecan-2 sequence under control of the 2.3-kb fragment of collagen I promoter that drives transgene expression in osteoblasts. Transgenic mice showed increased number of cells with high level of syndecan-2 near the bone surface (Figure 1a). We collected BMCs from WT or Coll-Synd2 mice to further determine the levels of syndecan-2 by flow cytometry (Figures 1b and c). Only very few of the early osteoblast precursors with CD51 and Sca-1 at their surface (CD51+Sca-1+ cells) expressed syndecan-2 in WT as in Coll-Synd2 mice. The median fluorescence intensity of syndecan-2 labeling was not significantly modified when considering the whole BMC population from WT or Coll-Synd2 mice (Figure 1c). At the opposite, the osteoblasts (CD51+Sca-1+ cells) in the marrow of transgenic mice had a significant increase in syndecan-2 levels as compared to osteoblasts from WT mice (Figure 1c). Increased expression of syndecan-2 in CD51+Sca-1+ cells was associated with a increased level of heparan sulfate chains (Figure 1d). Indeed, heparan sulfate increased along the bone surface (Figure 1e). Coll-Synd2 mice from three strains from different founders developed normally without any significant differences in bone mineral density (Supplementary Figure 1). We used the transgenic strain with the highest syndecan-2 expression for micro-CT analyses to assess the effect of increased GAG level on
bone mass in 2- (young) and 4-month-old (mature) mice (Supplementary Figure 1). Trabecular bone volume and number of trabeculae tended to increase in young transgenic males but were significantly higher in young females and in mature Coll-Synd2 male and females than in WT mice (Figures 2a–c and Supplementary Figure 2). Trabecula thickness was unchanged and trabecula separation was reduced in transgenic mice, especially at 4 months (Figure 2c). No modification of the cortical bone was observed in transgenic mice. Hence, increased level of the osteoblastic HSPG modified trabecular bone architecture.

Syndecan-2 overexpression in osteoblasts inhibited bone resorption. In adults, bone quantity results from the remodeling process, the first step of which is bone resorption by osteoclasts. Coll-Synd2 mouse bone showed a striking disappearance of osteoclasts with tartrate-resistant acid phosphatase (TRAP) activity (Figure 3a). The proportion of osteoclastic surface (Oc.S/BS) was significantly reduced in 2- and 4-month-old mice. Data are mean ± S.E.M. (n = 5 at 2 months; n = 10 at 4 months). * indicates significant difference between WT and Coll-Synd2 (P < 0.05)

Syndecan-2 in osteoblasts modulated bone formation. Syndecan-2 was found to be involved in signaling that favors osteoblastic activity. Here, we studied formation parameters using toluidine blue staining of osteoid tissue and osteoblasts (Figure 4a) and tetracycline and calcine injections to visualize the dynamic mineralization process (Figures 4b and c). The extent of bone surface with new matrix deposit, osteoid surface, number of active osteoblasts and mineralized surfaces was not significantly modified in 2-month-old Coll-Synd2 mice but were strongly reduced in 4-month-old Coll-Synd2 mice as compared to WT mice (Figures 4a and b). The mineral apposition rate was not significantly modified (Figure 4b). As a result, the global bone formation rate was decreased in 4-month-old Coll-Synd2 mice (Figure 4c). Hence, osteoblastic GAGs did not alter osteoblastic activity but downregulated osteoblast number. This latest effect did not appear to be related to altered proliferation or differentiation capacity of osteoblastic precursors as shown by BrdU incorporation and in vitro mineralization assay in mesenchymal cells from Coll-Synd2 or WT bone marrow (Supplementary Figure 3). However, the formation of ALP* colonies (CFU–ALP*) was significantly impaired in cultures of BMCs from Coll-Synd2 mice as compared with WT mice (Figure 4d). In addition, the expression of the osteoprogenitor marker RUNX2 was significantly decreased in bone marrow extracts from transgenic mice (Figure 4e). Thus, GAGs at the bone surface increased osteoblast activity in younger mice, reduced osteoblast number in older mice and altered the pool of osteoblast precursors in bone marrow.

Osteoblastic syndecan-2 promoted stromal-cell apoptosis. Survival of osteoblasts and their precursors may affect global osteogenic activity. Syndecan-2 overexpression decreased cell survival and increased effector caspase activity of proliferating stromal C3H10½ cells but not confluent cells (Supplementary Figure 4); therefore, syndecan-2 overexpression may be associated with increased apoptosis. Consistently, the mRNA level of anti-apoptotic Bcl2 was decreased in bone marrow extracts and increased in bone cells of Coll-Synd2 mice; the level of the pro-apoptotic Bax was unchanged in BMCs and increased in bone cells (Figures 5a and b). TUNEL assay showed a significant increase in the rate of apoptotic cells within the bone marrow of Coll-Synd2 vertebra (Figures 5c and d). However, the number of osteocytes and rate of apoptotic osteocytes did not differ between control and transgenic mice (Supplementary Figure 5). These results support syndecan-2 not affecting the survival of mature osteoblasts but increasing the apoptosis of BMC populations. Accordingly, Annexin V
binding was increased only in CD45−Sca-1+ mesenchymal progenitor populations but not in CD45−Sca-1− mature cells isolated from Coll-Synd2 bone marrow (Figure 5e). Increased apoptosis was associated with a decreased rate of mesenchymal progenitors (CD45−Sca-1+) in the bone marrow of Coll-Synd2 mice (Figure 5f). Syndecan-2 may prevent osteoblasts from supporting the survival of mesenchymal stem/precursor cells in bone marrow.

Osteoblastic syndecan-2 had an impact on pro-osteogenic vasculature. Increased apoptosis of BMCs was observed as soon as at 2 months in transgenic mice and did not alone explain how syndecan-2 overexpression differently influenced the bone formation at 2 and 4 months. To further address this question, we examined whether syndecan-2 overexpression did modify the endothelium within the bone marrow using CD31 and Endomucin markers to study a pro-osteogenic endothelium that was previously shown to be characterized by high expression of CD31 and Endomucin (CD31highEndomucinhigh cells; Figure 6a).22 We selected marrow cell populations that did not express lineage markers (Lin− cells). The proportion of Lin− cells that expressed CD31 was similar in WT and Coll-Synd2 mice (Figure 6b). At the opposite, the proportion of Lin− cells that expressed Endomucin was significantly decreased in the marrow of 12-week-old transgenic mice as compared to WT mice at the same age (Figure 6c). The proportion of CD31highEndomucinhigh cells was increased in 6-week-old but decreased in 12-week-old Coll-Synd2 mice (Figure 6d). Analyses by immunofluorescence illustrated that syndecan-2 overexpression did not prevent the loss of Endomucin+ endothelium within the marrow of 4-month-old mice (Figure 6e).

Syndecan-2 overexpression in osteoblasts decreased Wnt/β-catenin signaling. We next investigated whether the functions of osteoblastic GAGs were related to the modulation of Wnt activity. Basal and Wnt3a-induced activity of the TOPFlash reporter plasmid was reduced in syndecan-2-overexpressing C3H10½ cells (Supplementary Figure 6). This inhibitory effect was dependent on syndecan-2-associated GAGs since overexpression of a mutated syndecan-2, in which the three GAG-binding serines were replaced by alanines, did not induce the same modification of the TOPFlash activity (Supplementary Figure 6). Moreover, inhibition of sulfation of GAG chains with sodium chlorate increased basal and Wnt3a-dependent TOPFlash activity (Supplementary Figure 6). Therefore, the osteoblastic syndecan-2 appears to function as a Wnt signaling inhibitor. Consistently, the expression of the Wnt target genes Axin2, WISP and β-catenin was lower in bone extracts from Coll-Synd2 than in WT mice (Figure 7a). Syndecan-2-dependent changes in the expression of specific Wnt inhibitors, sFRP-1, DKK1 and sclerostin, could not explain this altered Wnt signaling in Coll-Synd2 mice (Figure 7b). Syndecan-2 may alter Wnt signaling by intracellular routes, as was previously shown.15,16 Using an anti-phospho-GSK3 (Tyr279/Tyr216) antibody to label the active form of the kinase, serum-starved C3H10½ control cells showed inactivated GSK3 by the addition of serum; in contrast, GSK3 was activated in syndecan-2-overexpressing C3H10½ cells (Supplementary Figure 3).
Syndecan-2 overexpression was associated with enhanced phospho-GSK3 staining in osteoblasts along the bone surface of transgenic mice (Figure 7c). As a possible result of inhibition of the Wnt/β-catenin pathway, the expression of Wnt-dependent genes such as Wnt3a, Wnt11 and RSPO-2 were reduced in bone extracts of transgenic mice (Figure 7d). Decreased level of RSPO-2 in the osteoblastic microenvironment in ColI-Synd2 vertebra was confirmed by immunohistochemistry (Figure 7e). Increased level of GAG at the cell surface could also enhance the trapping capacities of the osteoblasts. Indeed, pre-incubation with C3H10½ cells that overexpressed syndecan-2 significantly reduced the ability of Wnt3a-containing medium to induce axin expression (Supplementary Figure 7). This effect was abolished when Wnt3a-containing medium was pre-incubated on C3H10½ cells that overexpressed the mutated syndecan-2 lacking heparan sulfate modifications (Supplementary Figure 7). To determine whether the modification of Wnt effectors around transgenic osteoblasts may contribute to the altered BMCs, we co-cultured WT or ColI-Synd2 osteoblasts in porous inserts with mesenchymal cells from WT bone marrow. BMC apoptosis was greater with syndecan-2-overexpressing osteoblasts than WT osteoblasts; the pro-apoptotic effect was rescued when the medium was supplemented with recombinant Wnt3a-containing medium (Figure 6f).

Discussion

Synthetic GAGs have been considered promising compounds to improve biomaterial functions in the bone. To further define the usefulness of GAGs as an anabolic support, we investigated the function of endogenous cellular GAGs during bone remodeling by using a transgenic mouse model with increased syndecan-2 expression in osteoblasts. High
Syndecan-2 overexpression affected bone formation depending on the age of mice. It did not alter osteoblast activity but reduced osteoblast number. Syndecan-2 has been described as a promoter of osteoblast adhesion to the matrix and a modulator of matrix deposition. Our results are consistent with reports showing that heparan sulfate has an anabolic effect during bone regeneration in rat models of long bone or cranial defects. During fracture repair, the ossification occurs through a callus formation and resembles the endochondral ossification process. In contrast, in mature animals, bone formation is part of the remodeling process and depends on coupling signals between osteoclasts and osteoblasts. The decreased bone formation rate in our 4-month-old transgenic mice probably resulted from a missing osteoclastic signal that impaired recruitment of osteoblast precursors. Increased apoptosis of mesenchymal precursors may also account for the reduced number of osteoblasts in older transgenic animals. Indeed, heparan sulfate GAGs located at the interface between osteoblasts and BMCs may prevent osteoblasts, supporting the survival of BMC populations. Osteoblastic GAGs appear to affect specific BMC populations because the level of the apoptosis marker, Annexin V, was found to be associated with Sca-1+CD45− but
not more mature CD45−Sca-1− BMCs from Colli-Synd2 mice. Syndecan-2 overexpression did not affect apoptosis of other cell populations such as CD11b+ or CD19+ B cells (data not shown). In another hand, syndecan-2 overexpression modified bone marrow vasculature. These results are consistent with previous data showing that syndecan-2 is involved in neo-angiogenesis during development.18 Here we show that syndecan-2 specifically promoted the CD31highEndomucinhigh endothelium in youngest mice. This type of vessels mediates growth of the bone vasculature and supports osteogenesis.22 Hence, preserved bone formation in 2-month-old transgenic mice, despite osteoprogenitors’ apoptosis, can be related to an increased pro-osteogenic endothelium. Our results showed that the proportion of CD31highEndomucinhigh cells was increased in younger transgenic animals, whereas it was strongly decreased in mature transgenic mice. In another hand, the Endomucin+ bone vasculature decreased with age in WT mice, and this loss was not prevented in Colli-Synd2 mice. Hence, the decreased bone formation in 4-month-old Colli-Synd2 mice could be related to the reduction of the supportive action of CD31highEndomucinhigh vasculature. Different mechanisms could be involved in this dual action of syndecan-2 on angiogenesis. Inhibition of bone remodelling in transgenic mice probably induced the maintenance of old
osteoblasts with altered activity at the bone surface. Thus, syndecan-2 may be indirectly responsible for the modification of angiogenic factor production by osteoblasts. In another hand, shedding of syndecan-2 might be increased in older mice. This would contribute to switch the pro-angiogenic action of the proteoglycan into inhibition.19

Inhibition of Wnt signaling in osteoblasts contributed to the pro-apoptotic effect of syndecan-2 overexpression. Syndecan-2 expression modified intracellular signaling that affects the Wnt/β-catenin–T-cell factor pathway. In particular, syndecan-2 deregulated the GSK3 pathway, which may be related to the inhibitory effect of syndecan-2 on the PI3K level, as was previously reported in osteosarcoma cells.15 Wnt ligands secreted by osteoblasts have a crucial role in bone homeostasis.37 Therefore, inhibition of the expression of Wnt target genes, including Wnt ligands and RSPO, likely contributed to the altered microenvironment of osteogenic cells. However, Wnt and RSPOs are heparin-binding molecules. Our results suggest that high levels of proteoglycans may result in exaggerated capture of Wnt and other factors that are required to support Wnt signaling in BMGs. In the same way, sulfated hyaluronan coated on biomaterials was proposed to promote osteoblast function by binding sclerostin.5 Although the expression of Wnt inhibitors such as DKK1 and sFRP-1 was decreased in Coll-Synd2 mice, we cannot rule out that high levels of syndecan-2-conjugated heparan sulfate chains may promote sFRP-1 activity because heparin is responsible for sFRP-1 accumulation and stabilization.38 Moreover, RSPO is a DKK1 inhibitor.39,40 Hence, decreased RSPO level could also favor DKK1 activity in the osteoblastic environment. Many genetic models have proven that Wnt signaling is required for stem-cell commitment, differentiation and osteoprogenitor survival. However, the physiological requirement for the extinction of Wnt signaling for the terminal maturation of osteoblasts was proposed from data showing that aberrant stabilization of β-catenin in precursors prevented the terminal step of osteoblastic maturation.41,42 Overactivation of β-catenin in osteocytes and mature osteoblasts results in decreased bone quality and growth.43 Hence, inhibition of Wnt signaling can affect precursor cells within bone marrow and at the same time promote osteoblast activity.

In contrast with our results, heparan sulfate was shown to enhance the differentiation of osteoprogenitors and promote the proliferation of mesenchymal stem cells in culture systems.44,45 Moreover, osteoporosis was found as a side effect of treatment with heparin that stimulated osteoclastogenesis by inhibiting OPG.46,47 These discrepancies may be due to the different composition of the GAGs used in previous studies because the degree of sulfation affects the biological activity of these molecules.27 Osteoblastic GAGs should be better characterized at different ages and in particular pathological conditions to determine the optimal composition for GAG mimetics. Another way to interpret the different effects of GAG supply is that exogenous GAG could interfere or compete with the endogenous ones. Whether GAGs associated with or solubilized from a biomaterial could alter normal bone remodeling remains unknown.

Conclusions
The Coll-Synd2 mouse model provided new insights into the specific activities of syndecan-2-conjugated heparan sulfate GAGs during bone remodeling. These GAGs at the surface of osteoblasts were key constituents in the osteoblast environment and controlled osteogenesis, osteoclastogenesis and bone remodeling in our model. Especially, syndecan-2 controlled the ability of osteoblasts to support other BMC populations such as stromal cells and mesenchymal precursors. The proteoglycan also modulated angiogenesis, altering a specific endothelium with pro-osteogenic properties. Osteoblastic GAGs were involved in regulating Wnt signaling and controlling Wnt effectors’ production. Beyond promoting osteogenic properties, GAGs in biomaterials should provide an environment that can support stromal and hematopoietic cells of bone marrow.

Materials and Methods
Animals. The entire protocol was performed in accordance with French Government Animal Welfare Policy and European Directive 86/609/EEC and was approved by the Ethics, Animal Care and Experimentation Committee of the Institute of Health and Medical Research at Lariboisiere-Villemin (Paris, France). C57BL6-B6D2 Tg(Coll(2.3)-SCD2) (Coll-Synd2) mice were generated as previously described by using the construct with N-cadherin cDNA replaced by human syndecan-2 cDNA.48 The mouse genotypes were determined by PCR amplification of tail DNA with 5’TGTATCTCTCCTGGCTG-3’ and 5’AGGCAC TTTGATGGTCG-3’ as primers. Animals were not randomized. WT are littermate controls.

Histomorphological analyses of the bone. Micro-CT analyses involved use of a SKYSCAN 1272 scanner (Bruker, Coventry, UK). Bones were placed vertically and scanned with the settings: aluminum filter, 0.5 mm; resolution, 6 μm; energy, 70–90 kV; intensity, 100 μA; and integration time, 170 ms. Reconstruction of femurs and analysis of bone volume involved the use of CTanalyser. 3D illustrations were created with DataViewer and 3D-Visualization software (CTvol). The histomorphometric variables were recorded in compliance with the recommendation of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee.49 Given this recommendation and the type of mouse strain, five mice were included in each group for μCT analyses at 2 months and 10 animals/groups at 4 months. The bone formation and resorption variables were measured in sections of methyl methacrylate-embedded femurs stained with toluidine blue to show osteoid tissues and osteoblasts or with naphthol 3-hydroxy-2-naphthoic acid 4-chloro-2-methylamylidine (ASTR) phosphate for detecting mature osteoclasts with TRAP activity. Five animals/group were analyzed by two different investigators. One was blinded to the group allocation during the analysis. To evaluate bone formation rate, mice were intra-peritoneally injected with double fluorescent labeling of tetracycline (20 mg/kg) and calcein (10 mg/kg; Sigma, St. Louis, MO, USA) at 5 days and 1 day before being killed. Measurements involved use of a polarizing microscope (Nikon, Tokyo, Japan) with a Retiga 2000 R (Q Imaging, Surrey, BC, Canada) and a software package developed for bone histomorphometry (Microvision, Lisses, France).

Immunohistochemical analysis. Vertebrae from WT and Coll-Synd2 mice were fixed in 4% paraformaldehyde, decalcified and embedded in paraffin. Sections of 5 μm were treated with 10 mM citrate buffer, pH 6, at 70 °C, saturated with goat immunoglobulin, anti-syndecan-2, (366200, Invitrogen, Thermofisher, Montigny le Bretonneux, France), anti-R-spondin-2 (C-12, Santa Cruz Biotechnology, Dallas, TX, USA) or anti-heparan sulfate (F58-10E4; Amsbio, Cambridge, MA, USA), and then incubated with a biotinylated secondary antibody. Sections were counterstained with methyl green and observed under a polarizing microscope (Nikon) with a Retiga 2000 R camera (Q Imaging). For immunofluorescence of syndecan-2, activated glycogen synthase kinase 3 (GSK3) or Endomucin, sections were incubated with the antibody anti-syndecan-2 and anti-phospho-GSK3 (Tyr279/ Tyr216; 5G-2F; Millipore, Guayancourt, France), or with anti-endomucin (V7C7) then...
with DyLight488- and DyLight650-conjugated anti-immunoglobulin antibodies (Pierce, Thermofisher, Waltham, MA, USA). Nuclei were stained with DAPI. Sections were observed under a fluorescent microscope with the Apotome 1 system (Zeiss, Oberkochen, Germany) interfaced with Axiovision software and a x20 objective.

TUNEL assay. Paraffin-embedded vertebrae sections from 4-month-old mice were used for TUNEL assay (ApopTag Plus Peroxidase four to eight fields in sections using the semi-automated software Bondolab (MicroVision)). Results are presented as ratio of apoptotic BMCs to total cells or ratio of total or apoptotic osteocytes to the measured bone surface.

Flow cytometry. BMCs were flushed from long bones, and red blood cells were lysed. All staining steps were performed at 4 °C in phosphate-buffered saline containing 1% fetal calf serum (FCS) and 2 mM EDTA with the antibodies anti-CD45 (30F11) and anti-Sca-1 (D7; BD Biosciences, Plymouth, UK). Cells were first labeled with cell-surface antibodies, and then with Annexin V according to the manufacturer's procedure (BD Biosciences). To analyze syndecan-2 expression in osteoblastic cells we used anti-CD51 (RMV-7, Biologend, San Diego, CA, USA) as osteoblast marker,51 anti-SCA-1 (D7, Miltenyi Biotec, Bergisch Gladbach, Germany) as precursor marker51 and anti-syndecan-2 (305515, R&D Systems, Minneapolis, MN, USA). To label endothelial cells we used anti-CD31 (390, BD Biosciences) and anti-endomucin (eBioV7C7, eBioscience). To exclude mature hematopoietic lineages from the analysis, the marrow cells were labeled with a Lineage Cell Detection Cocktail-Biotin that contains biotin-conjugated monoclonal antibodies against the following: CD5, CD11b, CD45R, Anti-7-4, Anti-Gr-1 (Ly6G/C) and Anti-Terr-119. Cells were analyzed by using FACs Canto II, and the BD FACSDiva software (BD Biosciences).

RNA preparation from bone tissues. For each animal, BMCs were collected by centrifugation of long bones and suspended in 1 ml Trizol reagent (Invitrogen). Bone marrow-free bones were cut into pieces, immediately frozen in dry ice and ground with Trizol.

Cell cultures. BMCs were flushed from long bones and cultured as described.52 Osteoblasts were obtained by migration from trabecular fragments of long trabecular bone.53 Mature pluripotent mesenchymal C3H10T1/2 cells were obtained from the ATCC, and then infected with the control or syndecan-2-coding lentiviral vector as described.54 Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; DMEM/FCS/P/S).

Ex vivo osteoclastogenesis assay. Spleen or BMCs were isolated from 10-week-old WT and Coll2Synd2 male mice. Cell suspensions were prepared by using a 40-μm nylon mesh cell strainer. After red blood cell lysis, cells were cultured in complete α-MEM medium with 10 μg/ml dihydroxyvitamin D3 and 50 μM ascorbic acid or with macrophage-colony-stimulating factor (25 ng/ml) and RANKL (50 ng/ml) culture medium on day 4 of culture. After 11 days, cell colonies were stained for ALP activity by using bromochloroindoyl-phosphate/nitroblue tetrazolium chloride (Basser et al. 1983). Cells were assayed with BMCs derived from femurs and tibiae of 10-week-old WT and Coll2Synd2 mice, osteoblasts from WT or Coll2Synd2 bones were plated in porous inserts (Nunc, Waltham, MA, USA) and cultured to confluence. BMCs from WT mice were cultured on sterile μm nylon mesh cell strainer. After red blood cell lysis, cells were cultured in complete α-MEM medium with 10% FCS and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; DMEM/FCS/P/S) containing 15% L (control) or Wnt3a-CM for 72 h. The inserts were then removed and 0.5 μM SYTOX Orange nucleic acid stain (Life Technologies, Thermofisher, Waltham, MA, USA) was added to the medium. BMCs exposed to UV light were a positive control for apoptosis induction. The cells were washed and fixed with 4% paraformaldehyde in phosphate-buffered saline and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). The ratio of Sytox- to DAPI-positive cells was measured under a fluorescence microscope with the Apotome 1 system (Zeiss) and Axiovision software.

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RT-PCR analyses. Total RNA from bone or marrow extracts of 4-month-old mice was reverse-transcribed. Gene expression was analyzed by quantitative real-time PCR (RT-qPCR) with SYBR Green, Light-Cycler 480 (Roche Molecular Diagnostics, Pleasanton, CA, USA), and the following forward and reverse primers: HPRT1: 5′-TGAAGGCGCTGCGATGTGA-3′; 5′-GGTGCTCGAGAGCTTGTA GA-3′; Actin: 5′-GGCTGTATCCCTCCCTCATCG-3′; 5′-CACATGGTGTAACAGTCGATGTG-3′; hSyndecan-2: 5′-TTGCTCTTCTCTTCATTTGG-3′; 5′-CCTCATCCTTCTTCTCTC-3′; β-catenin: 5′-GAGCAAGCTTTTGTTAGA-3′; 5′-TTGGAGAGCAGTCCAGACA-3′; Wnt3a: 5′-CTTAGTGCTCTGGACGCTGTA-3′; 5′-ACTGCTCAGAGAGGACTGCC-3′; Wnt11: 5′-CAGAGTCCCAACGCAATAAAG-3′; 5′-TCGAGGAGGCCGCACTGAG-3′; Akin2: 5′-GCCATTGGCTCCCCACT-3′; 5′-CCAGTCAGAGCAGTCGAC-3′; Wap1: 5′-GGTGACATCAACTACACATGAA-3′; 5′-AAGTGCTTGTCCTCCTCTG-3′; Rspo-2: 5′-CTGCTAGGTTCCTCCTCCTG-3′; 5′-ACATGGCCGCTCTTCTCAGAA-3′; SOX9: 5′-GGAATTGCAGCGAGTCGAC-3′; 5′-CCCCGTTCGTTGCTGTT-3′; DKK1: 5′-CCCGGAACTAGTCTGAAA-3′; 5′-CCGAAGGCATTGTGCTT-3′; SFRP-1: 5′-GCCACAACTGCTGGCTACAA-3′; 5′-ACTCTCCTGACCTGCTGGT-3′; RANKL: 5′-GAGTACTATTGGACCCGATT-3′; 5′-GGCCACAGCCGCTCTCAG-3′; OPG: 5′-CCATCTGACTATTTCTGAAA-3′; 5′-AGTGGCTGAGCAGAAGAGT-3′; RUNX2: 5′-TGGACCTTTTGGCTCAGGT-3′; 5′-AGGTGGAGCCACAGATAG-3′; Bcl2: 5′-GATCTGACAGCCGCTACATG-3′; 5′-GGGGCCATATGCTGCACCACAA-3′; Bax: 5′-TGATAGGCCTGCTTGTCT-3′; 5′-GGTCCGAAGTGAAGAGGAA-3′.

Results are presented as mean ± S.E.M. All data are expressed as mean ± S.E.M. The significance level was set at P < 0.05. Two-tailed Student's t-test for unpaired samples was used for most statistical analyses except for the measures of syndecan-2 and heparan sulfate overexpression, for which only the increase was significant. The measures of cell apoptosis and Stat3 activation were tested using one-tailed Student's t-test. The n values in the legends indicate numbers of independent biological samples used for the analyses.

Conflict of Interest
The authors declare no conflict of interest.

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