Tissue inhibitor of metalloproteinase-1 (TIMP-1) regulates mesenchymal stem cells through let-7f microRNA and Wnt/β-catenin signaling

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Tissue inhibitor of metalloproteinases 1 (TIMP-1) is a matrix metalloproteinase (MMP)-independent regulator of growth and apoptosis in various cell types. The receptors and signaling pathways that are involved in the growth factor activities of TIMP-1, however, remain controversial. RNA interference of TIMP-1 has revealed that endogenous TIMP-1 suppresses the proliferation, metabolic activity, and osteogenic differentiation capacity of human mesenchymal stem cells (hMSCs). The knockdown of TIMP-1 in hMSCs activated the Wnt/β-catenin signaling pathway as indicated by the increased stability and nuclear localization of β-catenin in TIMP-1–deficient hMSCs. Moreover, TIMP-1 knockdown cells exhibited enhanced β-catenin transcriptional activity, determined by Wnt/β-catenin target gene expression analysis and a luciferase-based β-catenin–activated reporter assay. An analysis of a mutant form of TIMP-1 that cannot inhibit MMP indicated that the effect of TIMP-1 on β-catenin signaling is MMP independent. Furthermore, the binding of CD63 to TIMP-1 on the surface of hMSCs is essential for the TIMP-1–mediated effects on Wnt/β-catenin signaling. An array analysis of microRNAs (miRNAs) and transfection studies with specific miRNA inhibitors and mimics showed that let-7f miRNA is crucial for the regulation of β-catenin activity and osteogenic differentiation by TIMP-1. Let-7f was up-regulated in TIMP-1–depleted hMSCs and demonstrably reduced axin 2, an antagonist of β-catenin stability. Our results demonstrate that TIMP-1 is a direct regulator of hMSC functions and reveal a regulatory network in which let-7f modulates Wnt/β-catenin activity.

plasticity | osteogenesis | canonical Wnt signaling

Tissue inhibitor of metalloproteinases 1 (TIMP-1) is a glycoprotein with a relative molecular weight of 28,500 that is ubiquitously expressed in numerous human cells and tissues. TIMP-1 belongs to a group of four endogenous inhibitors (TIMP-1–TIMP-4) that control the activity of matrix metalloproteinases (MMP) and other metalloproteinases (1). In this respect, TIMP-1 is an important regulator of extracellular matrix turnover. TIMP-1 was discovered as a humoral protein that promoted the proliferation of human erythroid progenitor cells (2). Since then, TIMP-1 has been shown to exhibit multiple activities in the regulation of various biological processes such as cell growth, apoptosis, and differentiation that are independent of its metalloproteinase inhibitory activity (3). In this context, CD63, a member of the tetraspanin family, has been identified as a binding protein of TIMP-1 on the cell surface (4). However, little is known about the downstream mechanisms of TIMP-1–mediated cell signaling.

Previously, we reported the constitutively high expression of TIMP-1 in human mesenchymal stem cells (hMSCs) (5). This observation prompted us to hypothesize that autocrine TIMP-1 may influence basic stem cell functions in these cells. Bone marrow hMSCs are multipotent progenitor cells that are capable of differentiating into osteocytes, chondrocytes, adipocytes, and various nonmesodermal cell types, including neural cells (6–8). These features make hMSCs valuable candidates for cell-based therapies to regenerate tissues under various pathological conditions, such as skeletal trauma, bone loss, cardiac disorders, and neurodegenerative diseases (9–11). More recent findings provide evidence that hMSCs may also be used therapeutically to modulate immune response in patients with tissue injury, transplantation, and autoimmune disease because of their ability to release diverse cytokines and chemokines (12).

The canonical Wnt/β-catenin pathway appears to play important roles in multiple physiological and pathological processes in hMSCs (13). In the absence of Wnt ligands, cytoplasmatic β-catenin is constantly degraded through the activity of the axin complex (14). Activation of the Wnt/β-catenin pathway is characterized by the binding of Wnt ligands to specific receptors, which enables the stabilization of cytoplasmatic β-catenin. In the absence of the axin degradation complex, β-catenin is translocated into the nucleus where it interacts with lymphoid-enhancer–binding factor-1/T-cell factor-1 (LEF/TCF) transcription factors, resulting in the transcription of target genes such as cyclin D1 and membrane-type 1 matrix metalloproteinase (MT1-MMP) (14–16).

In recent years, noncoding RNA molecules known as microRNAs (miRNAs) have increasingly gained attention because of their important roles as posttranscriptional gene regulators in numerous biological processes such as development, stem cell regulation, and human disease (17, 18). miRNAs are mostly transcribed from intragenic or intergenic regions and undergo further processing by Drosha and other downstream ribonucleases before being converted into mature 18- to 25-nt miRNA transcripts that are capable of gene silencing. Several studies have demonstrated that specific miRNAs or miRNA expression profiles are associated with the intrinsic stem cell properties of self-renewal and pluripotency (19, 20). Less work, however, has focused on the roles of miRNAs in known regulatory networks that control cell fate and differentiation in response to endogenous and exogenous stimuli.

In the present study, we provide evidence that endogenous TIMP-1 acts as a suppressor of hMSC growth and osteogenic differentiation through negative modulation of Wnt/β-catenin signaling activity. This effect requires the interaction of TIMP-1 and CD63 on the cell surface and is independent of MMP-inhibitory

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activity. Furthermore, we found that the underlying regulatory network involved the miRNA let-7f as a key factor targeting the translation of axin 2, which promotes β-catenin degradation.

Results

Endogenous TIMP-1 Attenuates Growth and Osteogenic Differentiation. To investigate whether endogenously expressed TIMP-1 influences the proliferation and metabolic activity of hMSCs, we analyzed the effect of decreasing TIMP-1 biosynthesis in these cells, using siRNA-mediated knockdown (Fig. S1*A*). Using the CyQuant cell proliferation assay, we showed that hMSCs transfected with siRNA directed against TIMP-1 demonstrated a significant increase in proliferative activity after 3 d of incubation and a less marked increase in proliferative activity after 7 d of incubation than cells treated with a control siRNA (Fig. 1*A*). The results of a WST-1 proliferation assay demonstrated that TIMP-1–deficient hMSCs had robust augmentation of mitochondrial dehydrogenase activity after 3 and 7 d of cultivation relative to the control cells (Fig. 1*B*).

We then determined whether endogenous TIMP-1 influenced hMSC differentiation into the osteogenic lineage. hMSCs transfected with either siRNA targeting TIMP-1 or control siRNA were grown to subconfluency and subsequently cultivated in osteogenic differentiation media. After 14 d, microscopic analysis of the alizarin red-stained monolayers revealed higher levels of mineralization in TIMP-1 knockdown hMSCs than in control cells (Fig. 1*C*). To further evaluate the effect of TIMP-1 down-regulation on the osteogenic differentiation of hMSCs, the expression levels of specific differentiation markers (alkaline phosphatase, osteocalcin, and decorin) were assayed in cells grown under osteogenic conditions for 14 d. TIMP-1–deficient hMSCs demonstrated a 15-fold increase in alkaline phosphatase, a 7-fold increase in osteocalcin, and a 2-fold increase in the levels of decorin mRNA relative to control cells (Fig. 1*D*).

Together, these findings suggest that endogenous TIMP-1 acts as a negative regulator of hMSC growth and osteogenic differentiation.

TIMP-1 Reduces Wnt/β-Catenin Signaling Activity Independently of Its MMP Activity. To investigate the influence of endogenous TIMP-1 on hMSC gene expression in detail, we conducted microarray analysis using the Human Signal Transduction PathwayFinder Gene Array (SABiosciences), which evaluates transcriptional changes in 96 different genes associated with 18 signal transduction pathways. Microarray analysis was carried out in hMSCs 3 d after transfection with TIMP-1 siRNA or control siRNA. Down-regulation of TIMP-1 enhanced the mRNA expression of 16 genes in hMSCs, whereas only 1 gene (*bone morphogenetic protein 4*) exhibited reduced mRNA levels. The majority of genes up-regulated in the TIMP-1–deficient hMSCs are implicated in the Wnt signaling pathway, i.e., *WISP1* (*WNT-inducible signaling pathway protein 1*), *WISP2*, *WISP3*, *TCF7* (*transcription factor 7, T-cell specific*), *MYC* (*v-myc myelocytomatosis viral oncogene homolog*), *JUN* (*jun oncogene*), and *BIRC5* (*baculoviral IAP repeat-containing 5*, also termed survivin) (Table S1).

The activation of the Wnt/β-catenin pathway is characterized by (*i*) the stabilization of cytoplasmatic β-catenin, (*ii*) the translocation of β-catenin into the nucleus, (*iii*) the interaction of β-catenin with TCT/LEF transcription factors, and (*iv*) the increased expression of β-catenin target genes. Therefore, we investigated each of these features experimentally. Western blot analysis revealed that β-catenin protein levels were elevated in the hMSCs on days 3 and 7 after TIMP-1 knockdown compared with control cells (Fig. 2*A*). Immunocytochemical analysis demonstrated that hMSCs transfected with siRNA against TIMP-1 demonstrated strong nuclear staining of β-catenin and that this staining was weaker in cells transfected with control siRNA (Fig. 2*B*).

Furthermore, we used a *Gaussia* luciferase-based reporter system to investigate the activation of β-catenin target promoters. The β-catenin–activated reporter (BAR) system can be used to specifically monitor β-catenin activity on TCF/LEF-dependent gene transcription (e.g., *Gaussia* luciferase). hMSCs transfected with the BAR system (BAR-hMSCs) were treated with siRNA targeting TIMP-1 or control siRNA and cultivated for up to 7 d in the presence of Wnt3a to stimulate β-catenin activity. These studies revealed that β-catenin reporter activity was significantly

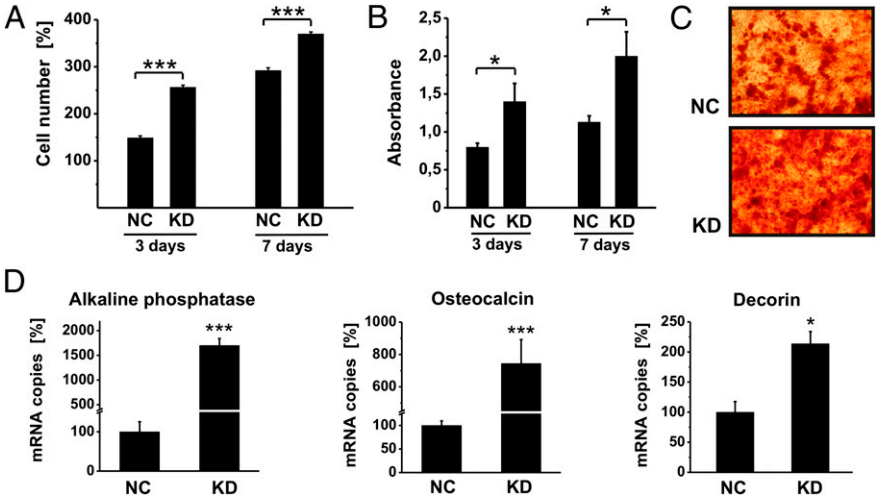

Fig. 1. TIMP-1 knockdown promotes proliferation, metabolic activity, and osteogenic differentiation in hMSCs. (*A*) Quantification of hMSC cells at 3 and 7 d after transfection with TIMP-1 siRNA (KD) or control siRNA (NC, set as 100%) using the CyQuant cell proliferation assay. (*B*) Analysis of mitochondrial dehydrogenase activity in the hMSCs at 3 and 7 d after transfection with TIMP-1 siRNA (KD) or control siRNA (NC) using the WST-1 assay. (*C, D*) hMSCs transfected with control siRNA (NC) or siRNA targeting TIMP-1 (KD) were incubated in osteogenic differentiation medium. (*C*) Representative microscopic images of cellular monolayer mineralization after alizarin red staining on day 14 of osteogenic differentiation (magnification 4×). (*D*) Relative mRNA levels of the differentiation markers alkaline phosphatase, osteocalcin, and decorin in the hMSCs on day 14 of osteogenic differentiation. Values were normalized to GAPDH mRNA. All results are given as mean values ± SD of triplicate measurements ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

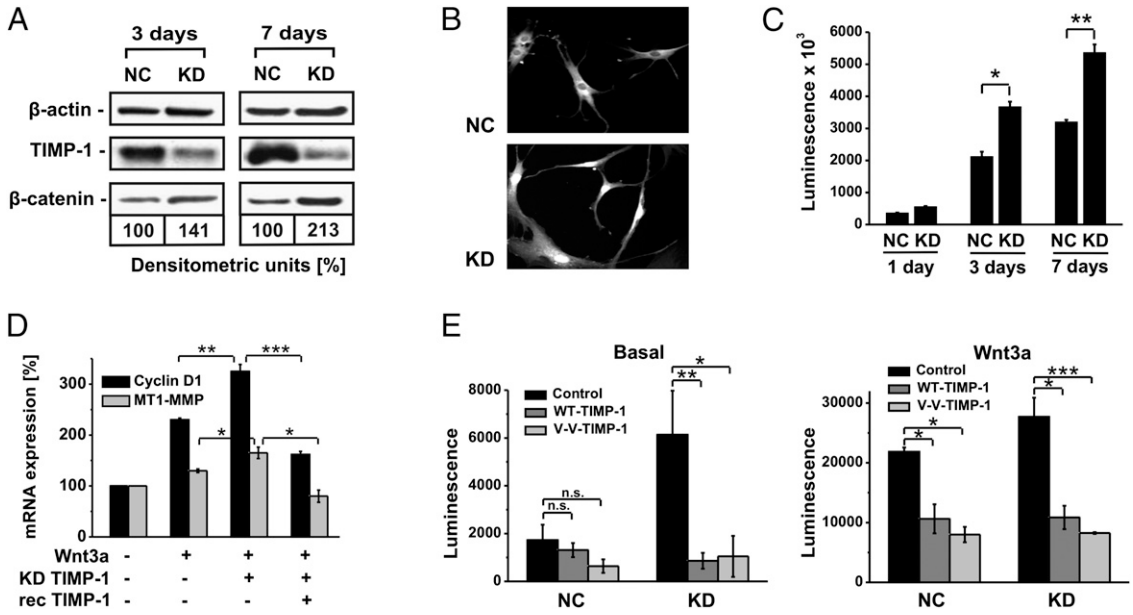

Fig. 2. TIMP-1 reduces Wnt/β-catenin activity in a MMP-independent manner. (*A*) Western blot analysis of β-catenin and TIMP-1 in protein extracts obtained from hMSCs transfected with siRNA targeting TIMP-1 (KD) or control siRNA (NC) on days 3 and 7 after transfection. For densitometric quantification, the amount of β-catenin present in cells transfected with control siRNA was set to 100% at each time point. Cellular β-actin was used as a loading control ($n = 3$). (*B*) Images of the immunocytochemical staining of β-catenin in hMSCs transfected with siRNA against TIMP-1 (KD) or control siRNA (NC) 3 d after transfection ($n = 3$) at magnification 10×. (*C*) TIMP-1 knockdown (KD) and control (NC) hMSCs were transiently transfected with the β-catenin–activated reporter (BAR) and cultivated for up to 7 d in the presence of Wnt3a (150 ng/mL) to stimulate Wnt/β-catenin activity. The activity of secreted *Gaussia* luciferase, which was transcribed due to β-catenin–dependent TCF/LEF signaling in the cells, was quantified in the conditioned medium at the indicated time points. (*D*) hMSCs transfected with TIMP-1 siRNA or control siRNA were cultured in the presence or absence of Wnt3a (150 ng/mL) and/or recombinant TIMP-1 (20 nM). After 7 d of incubation, the transcription levels of the Wnt/β-catenin target genes cyclin D1 and MT1-MMP were examined using qRT-PCR. The results are given as the percentage of change in mRNA expression relative to that in unstimulated control cells transfected with control siRNA. (*E*) The effects of wild-type TIMP-1 (WT-TIMP-1) and a non-MMP–inhibitory mutant form of TIMP-1 (V-V-TIMP-1) (each 20 nM) on β-catenin reporter activity in BAR-hMSCs without (NC) and with TIMP-1 knockdown (KD) in the absence (basal) and presence of Wnt3a (150 ng/mL) after 24 h of incubation. The data shown in *C*–*E* represent the mean ± SD of triplicate measurements ($n = 3$). n.s., not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

enhanced in hMSCs with impaired TIMP-1 biosynthesis relative to control cells over the 7-d period of measurement (Fig. 2*C*).

To confirm the inhibitory effect of TIMP-1 on β-catenin–dependent signaling in hMSCs, we performed qRT-PCR analysis of the Wnt/β-catenin target genes cyclin D1 and MT1-MMP, which contain TCF/LEF-binding sites in their promoter regions (15, 16). hMSCs transfected with siRNA targeting TIMP-1 or control siRNA were cultured for 7 d in the presence or absence of Wnt3a and recombinant TIMP-1. Wnt3a clearly up-regulated the basal transcription of cyclin D1 and, to a lesser extent, MT1-MMP. TIMP-1–deficient hMSCs demonstrated a further increase in cyclin D1 and MT1-MMP expression, which was abolished upon the addition of recombinant TIMP-1 (Fig. 2*D*). Taken together, these data indicate that TIMP-1 is a negative regulator of Wnt/β-catenin activity in hMSCs.

We conducted several experiments with a recombinant mutant form of TIMP-1 to clarify whether the effect of TIMP-1 on Wnt/β-catenin activity is dependent or independent of its MMP inhibitory activity. The mutant TIMP-1, referred to as Val-Val-TIMP-1, was engineered to contain two valine residues appended to the amino-terminal cysteine-1 of the normal wild-type (WT) TIMP-1. This modification results in a loss of inhibitory activity (Fig. S2) by disrupting the cysteine-1 interaction with the active zinc atom in the catalytic domain of MMPs (1, 21). BAR-hMSCs were cotransfected with siRNA against TIMP-1 or control siRNA and cultivated for 24 h in the absence or presence of Wnt3a, Val-Val-TIMP-1, and WT-TIMP-1. Luminometric quantification of reporter activity (*Gaussia* luciferase) revealed that the non-MMP–inhibiting Val-Val-TIMP-1 substantially inhibited both basal and Wnt3a-stimulated β-catenin signaling, similar to WT-TIMP-1. This effect was more pronounced in TIMP-1–deficient hMSCs than in control cells (Fig. 2*E*). These findings suggest that TIMP-1–mediated suppression of Wnt/β-catenin activity in hMSCs does not rely on the MMP inhibitory activity of TIMP-1.

CD63 Transduces TIMP-1–Mediated Effects on Wnt/β-Catenin Signaling. Cells were subjected to confocal microscopic analysis to determine whether CD63 binds to TIMP-1 on the surface of hMSCs. Immunofluorescence staining of nonpermeabilized hMSCs demonstrated the strong expression of both proteins on the plasma membrane. CD63 and TIMP-1 exhibited punctate costaining that was distributed unevenly on the cellular periphery, consistent with cell surface colocalization; this pattern was also documented by fluorescence intensity profiling analysis (Fig. 3*A*), and these findings were validated by coimmunoprecipitation analysis. Endogenous CD63 complexed with TIMP-1 was immunoprecipitated from hMSC lysates using an anti-CD63 antibody (Fig. 3*B*). After knockdown of CD63 in these cells (Fig. S1*B*), the amount of TIMP-1 that coprecipitated with the anti-CD63 antibody decreased (Fig. 3*B*). Moreover, Western blot and qRT-PCR analyses demonstrated that the CD63-deficient hMSCs had reduced levels of cell-associated TIMP-1 and elevated levels of soluble TIMP-1 in culture supernatants, but that there was no change in TIMP-1 mRNA expression (Fig. 3*C*). These results suggest that CD63 acts as a binding partner for TIMP-1 on the surface of hMSCs.

Next, we treated BAR-transfected hMSCs with siRNA against CD63 and observed a significant increase in TCF/LEF-dependent reporter activity in both the absence and presence of Wnt3a relative to cells that had been treated with control siRNA (Fig. 3*D*). Upon addition of recombinant TIMP-1 to CD63 knockdown hMSCs, the increase in β-catenin activity was less pronounced and

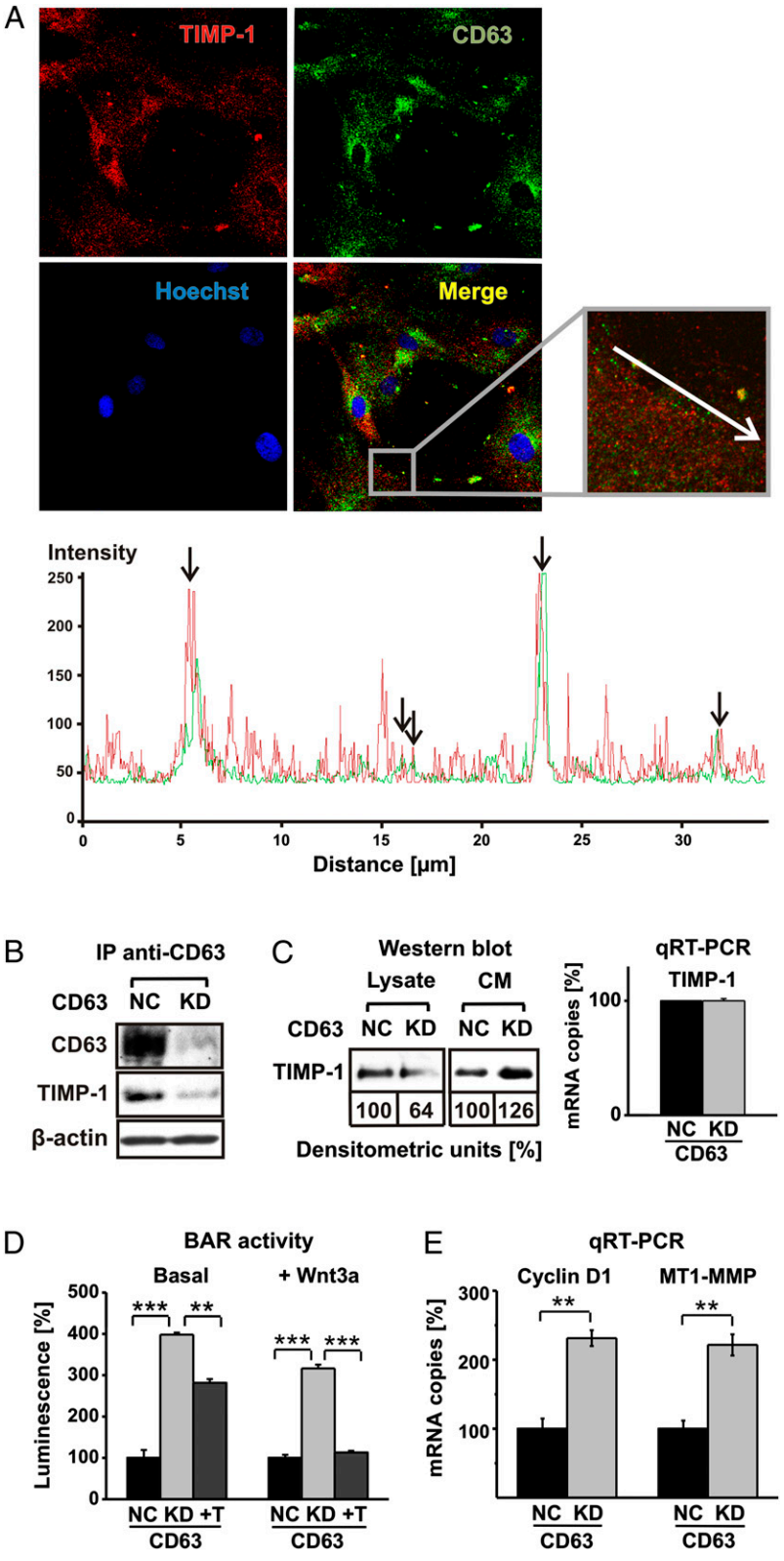

Fig. 3. TIMP-1 binding to the cell surface molecule CD63 affects Wnt/β-catenin signaling. (*A*) Confocal microscopic analysis of hMSCs costained with anti-TIMP-1/Alexa 543–conjugated secondary antibody (red staining), anti-CD63/Alexa 488–conjugated secondary antibody (green staining), and Hoechst 33342 (blue staining, for DNA labeling in cell nuclei). The merged image shows the overlay (yellow staining) of the two channels demonstrating TIMP-1 colocalization with CD63. The area marked by a square is shown in higher magnification to display the representative section (white arrow) used for fluorescence intensity profiling analysis. Intensity peaks representing the red (TIMP-1) and green (CD63) emission wavelengths indicate areas of colocalization (black arrows). (*B*) Anti-CD63 immunoprecipitates (IPs) obtained from hMSC lysates 3 d after transfection using a specific siRNA against CD63 (KD) or control siRNA (NC) were subjected to Western blotting, using anti-CD63 and anti-TIMP-1 antibodies. The detection of β-catenin in the cell lysates before immunoprecipitation served as a control. ($n = 3$). (*C*) hMSCs were transfected with siRNA against CD63 (KD) or control siRNA (NC). Equal amounts of protein were taken from the cell lysates and conditioned media (CM), and the presence of TIMP-1 was evaluated by Western blotting after 3 d of incubation. For densitometric quantification, the amounts of TIMP-1 present in the control cell lysate and the CM were set as 100%, respectively. TIMP-1 mRNA levels were determined using qRT-PCR after 24 h of incubation ($n = 3$). (*D*) BAR-hMSCs were transfected with siRNA targeting CD63 (KD) or a control siRNA (NC) and cultured for 3 d in the absence (basal) or presence of Wnt3a (150 ng/mL) and/or recombinant TIMP-1 (20 nM) (+T). β-Catenin reporter activity was quantified by measuring secreted *Gaussia* luciferase in the conditioned medium (CM). (*E*) hMSCs transfected with siRNA against CD63 (KD) or control siRNA (NC) were incubated for 3 d in the presence of Wnt3a (150 ng/mL), and the transcription levels of the Wnt/β-catenin target genes cyclin D1 and MT1-MMP were analyzed using qRT-PCR. The data shown in *D* and *E* represent the mean ± SD ($n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$.

almost absent in cells incubated without and with Wnt3a, respectively (Fig. 3*D*). Furthermore, the Wnt/β-catenin target genes cyclin D1 and MT1-MMP were up-regulated in CD63-deficient hMSCs (Fig. 3*E*).

When taken together, these findings demonstrate that CD63 is a receptor for endogenous TIMP-1 and implicate CD63 in the regulation of the Wnt/β-catenin signaling activity of hMSCs by TIMP-1.

The miRNA let-7f Regulates the TIMP-1-Mediated Inhibition of Wnt/β-Catenin Signaling. BAR-hMSCs were subjected to RNAi against Drosha (Fig. S1*C*) to determine whether TIMP-1–mediated

modulation of the Wnt/β-catenin activity in hMSCs is associated with changes in miRNA expression. Drosha is a key regulatory enzyme in miRNA biogenesis. At 3 d after transfection, Drosha-deficient hMSCs demonstrated a significant decrease in susceptibility to the TIMP-1 knockdown-mediated up-regulation of β-catenin activity (Fig. 4*A*).

To identify specific miRNAs involved in the regulation of hMSCs by TIMP-1, we silenced TIMP-1 expression and analyzed the subsequent transcriptional changes in 88 abundantly expressed and well-characterized miRNA sequences, using the Human miFinder RT^2 miRNA PCR Array (SABiosciences). Microarray analysis was carried out in hMSCs after 3 d of incubation following transfection with an siRNA targeting TIMP-1 or a control siRNA. The down-regulation of TIMP-1 in hMSCs modulated the transcription of numerous miRNAs, with the greatest increase observed in let-7f (Fig. 4*B* and Table S2). Quantification by qRT-PCR demonstrated an 18-fold augmentation of let-7f in TIMP-1–depleted hMSCs (Fig. 4*C*). Moreover, transcription of let-7f was increased 2- to 3-fold upon knockdown of CD63 in the cells (Fig. 4*C*).

Next, we studied the role of let-7f in more detail by transfecting hMSCs with let-7f miRNA (chemically synthesized, double-stranded RNA that mimics mature endogenous let-7f) and a let-7f miRNA inhibitor (chemically modified, single-stranded RNA designed to specifically bind and inhibit endogenous let-7f). The addition of the let-7f mimic to hMSCs slightly augmented β-catenin activity, whereas the inhibition of let-7f efficiently blocked

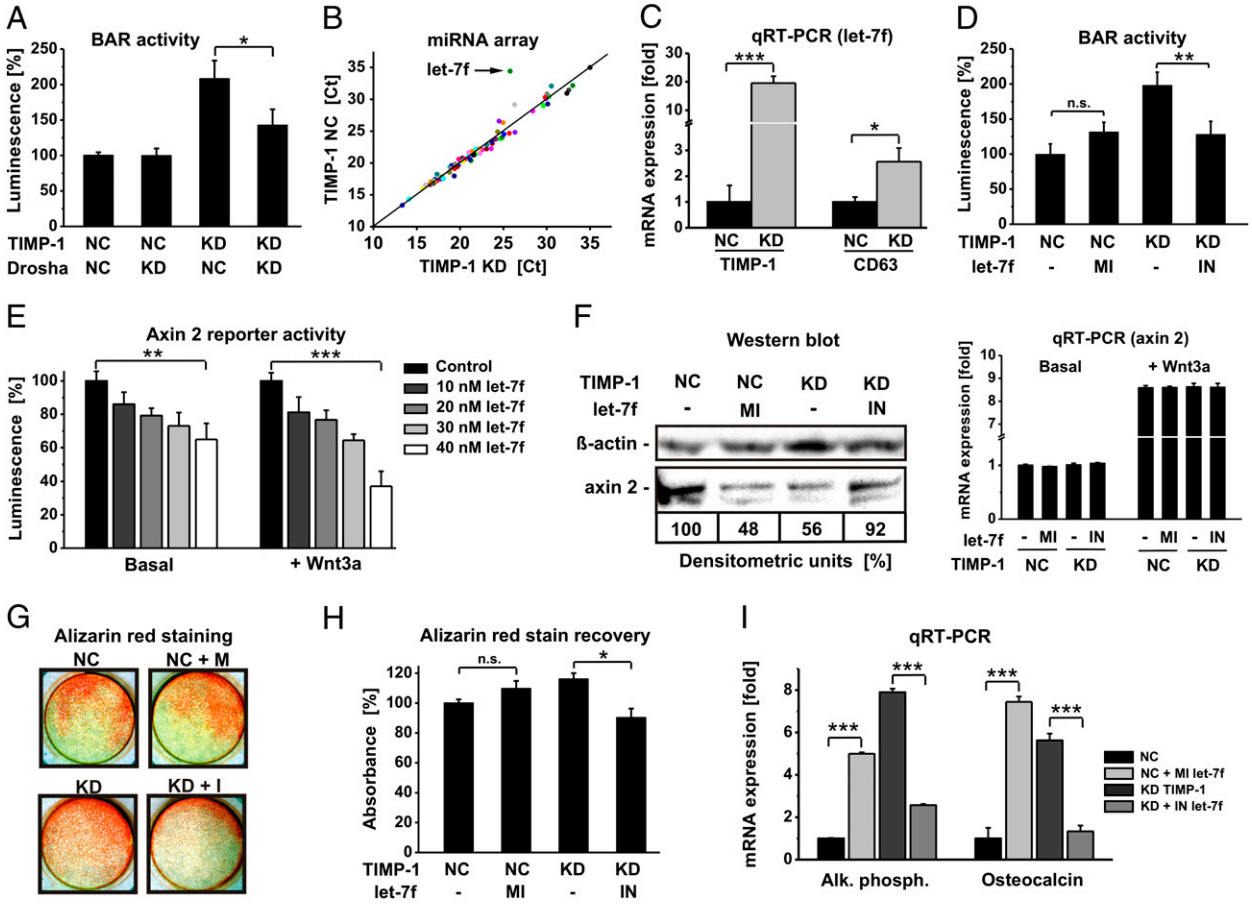

Fig. 4. The miRNA let-7f is implicated in the TIMP-1–mediated inhibition of β-catenin activity and osteogenic differentiation. (*A*) BAR-hMSCs were transfected with control siRNA (NC), siRNA targeting TIMP-1 (KD), and/or siRNA targeting Drosha (KD). After 3 d of culture, β-catenin reporter activity was determined by luminometric analysis of *Gaussia* luciferase in the culture supernatants. (*B*) hMSCs were transfected with control siRNA (NC) or siRNA targeting TIMP-1 (KD) and incubated for 3 d. RNA was collected and subjected to miRNA analysis, using PCR array technology. The results are shown in a diagram with the crossing point (*Ct*) values in TIMP-1 knockdown cells plotted against the respective *Ct*s in control cells and fitted well to a straight line, revealing that let-7f was the most up-regulated miRNA tested. (*C*) Quantitation of let-7f transcription in control cells (NC, set as 1) and hMSCs deficient (KD) in TIMP-1 and CD63 using qRT-PCR analysis. The values shown were normalized to GAPDH mRNA. (*D*) BAR-hMSCs were transfected with control siRNA (NC), siRNA against TIMP-1 (KD), a let-7f mimic (MI), and/or a let-7f inhibitor (IN). After 24 h of incubation, β-catenin reporter activity was quantified by luminometric measurement of secreted *Gaussia* luciferase in the conditioned medium (the control was set as 100%). (*E*) hMSCs were cotransfected with a *Renilla* luciferase reporter construct bearing the axin 2 3′-UTR and a let-7f mimic (10–40 nM) or control miRNA (40 nM). After 24 h, axin 2 reporter activity was quantified by luminometric measurement of *Renilla* luciferase in the cell extracts. (*F*) hMSCs were transfected with control siRNA (NC), siRNA against TIMP-1 (KD), a let-7f mimic (MI), and/or a let-7f inhibitor (IN) and cultivated in the presence of Wnt3a (150 ng/mL) to stimulate Wnt/β-catenin signaling. After 24 h, axin 2 protein expression was analyzed using Western blotting, and axin 2 mRNA expression was analyzed using qRT-PCR. β-Actin served as a loading control. For densitometric quantification, the amount of axin 2 protein present in the control cells (NC) was set as 100%. The qRT-PCR values shown were normalized to the GAPDH mRNA levels. (*G–I*) hMSCs transfected with control siRNA (NC), siRNA targeting TIMP-1 (KD), a let-7f mimic (MI), and/or a let-7f inhibitor (IN) were incubated for 14 d in osteogenic differentiation medium. (*G*) Representative images of cellular monolayer mineralization with alizarin red staining. (*H*) The relative absorbance of the alizarin red stain extracts (the NC was set as 100%). (*I*) Relative mRNA levels of the osteogenic markers alkaline phosphatase and osteocalcin were determined using qRT-PCR (the NC was set as 1). The values are normalized to the GAPDH mRNA levels. Data shown in *A*, *C–F*, *H*, and *I* are given as mean values ± SD of triplicate measurements ($n = 3$). n.s., not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the up-regulation of β-catenin signaling in TIMP-1–deficient hMSCs (Fig. 4*D*). These data indicate that let-7f participates in the TIMP-1–mediated regulation of β-catenin activity in hMSCs.

We then searched for potential let-7f targets, i.e., negative regulators of the Wnt/β-catenin signaling pathway that are complementary to (and therefore may be repressed by) let-7f. Using an *in silico* prediction algorithm, we discovered that let-7f may target axin 2 (axis inhibition protein 2), a cytoplasmatic protein that plays a key role in β-catenin degradation (22). The targeting was verified experimentally using a luciferase reporter assay and the 3′-untranslated region (UTR) of axin 2. When hMSCs were cotransfected with the reporter construct and increasing amounts of the let-7f mimic, the axin 2-specific luciferase activity was reduced in both unstimulated and Wnt3a-stimulated cells in a dose-dependent manner (Fig. 4*E*). Moreover, Western blot analysis demonstrated a clear decrease in the biosynthesis of axin 2 protein in the presence of the let-7f mimic and a rescue of axin 2 protein levels in TIMP-1 knockdown hMSCs upon inhibition of let-7f (Fig. 4*F*). Axin 2 mRNA expression was not affected by either a let-7f mimic or a let-7f inhibitor (Fig. 4*F*), indicating that let-7f regulates axin 2 at the posttranscriptional level. Taken together, these results provide evidence that let-7f promotes β-catenin activity in hMSCs by repressing the translation of axin 2.

Finally, we examined whether let-7f is involved in the regulation of osteogenic differentiation. Transfection with a let-7f mimic further increased cellular mineralization and osteogenic marker expression in hMSCs under osteogenic conditions. In contrast, the inhibition of let-7f activity by the transfection of a specific inhibitor abolished the increase of mineralization (Fig. 4 *G* and *H*) and osteogenic marker expression (Fig. 4*I*), as observed in TIMP-1–deficient cells grown in osteogenic media. These data indicate that let-7f expression in hMSCs promotes osteogenic differentiation.

Discussion

Our current study describes a mechanism by which TIMP-1 regulates essential stem cell functions. The inhibition of TIMP-1 expression in hMSCs significantly promoted cell growth and differentiation into adult cells of the osteogenic lineage, indicating that TIMP-1 is an endogenous attenuator of these processes. Previous reports have shown that TIMP-1 inhibits epithelial cell proliferation and osteoblastic differentiation (23, 24) but stimulates differentiation in hematopoietic progenitor cells, B cells, and Burkitt lymphoma cells (25–27). However, none of these studies has provided information regarding the underlying molecular mechanisms. Because TIMP-1 is secreted from hMSCs at relatively high levels in the absence of its major target protease MMP-9 (5), we hypothesized that TIMP-1 may also mediate signaling that affects gene expression and inhibits the growth and differentiation of these cells.

Our studies revealed that knockdown of TIMP-1 production in hMSCs up-regulates the stability, nuclear translocation, and promoter activity of β-catenin, demonstrating that TIMP-1 is an inhibitor of β-catenin–dependent signaling. The Wnt/β-catenin signaling pathway is closely associated with the growth and development of stem cells (13). In hMSCs, increased stability and/or activity of β-catenin has been shown to promote osteogenic differentiation, which may also occur independently of stimulation with Wnt (28, 29). In the present study, both endogenous TIMP-1 and exogenously added recombinant TIMP-1 acted as negative regulators of β-catenin activity in hMSCs, thereby preventing up-regulation of the β-catenin target genes cyclin D1 and MT1-MMP. Consistent with our data, TIMP-1 down-regulates cyclin D1 in human breast epithelial (MCF10A) cells (30).

We showed that TIMP-1 colocalizes with CD63 on the surface of hMSCs, which appears to be one extracellular mechanism by which TIMP-1 reassociates with the hMSC plasma membrane. In addition to CD63, other molecular structures appear to act as docking proteins for TIMP-1 on the plasma membrane of hMSCs; these structures will need to be identified by further studies. Nevertheless, CD63 plays an important role in the TIMP-1–mediated interference of the Wnt/β-catenin pathway. Functional depletion of CD63 in hMSCs significantly up-regulated Wnt/β-catenin activity even though TIMP-1 was still expressed in these cells. This result is consistent with previous reports demonstrating that CD63 can regulate the activity of the phosphatidylinositol 3-kinase, focal adhesion kinase, Src, and Akt signaling pathways, all of which have been implicated in the anti-apoptotic activity of TIMP-1 (31–33). Addition of an excess of TIMP-1 to CD63-depleted hMSCs attenuated the increase of Wnt/β-catenin activity in these cells, again indicating that CD63 is not the exclusive structure that interacts with TIMP-1 and thereby affects gene expression in these cells.

To further elucidate the intracellular molecular mechanism by which TIMP-1 suppresses the stability and activity of β-catenin in hMSCs, we focused on miRNAs, which have very recently been shown to play important roles in the self-renewal and differentiation of hMSCs (20). Functional disruption of the miRNA processing machinery significantly inhibited the increase in β-catenin activity in TIMP-1–depleted hMSCs, suggesting that precise levels of mature miRNAs are critical for the TIMP-1–mediated modulation of β-catenin. This finding is consistent with other recent data demonstrating that various miRNAs can act as positive or negative regulators of β-catenin transcriptional activity (34). Among the miRNAs that were up-regulated after knockdown of TIMP-1 biosynthesis in our hMSC experiments, let-7f exhibited the most pronounced increase. This is especially interesting because the let-7 family of miRNAs is thought to represent "pro-differentiation" factors with "antistemness" properties (35). For example, mature let-7 miRNAs are undetectable in embryonic stem cells but accumulate strongly after the onset of differentiation in these cells (36). Moreover, other studies in hMSCs have demonstrated that specific let-7 miRNAs are up-regulated during osteogenic differentiation and presumably down-regulate factors that inhibit osteogenesis (37, 38).

miRNAs can modulate β-catenin–dependent transcription in several ways, including the direct inhibition of β-catenin mRNA or the indirect inhibition of negative and positive regulators of β-catenin activity (34). Axin 2 is a negative regulator of the Wnt/β-catenin signaling pathway and was identified in silico and in vitro as a let-7f target in hMSCs. Similar to its homolog axin 1, axin 2 is thought to act as a scaffolding protein that organizes a multiprotein complex that can degrade β-catenin (39). Indeed, axin 2 translation is abrogated in TIMP-1–depleted hMSCs, concomitant with the increased β-catenin activity observed in these cells. Furthermore, axin 2 protein levels were rescued upon let-7f inhibition, confirming that axin 2 is a target of let-7f. Axin 2 down-regulation by let-7f may explain the direct increase in β-catenin activity by let-7f in hMSCs. Although additional miRNAs are clearly involved in the control of osteogenic differentiation in hMSCs, our findings indicate that let-7f is a key miRNA in this process.

In conclusion, we have demonstrated that TIMP-1 is a negative regulator of the growth and osteogenic differentiation of hMSCs and thus promotes a quiescent state in these cells. TIMP-1 binding to CD63 on the surface of hMSCs results in the repression of let-7f miRNA expression, thereby stabilizing the levels of axin 2 and subsequently promoting the degradation of β-catenin. Importantly, our results show that the functional depletion of TIMP-1 or an increase in let-7f accelerates osteogenic differentiation in hMSCs. A deeper knowledge of this regulatory network may improve the development of novel therapeutic approaches to enhance bone formation during pathological bone loss.

Experimental Procedures

Cultivation, Osteogenic Differentiation, and Alizarin Red Staining of hMSCs. hMSCs from bone marrow aspirates were purchased from Lonza as frozen vials of passage-one cells. Each hMSC lot was tested by Lonza for purity using

flow cytometry and for their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. The cells were positive for CD105, CD166, CD29, and CD44 and negative for CD14, CD34, and CD45.

hMSCs were cultured as described previously, using the mesenchymal stem-cell growth medium (MSCGM) BulletKit (Lonza) (8). For experiments under serum-free conditions, hMSCs were washed with serum-free medium and incubated in DMEM supplemented with 1% Nutridoma SP (Roche Applied Science) in the presence or absence of 20 nM human recombinant TIMP-1 (Peprotech), the null-inhibition mutant Val-Val-TIMP-1, or its active analog WT TIMP-1, both kindly provided by P. Nelson, Ludwig-Maximilians-University.

Osteogenic differentiation and alizarin red staining of hMSCs were performed as previously described (40). Alizarin red stain incorporation was quantified by extracting stained monolayers with a solution containing 50% ethanol, 10% methanol, and 2% SDS. Aliquots of the extracted dye were then transferred to 96-well plates and the absorbance at 405 nm was measured using a plate reader (Tecan). All studies were carried out with hMSCs from the fifth or sixth passage.

CyQuant Cell Proliferation Assay and WST-1 Assay for Cell Metabolic Activity. Cell proliferation was quantified using the CyQuant cell proliferation assay kit (Invitrogen) as previously described (40). Briefly, nucleic acids present in cell lysates were stained with fluorescent CyQuant GR dye before measurement at 480 nm (excitation) and 530 nm (emission). For each experiment, a standard calibration curve was generated by plotting the measured fluorescence values of the samples vs. the corresponding number of cells, which had been determined before staining using a hematocytometer.

The WST-1 assay (Roche Applied Science), which is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells, was conducted according to the manufacturer's protocol.

qRT-PCR Analysis of mRNA and miRNA Expression. RNA isolation, cDNA synthesis, and PCR were performed as described in ref. 5. qRT-PCR was carried out on a LightCycler (Roche Applied Science), using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science). For amplification of specific human transcripts, the LightCycler Primer Sets for alkaline phosphate, osteocalcin, decorin, cyclin D1, MT1-MMP, axin 2, and the housekeeping gene standard GAPDH were used following the manufacturer's instructions (Search LC).

The expression of select miRNAs was determined using the miScript PCR System (Qiagen), which converts miRNA into cDNA, which was detected using SYBR Green-based RT-PCR. The let-7f primer sequence is given in Table S3.

Transfection of hMSCs with siRNA, miRNA Mimics, and miRNA Inhibitors. RNA interference (RNAi) technology was used to generate specific knockdowns of TIMP-1, CD63, and Drosha expression in hMSCs. siRNA sense and antisense oligonucleotides targeting human TIMP-1 mRNA were designed in our laboratory according to the protocol of Reynolds et al. (41) and synthesized by Qiagen. siRNA against CD63, Drosha, and a nonspecific siRNA with no target in the human transcriptome (used as a negative control) were purchased from Qiagen. For studies of miRNA function and gene regulation, miScript miRNA mimics and miScript miRNA inhibitors of let-7f were used (Qiagen). The sequences of all siRNAs, miRNA mimics, and miRNA inhibitors used are listed in Table S3. hMSCs were transfected with miRNA mimics (20 nM) and miRNA inhibitors (20 nM) according to a previously described method for hMSC transfection with siRNA (5).

miRNA Target Prediction and Experimental Validation of Target Genes. We used the computational target prediction algorithm miRanda to identify mature gene transcripts that might be targeted by let-7f at their 3′-UTRs (42). Axin 2 was then experimentally verified as a let-7f target gene in hMSCs according to the protocol suggested by Huang et al. (43).

Reporter assays used a LightSwitch GoClone 3′-UTR Reporter Construct (BioCat) based on the 3′-UTR sequence of axin 2, which was cloned downstream of the *Renilla* luciferase gene. hMSCs grown in microtiter plates were cotransfected with the axin 2 reporter plasmid (100 ng) and either the let-7f mimic (10–40 nM) or the control siRNA (40 nM) (Qiagen). After 24 h of incubation, 100 μL of LightSwitch reagent (BioCat) was added to each well, and luminescence was measured using a plate-reading luminometer (Tecan).

The effect of let-7f on axin 2 was examined by Western blot analysis of axin 2 protein levels in hMSC extracts 24 h after treatment with the let-7f mimic or inhibitor. qRT-PCR analysis was performed to quantitate the level of axin 2 transcript in the hMSCs 1 d after incubation with the let-7f mimic or inhibitor.

Transcription-Based Reporter Assay of Wnt/β-Catenin Signaling. The activity of the Wnt/β-catenin signaling pathway, which culminates in the TCF/LEF-dependent regulation of target gene transcription, was monitored in the hMSCs, using a reporter assay. This assay was based on the BAR, which contains multimerized TCF/LEF DNA-binding sites, and the control reporter, found-unresponsive BAR (fuBAR) (44). We modified the BAR/fuBAR by cloning *Gaussia* luciferase as the reporter gene to allow the detection of the reporter in the supernatant; after its transcription is activated by β-catenin, *Gaussia* luciferase is secreted from the cells, so the cells do not have to be destroyed to assay its transcription.

hMSCs were transfected with the BAR (or fuBAR, which harbors mutant TCF-binding sites) *Gaussia* luciferase reporter plasmid and cultivated in the presence of geneticin (100 μg/mL) to eliminate nontransfected cells. Then, the BAR/fuBAR-hMSCs were used for the stimulation and/or knockdown experiments. Conditioned medium was collected after the indicated time intervals and analyzed for the presence of reporter protein, using the *Gaussia* luciferase assay kit (New England Biolabs) according to the manufacturer's protocol. The light intensity was determined using a plate-reading luminometer (Tecan). No *Gaussia* luciferase activity was detected in conditioned media from hMSCs transfected with the fuBAR system in any experiment performed in this study.

Western Blot and Coimmunoprecipitation Analyses. Protein extraction from the hMSCs, SDS/polyacrylamide gel electrophoresis, and Western blotting were performed as described in ref. 5. The blotted membranes were incubated overnight with monoclonal antibodies against β-actin (1:2,000) and TIMP-1 (1:500) (both from Abcam), β-catenin (1:500) (Santa Cruz Biotechnology), CD63 (1:1,000) (Millipore), or axin 2 (1:1,000) (Cell Signaling Technology) diluted in Tris-buffered saline containing 0.1% Tween-20 (TBS-Tween buffer) containing 5% BSA. The membranes were washed in TBS-Tween buffer and then incubated with peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) at a dilution of 1:8,000 in TBS-Tween for 30 min. Bound antibodies were detected using the enhanced chemiluminescence system (GE Healthcare Life Sciences). Recombinant protein standards (Invitrogen) were used for molecular mass determination.

Coimmunoprecipitation was performed to investigate protein interactions. Total hMSC lysates were prepared in lysis buffer as described in ref. 5. Aliquots (10 μg of protein) were incubated overnight at 4 °C with anti-CD63 (2 μg) (Millipore) or control IgG in TBS-Tween. The immunocomplexes were collected on protein A agarose beads (Invitrogen), following the manufacturer's instructions. Bound proteins/complexes were then eluted and subjected to Western blotting.

Immunocytochemistry and Confocal Microscopic Analysis. The subcellular localization of β-catenin protein in the hMSCs was examined by immunocytochemistry as previously described (8). Culture slides were incubated with mouse anti–β-catenin antibodies (Santa Cruz Biotechnology) at a dilution of 1:500 in a solution containing 0.5% Triton X-100 and 10% normal goat serum (NGS) for 2 h at 37 °C. After several washes in PBS, the culture slides were incubated with rhodamine-conjugated anti-rabbit IgG-R (Santa Cruz Biotechnology) at a dilution of 1:500 for 45 min at room temperature and visualized using an Olympus IX70 microscope equipped with an Olympus 20× objective lens (numerical aperture 0.55) at 20 °C.

For confocal microscopic analysis, the cells were seeded on Ibidi eight-well slides and incubated overnight with conditioned hMSC medium. The medium contained secreted TIMP-1 to saturate potential TIMP-1 surface docking receptors on the hMSCs. The cells were fixed with methanol-free 4% formalin and then washed and stained with monoclonal anti-TIMP-1 (Chemicon) (1:500) and anti-CD63 (Santa Cruz) (1:125) antibodies. Nuclei were fluorescently labeled with Hoechst 33342 (Thermo Scientific). All secondary antibodies were purchased from Invitrogen. Confocal images were obtained using a Zeiss LSM 510 microscope equipped with a 40× oil immersion objective. Pinhole diameters were adjusted to visualize 1.5-μm optical slices. To detect spots of colocalization of both proteins, fluorescence intensity profiles were obtained from representative images. Intensity peaks that were detected at both emission wavelengths indicated areas of colocalization.

Statistical Analysis. Statistical significance was assessed by comparing mean (±SD) values, using Student's *t* tests for independent groups. One-way analysis of variance followed by Dunnett's test was used to compare multiple groups. Significance was assumed for $P < 0.05$. Statistical analysis was conducted using Origin 8.0 software (OriginLab).

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