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RESEARCH ARTICLE

Histatin-1 is a novel osteogenic factor that promotes bone cell adhesion, migration, and differentiation

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Abstract

Histatin-1 is a salivary antimicrobial peptide involved in the maintenance of enamel and oral mucosal homeostasis. Moreover, Histatin-1 has been shown to promote re-epithelialization in soft tissues, by stimulating cell adhesion and migration in oral and dermal keratinocytes, gingival and skin fibroblasts, endothelial cells and corneal epithelial cells. The broad-spectrum activity of Histatin-1 suggests that it behaves as a universal wound healing promoter, although this is far from being clear yet. Here, we report that Histatin-1 is a novel osteogenic factor that promotes bone cell adhesion, migration, and differentiation. Specifically, Histatin-1 promoted cell adhesion, spreading, and migration of SAOS-2 cells and MC3T3-E1 preosteoblasts in vitro, when placed on a fibronectin matrix. Besides, Histatin-1 induced the expression of osteogenic genes, including osteocalcin, osteopontin, and Runx2, and increased both activity and protein levels of alkaline phosphatase. Furthermore, Histatin-1 promoted mineralization in vitro, as it augmented the formation of calcium deposits in both SAOS-2 and MC3T3-E1 cells. Mechanistically, although Histatin-1 failed to activate ERK1/2, FAK, and Akt, which are signaling proteins associated with osteogenic differentiation or cell migration, it triggered nuclear relocalization of β-catenin. Strikingly, the effects of Histatin-1 were recapitulated in cells that are nonosteogenically committed, since it promoted surface adhesion, migration, and the acquisition of osteogenic markers in primary mesenchymal cells derived from the apical papilla and dental pulp. Collectively, these observations indicate that Histatin-1 is a novel osteogenic factor that promotes bone cell differentiation, surface adhesion and migration, as crucial events required for bone tissue regeneration.

KEYWORDS
bone, differentiation, mesenchymal, osteogenic, salivary peptide, wound healing

Pedro Torres and Nadia Hernández contributed to this study.

1 | INTRODUCTION

Bone tissue is highly dynamic, as it undergoes continuous synthesis, remodeling and resorption, thereby depicting intrinsic potential to self-repair. However, this capacity is overwhelmed by several factors, including aging, chronic diseases and by certain pharmacological treatments. Thus, considerable research in bone regenerative medicine seeks for novel approaches to improve bone wound healing. These include reducing the inflammatory responses and stimulation of efficient callous formation and remodeling stages (Clark et al., 2017; Marsell & Einhorn, 2011). Ideally therapies should improve not only bone cell differentiation, but also conductive responses, such as cell surface adhesion, spreading and migration, as well as other processes required for successful wound healing, such as appropriate vascularization (Ghiasi et al., 2017; Marsell & Einhorn, 2011). In this context, the search of multifunctional molecules has gained interest, as they are expected to impact events contributing complementarily to bone wound healing.

Histatin-1 is a peptide secreted in various human body fluids (Melino et al., 2014; Torres et al., 2018). In saliva, Histatin-1 is known for its antimicrobial activity and maintenance of tooth enamel homeostasis (Khurshid et al., 2017; Melino et al., 2014). Recently, it was found that Histatin-1 promotes wound healing in soft tissues in vitro and in vivo, including oral mucosa, skin, and corneal epithelium. This raises the view that Histatin-1 behaves as a multifunctional molecule that evokes multiple responses in different cell types (reviewed in Torres et al. (2018); Van Dijk et al. (2018)). Specifically, Histatin-1 is a potent activator of cell stretching and migration in oral keratinocytes (Oudhoff et al., 2008), fibroblasts (Oudhoff, van den Keijbus et al., 2009), and endothelial cells (Torres et al., 2017; Van Dijk, Ferrando et al., 2017). Accordingly, Histatin-1 was shown to stimulate the re-epithelialization of engineered skin (Oudhoff, Kroeze et al., 2009), to promote vascular morphogenesis in vitro and angiogenesis in vivo (Torres et al., 2017) and to promote wound healing in vivo in epithelial corneal tissue (Oydanich et al., 2018). Moreover, a recent study from our laboratory showed that Histatin-1 protects cells committed to the osteoblastic lineage against zoledronic acid, which is a drug used to treat bone resorptive disorders (Castro et al., 2019). The latter is intriguing, because it extends the spectrum of cells that are sensitive to Histatin-1, raising the possibility that Histatin-1 impinges on aspects of bone cell biology. Another recent study explored a possible relationship between Histatin-1 and the osteogenic differentiation agent *all trans retinoic acid* in MC3T3-E1 preosteoblasts, showing that both molecules promote cell spreading and the activity of alkaline phosphatase (ALP), which is involved in bone mineralization (Sun, Shi, Ma et al., 2020). However, that study showed no alterations in ALP activity upon treatment with Histatin-1 alone, which might be due to the result of brief exposure times or usage of a single cell line. Interestingly, a more recent study showed that Histatin-1 increases the efficiency of bone morphogenetic protein-2 (BMP-2) in inducing ectopic bone formation in vivo (Sun, Shi, Shen et al., 2020), although the mechanisms underlying such effects have not been explored. Consequently, the basic question as to whether Histatin-1 behaves as a factor that promotes osteogenic differentiation and other bone cell responses, remains unclear. Here, we provide evidence that Histatin-1 is an osteogenic factor that promotes bone cell adhesion, migration, and differentiation. Namely, Histatin-1 increased surface adhesion and migration of SAOS-2 osteosarcoma cells, MC3T3-E1 preosteoblasts and primary mesenchymal cells derived from the dental pulp and tooth apical papilla. In line, Histatin-1 induced the expression of osteocalcin, osteopontin, and Runx2, events that were accompanied by increased expression and activity of ALP. These findings uncover a novel role for Histatin-1 in promoting osteogenic differentiation and function, opening new avenues to explore its effects in bone and pulpal regenerative medicine.

2 | MATERIALS AND METHODS

2.1 | Histatin-1 synthesis

All peptides were obtained by chemical synthesis, as previously described (Torres et al., 2017). Histatin-1 consisted on the sequence DpSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN, which is a phosphopeptide (phospho-Serine2), whereas the scrambled sequence SYGYKNpSPLYNHHDRFEDKYGHFEDHFSRRHKREYGHH, which consisted on a phosphopeptide (phospho-Serine2), was used as control peptide. The nonphosphorylated version of Histatin-1, consisting on the following sequence: DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN, was also used in selected experiments. Chemical purity and authenticity were confirmed by reverse phase high-performance liquid chromatography and mass spectrometry, whereas all samples were removed from remnant trifluoracetic acid by ion exchange and obtained as the acetate salt (0.1%–0.5% trifluoroacetic acid).

2.2 | Materials

Rabbit polyclonal anti-ALP was from Origene (Cat TA324563), mouse monoclonal anti-β-catenin was from Dako (Cat M3539). Goat anti-rabbit and goat anti-mouse antibodies coupled to HRP were from Bio-Rad Laboratories. Wnt-C59 is the inhibitor of porcupine (inhibitor II C59) and was from Calbiochem. Tissue culture medium, antibiotics and fetal bovine serum (FBS) were from Corning Mediatech. The EZ-ECL chemiluminescent substrate was from Pierce Chemical.

2.3 | Cell culture

SAOS-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-high glucose, supplemented with penicillin (10,000 U/ml) and streptomycin (10 μg/ml), 10% FBS. All cells were incubated at 37°C and 5% CO_2. MC3T3-E1 were maintained in minimum essential medium alpha modification with L-glutamine, supplemented with penicillin (10,000 U/ml) and streptomycin (10 μg/ml) and 10% FBS.

2.4 | Apical papilla and dental pulp samples

Premolars with incomplete rhyzogenesis from patients with an indication of extraction by orthodontic reasons were collected to assess the cellularity of the pulp chamber, root canal, and apical papilla zone. Primary cultures were obtained from tissue explants, as previously reported by our group (Caceres et al., 2014). To this end, explants were obtained from apical papilla of young immature premolars ($n = 5$; all donors signed informed consent in agreement to the Ethical Committee, Faculty of Medicine, Universidad de Chile; procedures were performed in accordance to the protocol 011-2018). Primary mesenchymal cells were obtained according to the method of Sonoyama et al. (2008) which allowed to the isolation of apical papilla residing cells from human immature permanent teeth. Cell cultures were characterized by phase contrast microscopy and immunofluorescence. While most cells were positive for vimentin, 10%–30% of cells were positive for STRO-1 (data not shown). These cells were capable to differentiate into bone-like cells following incubation with differentiating media (β-glycerophosphate, L-ascorbic acid, dexamethasone). Also, these cells depicted higher proliferation rates than primary gingival fibroblasts. Cells were maintained in DMEM containing 10% FBS and Normocin (Invivogen), at 37°C and 5% CO_2. All experiments were performed using cells expanded between passages three and eight, as higher passages increase variability and spontaneous differentiation.

2.5 | Immunofluorescence

Cells were grown for 24 h on glass coverslips and following each treatment, samples were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min, permeabilized with 0.1% Triton X-100/PBS for 15 min, washed with PBS and blocked with 5% bovine serum albumin/PBS. Samples were then incubated with primary antibodies overnight at 4°C, washed three times (PBS, 5 min) and incubated with secondary antibodies for 1 h, and then washed three times and mounted using the DAKO assembly medium. The samples were visualized by microscopy, using a Nikon C2 Plus confocal microscope.

2.6 | Migration assays

Cell migration was evaluated in Boyden chamber assays (Transwell Costar, 6.5-μm diameter, 8-mm pore size), as previously reported (Torres et al., 2017).

2.7 | Rab5-GTP, Rab7-GTP, and Rab11-GTP pulldown assays

Rab5-GTP, Rab7-GTP, and Rab11-GTP pulldown assays were performed as previously described (Mendoza et al., 2013; Reyes et al., 2020). Briefly, cells were lysed in a buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1% NP 40%, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors. Extracts were incubated for 5 min on ice and clarified by centrifugation (10,000*g*, 1 min, 4°C). Postnuclear supernatants were used for pulldown assays with 30 μg of GSH beads precoated with glutathione-S-transferase (GST)-Rab5-binding domain (R5BD) (Rab5-GTP pulldown), GST-RILP (Rab7-GTP pulldown), or GST-FIP (Rab11-GTP pulldown), per condition. Beads were incubated with supernatants for 15 min at 4°C in a rotating shaker. Thereafter, beads were collected, washed with lysis buffer containing 0.01% NP 40 and samples were analyzed by Western blotting.

2.8 | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot

Cells were washed with ice-cold PBS and homogenized in lysis buffer supplemented with protease and phosphatase inhibitors. Subsequently, the total protein extracts were subjected to denaturing polyacrylamide gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Resolved proteins were transferred to nitrocellulose membranes and used for Western blotting. Membranes were blocked with 5% milk in 0.1% Tween-TBS and then incubated with antibodies. Primary antibodies were detected with secondary antibodies conjugated with the enzyme horseradish peroxidase (HRP) and determined by using the chemiluminescence EZ-ECL system.

2.9 | Alizarin red staining

Induction of osteogenic/odontogenic differentiation was performed in mesenchymal cells derived from apical papilla and the dental pulp, under culture conditions of complete medium (DMEM) supplemented with 10% FBS, 1% penicillin–streptomycin) and either 10 μM Histatin-1 or differentiation agents (5 mM β-glycerophosphate, 50 mM L-ascorbic acid 2-phosphate and 10 nM dexamethasone). After 7 days of incubation with Histatin-1 or differentiation medium, cells were fixed and mineral nodules were stained with 1% alizarin red-S (A5533; Sigma-Aldrich) at pH 4.2 (Wang et al., 2019). Total mineralized material produced per well was quantified by extracting alizarin red-S from stained sites, upon addition of 2 ml cetylpyridinium chloride buffer (10% wt/vol) in 10 mM Na_2HPO_4 (pH 7.0) for 12 h at 37°C. Thereafter, optical density at 550 nm was measured, and, alternatively, the number of crystals per field, as well as the area per crystal, was calculated by using the *ImageJ* software.

2.10 | RNA extraction and analysis

Total RNA was extracted with TRIZOL (Invitrogen, Life Technologies). For complementary DNA (cDNA) synthesis, samples were

treated with RNase-Free DNase Kit (#M6101; Promega), quantified, and purity was verified for subsequent reverse transcription, using the cDNA Reverse Transcription Kit (Applied Biosystems). Runx2, osteocalcin, osteopontin, and GAPDH were quantified by quantitative reverse transcription polymerase chain reaction ($\Delta\Delta C_t$ method; Applied Biosystems), using the following forward and reverse primers, respectively: Runx2, 5′-GCCTTCAAGGTGGTAGCCC-3′ and 5′-CGTTACCCGCCATGACAGTA-3′; osteocalcin, 5′-GAAGCCCAGCGGTGCA-3′ and 5′-CACTACCTCGCTGCCCTCC-3′; osteopontin, 5′-GAAGTTTCGCAGACCTGACAT-3′ and 5′-GTATGCACCATTCAACTCCTCG-3′; ALP, 5′-GGATCTGACCCTCCCAGTCT-3′ and 5′-AGTGAGTGAGTGAGCAAGGC-3'; GAPDH, 5′-TCAACGACCACTTTGTCAAGCTCA-3′ and 5′-GCTGGTGGTCCAGGGGTCTTACT-3′.

2.11 | Statistical analysis

An exploratory data analysis was performed using descriptive statistics. *t*-test and analysis of variance were used (Tukey's post-hoc test). Values averaged from at least three independent experiments were compared. A value of significance of 5% or less ($p < 0.05$) was accepted as statistically significant. All statistical tests were performed using Stata 11.0 software.

3 | RESULTS

3.1 | Histatin-1 promotes bone cell adhesion, spreading, and migration

Recent studies indicated that Histatin-1 prevents both cytotoxicity and anti-migratory effects of the bone-resorptive drug, zoledronic acid, in SAOS-2 osteosarcoma and MC3T3-E1 preosteoblasts (Castro et al., 2019). Besides, it was shown that Histatin-1 promotes spreading of MC3T3-E1 cells on titanium surfaces (Van Dijk, Beker et al., 2017). These findings made us hypothesize that Histatin-1 is a factor that induces cell migration by itself, as well as cell adhesion and spreading over a physiological extracellular matrix. This is in line with those observations made in other cell types, including oral and skin keratinocytes, endothelial cells, and fibroblasts (Oudhoff et al., 2008; Oudhoff, van den Keijbus et al., 2009; Torres et al., 2017). To address this, SAOS-2 and MC3T3-E1 cells were exposed to physiological concentrations of Histatin-1 (10 μM) (Castro et al., 2019) and the different events were measured. First, as described in endothelial and epithelial cells (Oudhoff et al., 2008; Torres et al., 2017), Histatin-1 caused a 1.4-fold increase in cell adhesion, with significant differences at 30 min, as shown in SAOS-2 cells (Figure 1a,b). Next, the extent of cell spreading upon cell attachment was evaluated in both SAOS-2 and MC3T3-E1 cells. In doing so, following adhesion to fibronectin, the extent of cell spreading was increased by Histatin-1, as observed in both SAOS-2 and MC3T3-E1 cells (Figure 1c,d). Finally, using Transwell Boyden Chambers, the effects of Histatin-1 were evaluated in SAOS-2 and MC3T3-E1 cells, observing that in both cases, Histatin-1 induced a significant increase in cell migration (Figure 1e,f).

Although Histatin-1 is naturally found as a phosphoprotein, that is phosphorylated in Serine2, the migration-promoting effects of this peptide in soft tissue cells were previously demonstrated to be independent on that phosphorylation (Oudhoff et al., 2008). Accordingly, we observed that nonphosphorylated Histatin-1 triggered a similar response in promoting migration of SAOS-2 and MC3T3-E1 cells, with a similar magnitude of that observed with the phosphorylated peptide (Figure 1e,f). Hence, we conclude that the effects of Histatin-1 in bone cell adhesion, spreading, and migration are reminiscent of those effects observed in cells derived from soft tissues.

3.2 | Histatin-1 induces the expression of osteogenic differentiation markers and mineralization capacity in vitro

The observation that Histatin-1 increases cell adhesion, spreading, and migration in SAOS-2 and MC3T3-E1 cells are intriguing, because all these events are somewhat related to different functions in bone cells and the capacity to undergo osteogenic differentiation (Ghiasi et al., 2017; Marsell & Einhorn, 2011). Therefore, we first evaluated the mineralizing activity in vitro, by incubating cells for 7 days in the absence or presence of Histatin-1, and the formation of calcium deposits was assessed via alizarin red staining. In doing so, SAOS-2 cells, which depicted high basal mineralization, showed a significant increase in both number of calcium deposits and the area of mineralization, following treatment with Histatin-1 (Figure 2a). Alternatively, we evaluated the effects of Histatin-1 in MC3T3-E1 preosteoblasts, which depict low basal mineralization, but are sensitive to known osteogenic differentiation inducers (ascorbic acid, glycerophosphate, dexamethasone), augmenting formation of calcium deposits (Figure 2b). Although the extent of calcium deposit formation was not equivalent to that observed with the positive control, MC3T3-E1 depicted a modest, but significant increase in the extent of mineralization when compared with the control condition (Figure 2b).

Mineralization capacity is commonly attributed to the activity of the enzyme ALP. Thus, we evaluated the activity of ALP in SAOS-2 cells and found that it was increased by a 18%–22%, following 24 and 48 h treatments with Histatin-1 (Figure 2c). Since both mineralization and ALP activity were increased by Histatin-1, we next evaluated the expression of osteogenic differentiation markers in SAOS-2 and MC3T3-E1 cells. Surprisingly, unlike ALP activity, which showed a modest increase following Histatin-1 treatment, Western blot analysis showed a fourfold and twofold increase in ALP protein levels in SAOS-2 and MC3T3-E1 cells, respectively (Figures 3a and S1a). These differences might be due to the different protein extraction protocols used for determining enzyme activity and total protein content. On the other hand, we observed that Histatin-1

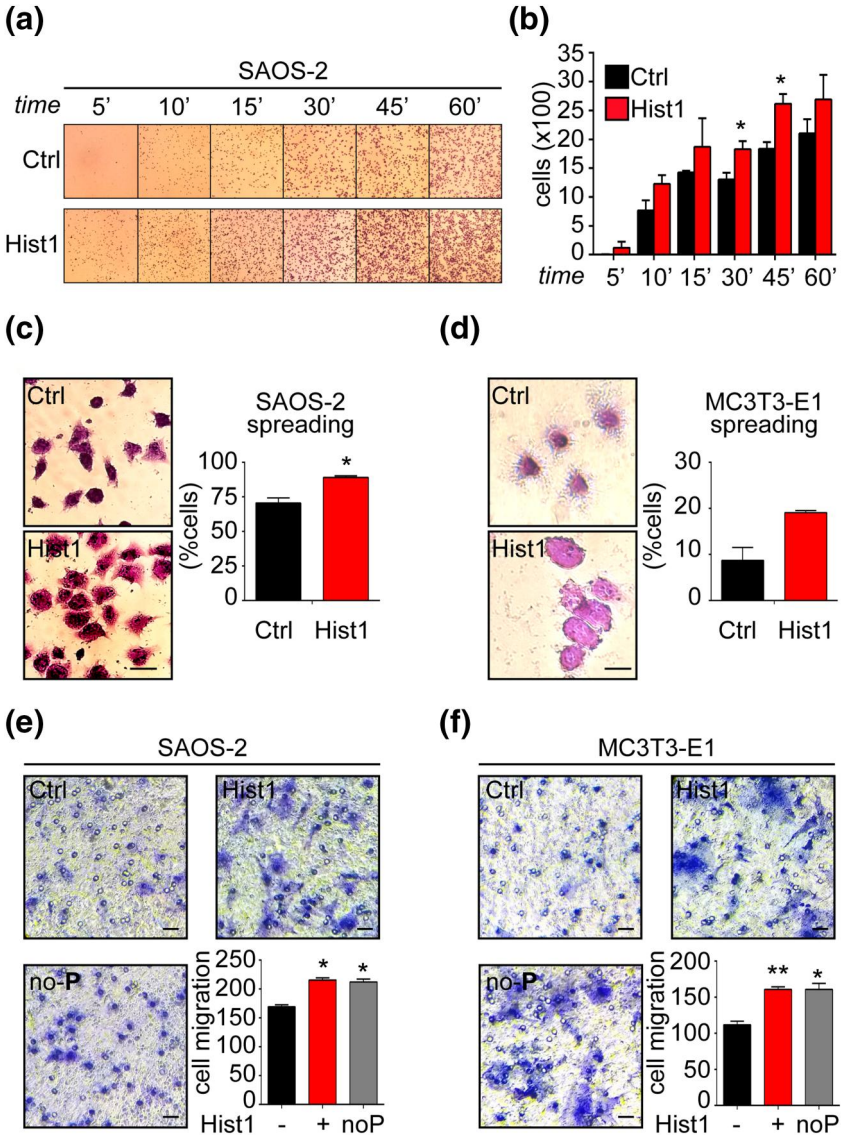
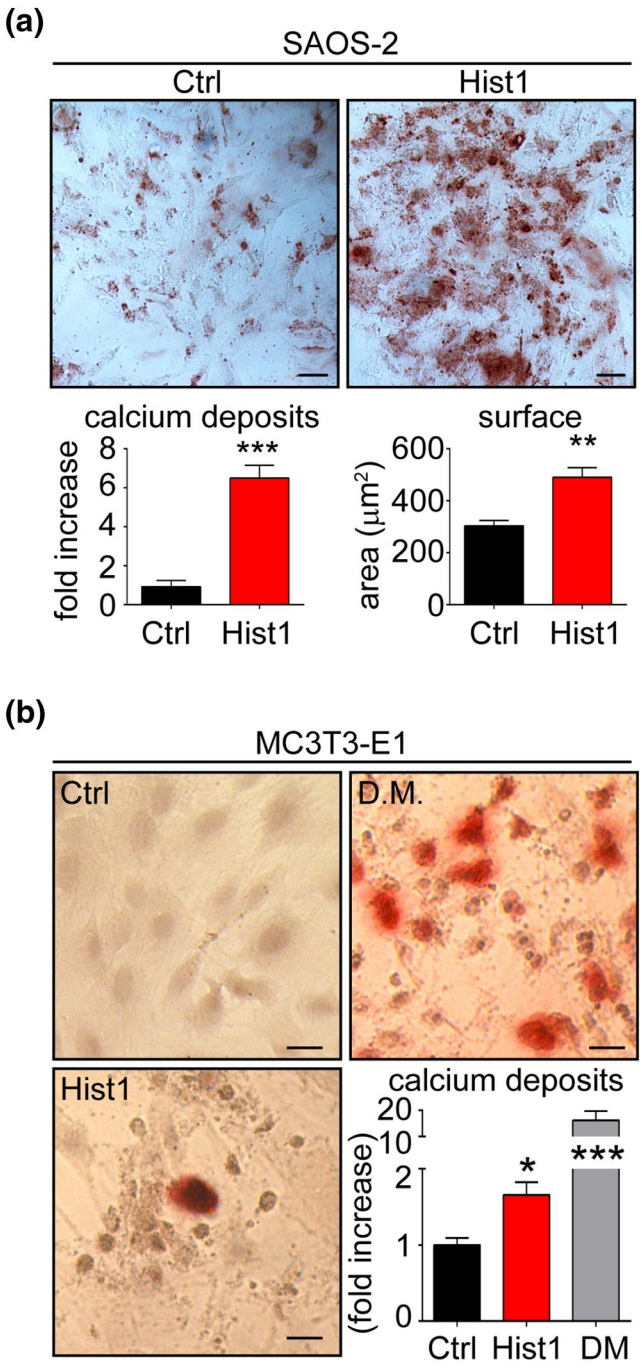


FIGURE 1 Histatin-1 stimulates bone cell adhesion, spreading, and migration. (a, b) SAOS-2 cells were resuspended and incubated for 30 min in serum-free medium containing or not 10 μM Histatin-1, then allowed to attach to fibronectin-coated plates (2 μg/ml) for different time points and stained with crystal violet. (a) Representative images are shown. (b) Quantification of three independent experiments (mean ± SEM; $^{*}p < 0.05$). (c, d) Cell spreading was evaluated in SAOS-2 (c) and MC3T3-E1 cells (d), following 30 min attachment to fibronectin-coated plates, as described in (a). Left panels show representative images, whereas right graphs indicate the percentage of cells undergoing spreading and shown as the average of three independent experiments (mean ± SEM; $^{*}p < 0.05$). (e, f) Cell migration was measured in Transwell chambers coated with 2 μg/ml fibronectin. SAOS-2 (e) and MC3T3-E1 cells (f) were allowed to migrate for 60 min in the presence of either 10 μM Histatin-1, 10 μM of nonphosphorylated Histatin-1 (no-P) or vehicle control (dH_2O). Cells that migrated were visualized by crystal violet staining. Representative images are shown and graphs represent the averages of three independent experiments (mean ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$)

phosphorylation was dispensable for ALP induction, because non-phosphorylated Histatin-1 triggered a similar increase in ALP protein level (Figure S1b). Alternatively, the expression of osteogenic genes was also evaluated, and found that Histatin-1 induced the expression of osteocalcin, Runx2, and ALP in MC3T3-E1 cells, although no significant fluctuations were observed in SAOS-2 cells, which could be due to the differentiated nature of this cell line (Figure 3b). Importantly, as control, MC3T3-E1 cells responded with a substantial induction of these genes when treated with a standard mixture of differentiation inducers, including ascorbic acid, glycerophosphate, dexamethasone (Figure S1c). Taken together, these data indicate that in nondifferentiated cells (preosteoblasts), Histatin-1 promotes the expression of osteogenic differentiation markers, further supporting the notion that Histatin-1 is a bone differentiating factor.



(c)

Time (h)	Increase (%)
0	-
24	21.88 ± 0.01 *
48	18.18 ± 0.01 *

FIGURE 2 Histatin-1 promotes mineralization in vitro. (a) SAOS-2 cells were incubated for 7 days in complete medium containing or not 10 μM Histatin-1, and then washed, fixed, and stained with alizarin red solution. Calcium deposits were visualized in an optic microscope. Upper panels show representative images and lower graphs represent the quantification of the amount of calcium deposits and mean area (μm²) per calcium deposit. Values are shown as the mean ± SEM (**$p < 0.01$; ***$p < 0.001$). (b) MC3T3-E1 cells were incubated for 7 days in complete medium containing 10 μM Histatin-1 or differentiation medium (DM: ascorbic acid, glycerophosphate, dexamethasone) as positive control, and then analyzed as described in (a). Representative images are shown and graph represents the amount of calcium deposits (mean ± SEM; *$p < 0.05$; ***$p < 0.001$). (c) SAOS-2 cells were treated or not with 10 μM Histatin-1 for 24 and 48 h, and the activity of alkaline phosphatase was measured by metabolization of the substrate *p*-nitrophenilphosphate, following absorbance at 405 nm. Data were averaged from three independent experiments and shown as residual activity (% increase with respect to the control condition; mean ± SEM; *$p < 0.05$)

3.3 | Signaling pathways triggered by Histatin-1 in bone cells

In order to determine which signaling pathways are triggered by Histatin-1 in bone cells, we focused on signaling proteins involved in bone cell adhesion, migration, or differentiation, and particularly those proteins previously shown to be activated by Histatin-1 in keratinocytes and endothelial cells (Oudhoff et al., 2008; Oudhoff, Kroeze et al., 2009; Torres et al., 2017). To this end, SAOS-2 cells were challenged with Histatin-1 and whole cell lysates were prepared for subsequent Western blot analyses of active and total protein levels of focal adhesion kinase (FAK), extracellular signal regulated kinase 1/2 (ERK1/2) and protein kinase B (Akt). Unexpectedly, neither of these proteins was affected by treatment with Histatin-1 (Figure 4a). Thereafter, since Histatin-1 is known to activate the small GTPases Rac1 and Rab5 in endothelial cells, in an event that is required for Histatin-1-induced cell migration (Torres et al., 2017), we evaluated the activity of these GTPases in bone cells, following treatment of with Histatin-1. As for FAK, ERK1/2, and Akt, neither Rab5-GTP nor Rac1-GTP levels were affected by treatment with Histatin-1 (Figure 4b). Finally, we attempted to evaluate alternative signaling pathways, which have not been related yet with Histatin-1, but of relevance in osteogenic differentiation. In this context, β-catenin-dependent signaling was interesting, because it is a transcriptional factor that upon stabilization and relocalization to the nucleus, allows osseous differentiation and bone cell migration (Teufel & Hartmann, 2019). Surprisingly, treatment of SAOS-2 cells with Histatin-1 provoked both stabilization of β-catenin (Figure 4c) and nuclear accumulation of this protein, as detected by using antibodies against both total and nonphosphorylated, transcriptionally active β-catenin (Figure 4d). In agreement with these findings, inhibition of β-catenin signaling with Wnt-C59, which was previously reported to interfere with nuclear accumulation of β-catenin (Reyes et al., 2020), prevented Histatin-1-dependent increase of ALP levels (Figure 4e). Thus, we conclude that Histatin-1 promotes the activation of β-catenin signaling in bone cells and that this is required for osteogenic differentiation.

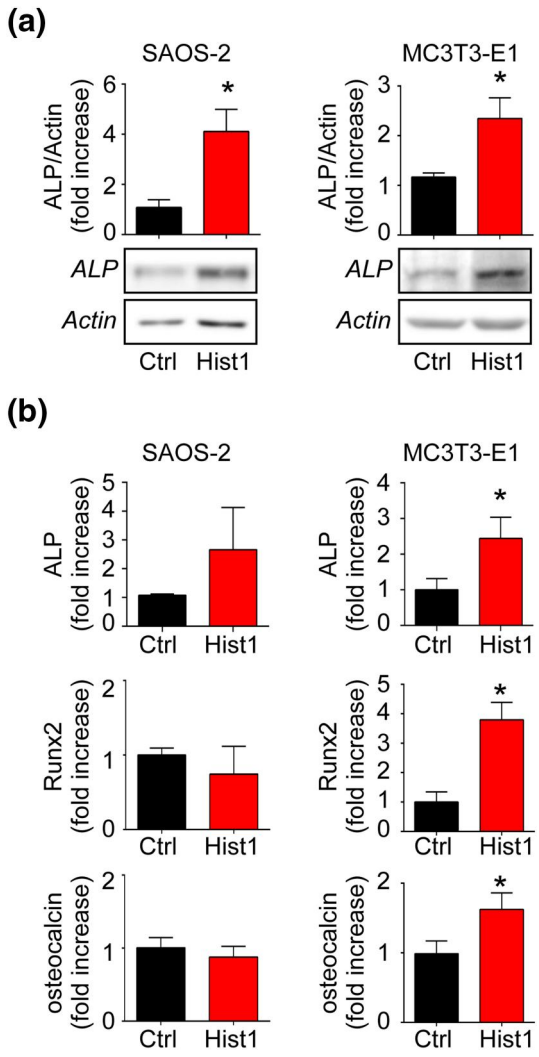


FIGURE 3 Histatin-1 increases the expression of osteoblastic differentiation markers. (a) SAOS-2 and MC3T3-E1 cells were incubated in the absence or presence of 10 μM Histatin-1 for 24 h, and whole cell lysates were prepared for Western blot analysis of alkaline phosphatase (ALP) and actin. Representative Western blot images are shown, and relative levels of ALP were quantified by scanning densitometry and normalized to actin. Graphs represent the average from three independent experiments (mean ± SEM.; $^*p < 0.05$). (b) SAOS-2 and MC3T3-E1 cells were treated or not with 10 μM Histatin-1 for 24 h or 7 days, respectively. Total RNA was extracted, for subsequent RT-qPCR analysis of Runx2, osteocalcin, and ALP. Relative abundance of all screened mRNAs was normalized to GAPDH, by using the $\Delta\Delta C_t$ method. Graphs show data obtained by averaging three independent experiments (mean ± SEM; $^*p < 0.05$). Extended data with controls are shown in Figure S1c. mRNA, messenger RNA; RT-qPCR, quantitative reverse transcription polymerase chain reaction

3.4 | Histatin-1 stimulates cell adhesion, migration, and differentiation in primary mesenchymal cells

To evaluate whether the osteogenic differentiation effects of Histatin-1 could be extended to other cell populations that are not necessarily committed with the osteogenic lineage, primary cultures of mesenchymal cells were obtained from two different sources, dental pulp and tooth's apical papilla. These tissues were chosen, because both are known to contain mesenchymal cell populations with the capacity to differentiate towards different lineages, including osteoblast-like cell types (Aydin & Sahin, 2019; Sonoyama et al., 2008; Sybil et al., 2019). After approval of the Ethics Committee of the Faculty of Medicine, Universidad de Chile, dental pulp and apical papilla were obtained from healthy donors, undergoing tooth extraction for orthodontic reasons, and primary cultures derived from them were classified according to their origin as dental pulp mesenchymal cells or apical papilla mesenchymal cells (APMCs; Figure 5a). First, we assessed the effects of Histatin-1 on mesenchymal cell adhesion, spreading and migration and, although no significant changes were observed in cell spreading (Figure 5b), Histatin-1 induced a significant increase in cell adhesion (1.3-fold; Figure 5c) and in cell migration (2.5-fold), when compared with the nontreated condition (Figure 5d). Importantly, as control, a nonrelated scramble peptide was not able to induce cell migration, further indicating that the effects are specific for Histatin-1 peptide sequence (Figure 5d). Next, messenger RNA levels encoding for osteogenic differentiation genes were determined and observed that treatment with Histatin-1 induced a 1.6-fold increase in osteopontin and 1.5-fold increase in Runx2 levels (Figure 5e). Thereafter, to evaluate the possibility that Histatin-1 stimulates the mineralizing capacity in vitro, formation of calcium deposits was assessed in APMCs. As anticipated, treatment of APMCs with Histatin-1 caused a significant increase in both amount and size of calcium deposits (Figure 5f). Finally, in an attempt to extend these observations to an alternative model, primary mesenchymal cells derived from the dental pulp were used and observed that, as for APMCs, Histatin-1 provoked a substantial increase in the formation of calcium deposits (Figure 5g). Therefore, we conclude that Histatin-1 is a novel factor that promotes the acquisition of osteogenic properties in primary cultures of mesenchymal cells derived from apical papilla and dental pulp.

4 | DISCUSSION

A few studies have explored a possible relationship between Histatin-1 and bone cell activity, but none of them demonstrated any osteinductive effects of this peptide. Specifically, a recent study from our group showed that Histatin-1 decreases cytotoxicity of zoledronic acid, an antiresorptive drug, in MC3T3-E1 preosteoblasts and SAOS-2 osteosarcoma cells (Castro et al., 2019). The remaining two studies evaluated the effects of Histatin-1 on MC3T3-E1 cells (Sun, Shi, Ma et al., 2020; Van Dijk, Beker et al., 2017). Specifically, in one study, Histatin-1 was shown to increase cell adhesion to titanium surfaces (Van Dijk Beker et al., 2017), whereas the other study showed that Histatin-1 increases the effects of all-*trans* retinoic acid on the activity of ALP, which is an enzyme associated with matrix mineralization (Sun, Shi, Ma et al., 2020). However, the latter study focused on the effects of all-*trans* retinoic acid and the combined effects of this molecule with Histatin-1, but not about Histatin-1

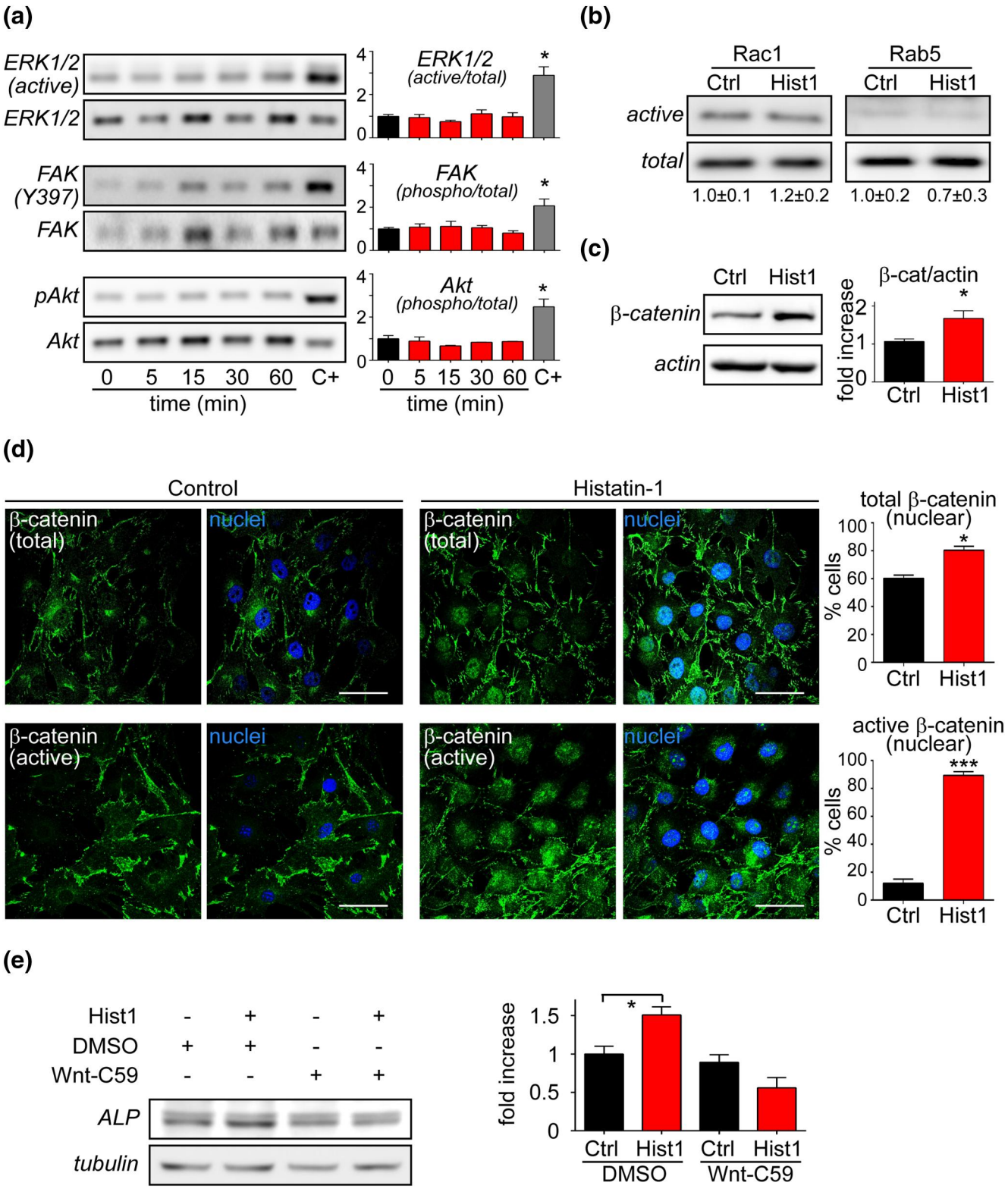


FIGURE 4 Signaling pathways triggered by Histatin-1 in bone cells. (a) SAOS-2 cells were treated with 10 μM Histatin-1 for 0, 5, 15, 30, and 60 min, as indicated, and whole-cell lysates were prepared for Western blot analysis. Cells treated with 10% serum for 15 min were used as positive control (C+). ERK1/2, phosphorylated ERK1/2, FAK, phosphorylated FAK on Y397, Akt, phosphorylated Akt (pAkt) and actin were detected with specific antibodies. Representative images are shown (left panels). Relative phosphorylated ERK, FAK, and Akt levels were normalized to their total counterparts and shown as fold increase (three independent experiments; mean ± SEM; $^*p < 0.05$). (b) SAOS-2 cells were treated with 10 μM Histatin-1 for 60 min, whole-cell lysates were prepared, and Rab5-GTP levels were determined by GST-R5BD pull-down assay. Rab5-GTP levels were normalized to total Rab5 and shown as relative levels with respect to nontreated cells (numbers below each panel indicate the mean ± SEM; ns, nonsignificant; three independent experiments). Representative Western blot images are shown for

alone, therefore failing to observe any effects of Histatin-1 in ALP activity. This could be due to the fact that the study was conducted in a single short time of incubation (2 h), which is insufficient to observe fluctuations in ALP activity (Sun, Shi, Ma et al., 2020). Thus, no study has shown or suggested a positive effect of Histatin-1 in bone cell differentiation or function, and hence, no potential therapeutic use of Histatin-1 as a bone regeneration agent has been proposed. Strikingly, as for bone cells, a possible relationship between Histatin-1 or other Histatins and mesenchymal cells derived from dental pulp or apical papilla, has not been explored, thereby opening several possibilities for therapies involving these tissues.

Interestingly, a more recent study by Sun et al (Sun et al., 2020) showed that Histatin-1 improves osteoinductive properties of BMP-2 in a preclinical model. However, that study was conceived on the basis that Histatin-1 is a proangiogenic factor, but not that the peptide itself was osteoinductive. Rather, the study by Sun et al. explored the effects of Histatin-1 and its combination with BMP-2 in ectopic bone formation in vivo. By using that model, unlike its combination with BMP-2, Histatin-1 alone failed to induce ectopic bone formation, as observed by micro-computerized tomography and histological analyses, although immunohistochemical analysis showed that Histatin-1 increases the presence of osteogenic markers. Nevertheless, those experimental approaches do not permit concluding that Histatin-1 stimulates osteogenic differentiation, but rather that Histatin-1 increases the presence/recruitment of osteogenic cells in the model of ectopic bone formation. In this scenario, our work addresses this gap of knowledge, as we show that Histatin-1 directly promotes the acquisition of bone differentiation markers in preosteoblastic and osteoblast cells, as well as in primary mesenchymal cells.

Collectively, our findings uncover a novel role for Histatin-1 in promoting osteoinduction, which paves new avenues to explore its effects in bone regeneration. It should be mentioned that, although the data shown here indicate a moderate osteoinductive effect of Histatin-1, as compared with standard osteoinductive factors, such as BMP-2 (Sun, Shi, Shen et al., 2020), this does not necessarily imply that the performance of Histatin-1 in bone regeneration is moderate or poor at the clinical level. This is because bone regeneration relies on multiple cellular and molecular phenomena regulated in a temporal and spatial manner (Marsell & Einhorn, 2011). Specifically, the sole migration of precursor cells to the injured site (osteoconduction) is not sufficient to assure efficient tissue regeneration, as these cells will eventually require differentiating into preosteoblasts and osteoblasts. Other events, such as angiogenesis, are also critical to the bone regeneration process (Dickson et al., 1994; Gong et al., 2019), and, considering that Histatin-1 is a versatile peptide that stimulates both angiogenesis (Torres et al., 2017) and osteoinduction (this study), it might be expected to have a good performance in preclinical and clinical settings. Likewise, another aspect that should be evaluated in a potential clinical application, is the specificity of Histatin-1, since bone regeneration should be restricted to the site of the injury, and not ectopically, as it has been described for BMP-2 (James et al., 2016). This scenario must be confirmed by using appropriate models of bone regeneration for instance, an orthotopic model. In addition to its proangiogenic activity (Torres et al., 2017), Histatin-1 meets other advantages, such as its noninflammatory nature (Oudhoff et al., 2010) and structural simplicity (this peptide consists of a 38-amino acid sequence), as well as the dispensability of its phosphorylation to promote bone cell migration and osteogenic differentiation (this study). Hence, the data provided in this study indicate that Histatin-1 is an osteogenic peptide, with potential as a coadjuvant element in bone regenerative medicine and in dental pulp regeneration.

5 | CONCLUSION

We conclude that Histatin-1 is a novel osteogenic factor that promotes bone cell adhesion, spreading and migration, as well as osteoblastic differentiation and mineralization in vitro, with potential impact to the field of bone regenerative medicine.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

total and GTP-bound Rab5. For measurements of Rac1 activity, same procedure was achieved, but using a GST-PBD pull-down assay instead. Representative Western blot images are shown and numbers below each panel indicate relative Rac1-GTP levels normalized to total Rac1 (mean ± SEM; ns, nonsignificant; three independent experiments). (c) SAOS-2 cells were treated or not with 10 μM Histatin-1 for 24 h, and whole cell lysates were prepared for Western blot analysis of β-catenin and actin. Representative Western blot images are shown, and relative levels of β-catenin were normalized to actin. Graphs represent the average from three independent experiments (mean ± SEM; $^{*}p < 0.05$). (d) SAOS-2 cells were treated or not with 10 μM Histatin-1 for 24 h, then processed for immunofluorescence of total and nonphosphorylated (transcriptionally active) β-catenin. Left panels show representative confocal microscopy images of total and active β-catenin, whereas nuclei were stained with DAPI. Upper and lower right graphs show the percentage of cells with nuclear accumulation of total and active β-catenin, respectively (mean ± SEM; $^{*}p < 0.05$; $^{***}p < 0.001$). (d) SAOS-2 cells were treated or not with 10 μM Histatin-1 in the presence of either 50 μM Wnt-C59 or DMSO (vehicle, control) for 24 h, and whole cell lysates were prepared for Western blot analysis of alkaline phosphatase (ALP) and tubulin. Representative Western blot images are shown, and relative levels of ALP were quantified and normalized to tubulin. The graph represents the average obtained from three independent experiments (mean ± SEM; $^{*}p < 0.05$). Akt, protein kinase B; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal regulated kinase 1/2; FAK, focal adhesion kinase; GST, glutathione-S-transferase

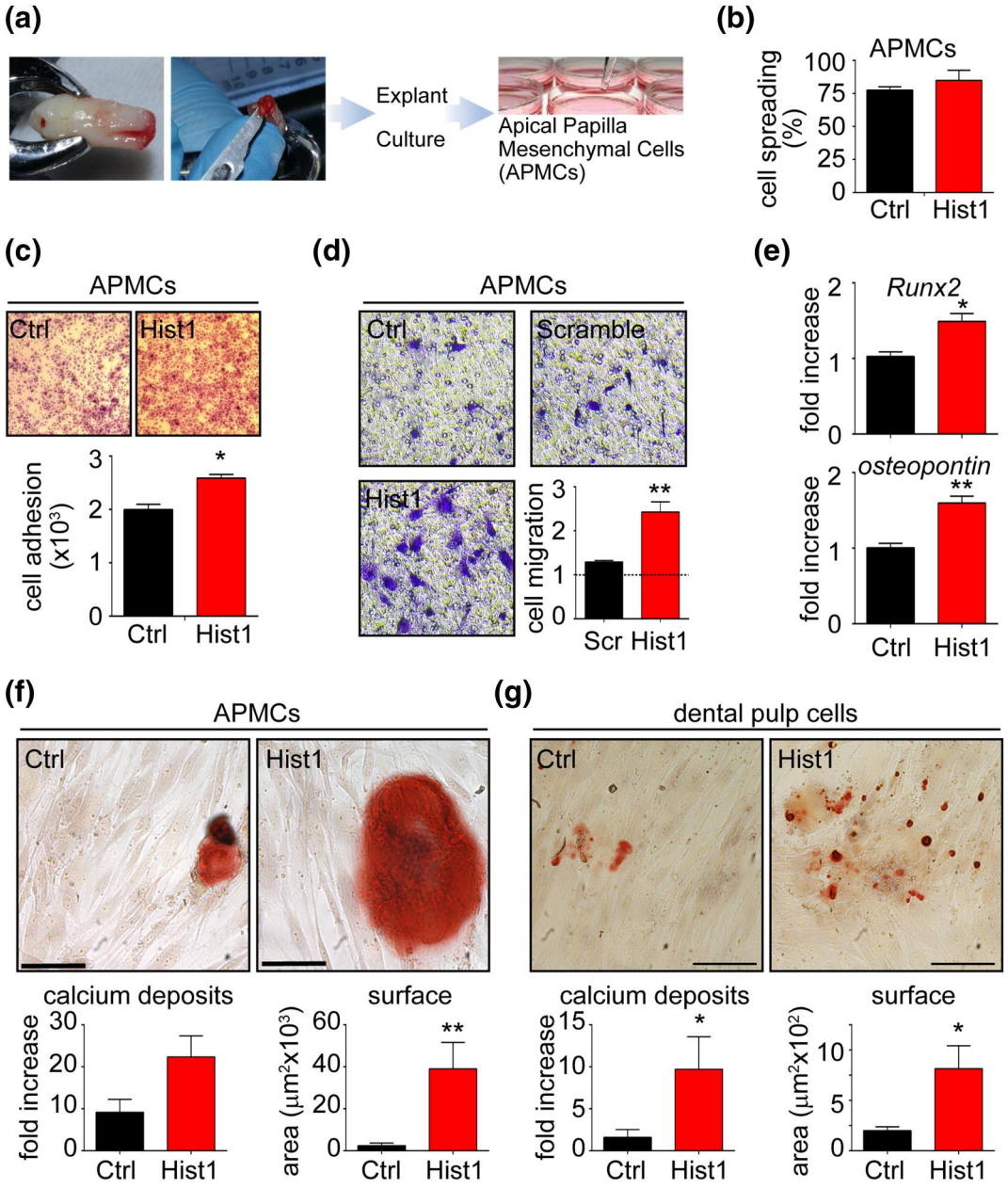

FIGURE 5 Histatin-1 induces mesenchymal cell adhesion, migration and osteogenic differentiation markers. (a) Primary cultures of mesenchymal cells from tooth's apical papilla (APMCs, apical papilla mesenchymal cells) were obtained from fresh premolars and third molars extracted for orthodontic reasons. (b) APMCs were allowed to adhere to fibronectin-coated plates (2 µg/ml) for 30 min and cell spreading was visualized by crystal violet staining. Data indicate the percentage of cells undergoing spreading (mean ± SEM; nonsignificant; three independent experiments). (c) APMCs were collected, resuspended in serum-free medium containing or not 10 µM Histatin-1 and then allowed to attach to fibronectin-coated plates (2 µg/ml) for 60 min. Cell adhesion was detected by crystal violet staining (upper panels) and data were quantified from three independent experiments (mean ± SEM; $^{*}p < 0.05$). (d) APMCs migration was measured in Transwell chambers coated with 2 µg/ml fibronectin, for 60 min. Conditions included 10 µM Histatin-1, 10 µM of scrambled sequence peptide or vehicle control (dH_2O). Cells that migrated were visualized by crystal violet staining and cell migration was quantified and normalized with respect to the vehicle control condition (dotted line). Graph represents the average of three independent experiments (mean ± SEM; $^{**}p < 0.01$). (e) APMCs were treated or not with 10 µM Histatin-1 for 7 days and total RNA was extracted, for subsequent RT-qPCR analysis of Runx2 and osteopontin. Relative abundance of mRNAs was normalized to GAPDH, by using the $\Delta\Delta C_t$ method. Data were obtained from three independent experiments (mean ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$). (f) APMCs were incubated for 7 days in complete medium containing or not 10 µM Histatin-1, then washed, fixed, and stained with alizarin red solution. Calcium deposits were visualized in an optic microscope (upper panels) and quantified as the amount of calcium deposits and mean area (μm^2) per calcium deposit. Values are shown as the mean ± SEM ($^{**}p < 0.01$). (g) Primary cultures of mesenchymal cells derived from dental pulp (dental pulp mesenchymal cells) were obtained from premolars and incubated for 7 days in complete medium containing or not 10 µM Histatin-1 and analyzed for calcium deposit formation, as described in (f). Values are shown as the mean ± SEM ($^{*}p < 0.05$). mRNA, messenger RNA

AUTHOR CONTRIBUTIONS

Pedro Torres, Nadia Hernández, and Vicente A. Torres designed research, performed research, analyzed data, and wrote the paper; Carlos Mateluna, Patricio Silva, Montserrat Reyes, Luis Solano, Sebastián Venegas, Kamran Nazmi, and Mauricio Garrido, and Mónica Cáceres performed research and analyzed the data; Floris J. Bikker, Jan G. M. Bolscher, Alfredo Criollo, Mónica Cáceres, and Vicente A. Torres contributed with reagents and analytic tools and reviewed the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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