E-selectin ligand 1 regulates bone remodeling by limiting bioactive TGF-β in the bone microenvironment

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TGF-β is abundantly produced in the skeletal system and plays a crucial role in skeletal homeostasis. E-selectin ligand-1 (ESL-1), a Golgi apparatus-localized protein, acts as a negative regulator of TGF-β bioavailability by attenuating maturation of pro–TGF-β during cartilage homeostasis. However, whether regulation of intracellular TGF-β maturation by ESL-1 is also crucial during bone homeostasis has not been well defined. Here, we show that $Esl-1^{-/-}$ mice exhibit a severe osteopenia with elevated bone resorption and decreased bone mineralization. In primary culture, $Esl-1^{-/-}$ osteoclast progenitors show no difference in osteoclastogenesis. However, $Esl-1^{-/-}$ osteoblasts show delayed differentiation and mineralization and stimulate osteoclastogenesis more potently in the osteoblast–osteoclast coculture, suggesting that ESL-1 primarily acts in osteoblasts to regulate bone homeostasis. In addition, $Esl-1^{-/-}$ calvaria exhibit an elevated mature TGF-β/pro–TGF-β ratio, with increased expression of TGF-β downstream targets (plasminogen activator inhibitor-1, parathyroid hormone-related peptide, connective tissue growth factor, and matrix metallopeptidase 13, etc.) and a key regulator of osteoclastogenesis (receptor activator of nuclear factor κB ligand). Moreover, in vivo treatment with 1D11, a pan–TGF-β antibody, significantly improved the low bone mass of $Esl-1^{-/-}$ mice, suggesting that elevated TGF-β signaling is the major cause of osteopenia in $Esl-1^{-/-}$ mice. In summary, our study identifies ESL-1 as an important regulator of bone remodeling and demonstrates that the modulation of TGF-β maturation is pivotal in the maintenance of a homeostatic bone microenvironment and for proper osteoblast–osteoclast coupling.

1D11 antibody | osteoblast mineralization | osteoporosis

Bone is a highly dynamic tissue that undergoes continuous remodeling. Osteoblasts (OBs) and osteoclasts (OCs), the two cell types responsible for bone formation and bone resorption, respectively, are tightly coupled by a set of paracrine factors acting within the bone microenvironment. The dysregulation of OB–OC coupling is one of the most common causes of osteopenia/osteoporosis or other metabolic bone diseases (1).

Transforming growth factor β (TGF-β) is abundantly produced in bone. It plays a crucial role in regulating the differentiation of both OBs and OCs and mediating OB–OC coupling during bone remodeling (2, 3). Therefore, manipulating TGF-β signaling in bone offers an attractive strategy for the treatment of bone diseases, such as osteoporosis and osteolytic bone metastasis of cancers (4, 5). Recent data show that TGF-β can play either a catabolic or anabolic role in bone homeostasis, suggesting that TGF-β signaling acts in a context- and dose-dependent manner (4, 6–10). This emphasizes the need for a precise regulation of TGF-β bioavailability to maintain balanced bone remodeling. TGF-β is initially synthesized as an inactive precursor (pro–TGF-β), which undergoes cleavage/maturation by the furin protein convertase, at a specific site between the latency domain (LAP) and ligand domain. This process occurs in the Golgi apparatus and/or ECM, and is a prerequisite for TGF-β activity (2, 11–13). After cleavage, the LAP and TGF-β ligand continue to associate noncovalently within the small latency complex (SLC), which may further associate with latent TGF-β binding proteins covalently to form the large latency complex (LLC) (2, 11–13). These latent

forms of TGF-β are secreted and deposited in the bone matrix, and subsequently activated by proteases, integrin, and glycosidase, etc. (11). Multiple lines of in vivo evidence have shown that dysregulated TGF-β expression, secretion, or activation from latency leads to defective bone remodeling (6, 7, 14). However, to date, to what extent bone homeostasis is modulated at the level of TGF-β maturation in the cell has not been fully defined.

We previously reported that E-selectin ligand-1 (ESL-1) (also referred to as Cysteine-rich fibroblast growth factor receptor, golgi apparatus protein 1, Latent TGF–β complexed protein-1 or MG160) (15–18) is an important regulator of TGF-β maturation in the Golgi apparatus during cartilage homeostasis (19). Loss of ESL-1 in mice led to accelerated TGF-β maturation and elevated TGF-β signaling in the growth plate. The enhanced TGF-β signaling results in a distinctive chondrodysplasia partly by disrupting the balance between parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (IHH), thus impairing proliferation and terminal differentiation of chondrocytes in the growth plate (19). This mechanism was evolutionarily conserved and was functionally demonstrated in *Xenopus* gastrulation (19). In the present study, we found that $Esl-1^{-/-}$ mice develop severe osteopenia with impaired mineral deposition and elevated bone resorption. Moreover, our data indicate that, during bone remodeling, ESL-1 primarily acts within the OBs to maintain proper TGF-β bioavailability in the bone microenvironment, thus enhancing OB differentiation as well as restricting overactivation of OCs. Overall, this study suggests that attenuation of TGF-β maturation by ESL-1 is an important mechanism to maintain a balanced bone homeostasis.

Results

Loss of ESL-1 Leads to Severe Osteopenia in Mice. Our previous studies have shown that ESL-1 regulates chondrocyte differentiation by modulating TGF-β bioavailability in the cartilage (19). Interestingly, $Esl-1$ is highly expressed in bone tissue and is localized in the Golgi apparatus of primary OBs (Fig. S1). Because TGF-β also plays a critical role in bone homeostasis, we further explored the extent to which ESL-1 is involved in bone homeostasis.

$Esl-1^{-/-}$ mice at postweaning age exhibit a markedly increased X-ray translucency throughout the skeleton, suggesting a low bone mass phenotype (Fig. 1*A*). Micro-computed tomography (μCT) analysis revealed that loss of ESL-1 leads to significant reduced bone volume/tissue volume (BV/TV), decreased trabecular number (Tb.N), and increased trabecular separation (Tb.Sp) in the distal femurs of 1-mo- and 3-mo-old mice, compared with wild-type (WT) littermates (Fig. 1*B* and Table 1). Moreover, dramatic
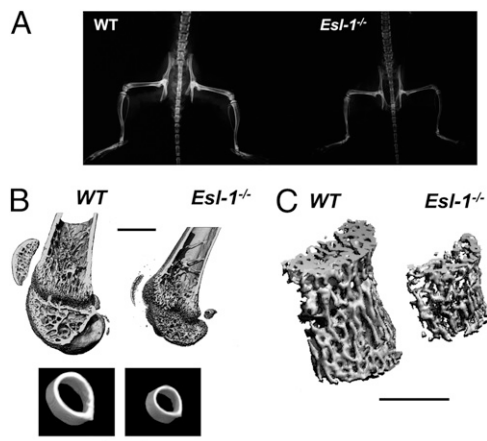
Fig. 1. ESL-1 is expressed in OBs and loss of ESL-1 leads to a severe osteo-porotic phenotype. (*A*) One-month-old $Esl\text{-}1^{-/-}$ skeleton shows increased X-ray translucency. (*B*) μCT images of $Esl\text{-}1^{-/-}$ vs. WT femurs. (*Upper*) Longitudinal sections of 1-mo-old male distal femurs. (*Lower*) The transverse sections right below the minor trochanter of the same femurs. (*C*) Decreased trabecular bone of 3-mo-old vertebra L4. (Scale bars: 1 mm.)

decreases in the BV/TV and Tb.Th are also identified in 3-mo-old lumbar vertebrae 4 (L4) (Fig. 1*C* and Table 1). In addition, cortical thickness (Cortical.Th) in $Esl\text{-}1^{-/-}$ mice is significantly decreased. The μCT data also revealed that 1-mo $Esl\text{-}1^{-/-}$ mice have significantly decreased midfemur cortical diameter (WT vs. $Esl\text{-}1^{-/-}$: 1.29 vs. 1.01 mm, a 22% decrease), and lower bone mineral density (BMD) in the midfemur cortical bone (WT vs. $Esl\text{-}1^{-/-}$: 890.6 vs. 820.3 mg HA/ccm, a 8.5% decrease) (Fig. 1*B*, Table 1, and Fig. S2 *A* and *B*). The decreased BMD, Cortical.Th, and cortical diameter suggest that $Esl\text{-}1^{-/-}$ mice may have defective mechanical properties. This notion is further supported by a three-point bending test on 1-mo femurs. As expected, the reduction in bone mass resulted in significantly diminished maximum load and energy to failure for the $Esl\text{-}1^{-/-}$ animals. However, the ultimate strength is not significantly changed in the $Esl\text{-}1^{-/-}$ femurs, and the elastic modulus is increased (Table S1). These data indicate that loss of ESL-1 results in an alteration in the composition of bone matrix, which affects the material properties of the bone. In summary, the early onset and sustained low bone mass in both the long bones and vertebrae of $Esl\text{-}1^{-/-}$ mice suggests that ESL-1 is an important regulator of bone homeostasis, acting in both axial and appendicular skeleton.

$Esl\text{-}1^{-/-}$ Low Bone Mass Is Caused by Abnormal Activity of both OBs and OCs. The low bone mass phenotype can be a result of impaired bone formation, overactive bone resorption, or a combinatorial effect of each. To differentiate among these possibilities, trabecular parameters of 3-mo-old male $Esl\text{-}1^{-/-}$ vs. WT mouse

vertebra (L4) were assessed by bone histomorphometry (Fig. 2). We found that the OB surface per bone surface (Ob.S/BS), and the number of OBs per bone perimeter (N.Ob/B.Pm) are not significantly changed, suggesting that the proliferation of OBs is unaffected and does not account for the osteopenia in $Esl\text{-}1^{-/-}$ mice (Fig. 2*A*). Next, to understand whether loss of ESL-1 influences osteoblastic activity in the production and mineralization of bone matrix, we examined OB function by calcein double-labeling analysis. The bone formation rate per bone surface (BFR/BS) shows a trend toward a decrease in the $Esl\text{-}1^{-/-}$ mice, although not statistically significant. However, the mineralization apposition rate (MAR) is significantly decreased in the KO mice, suggesting that loss of ESL-1 decreases OB activity in producing mineralized bone matrix (Fig. 2*B*). Moreover, we found that the OC surface/bone surface (Oc.S/BS) and OC number/bone perimeter (N.Oc/B.Pm) are increased by ~30% in the mutant mice (Fig. 2*C*). These data suggest that loss of ESL-1 leads to an overall increase in OC differentiation. Overall, our results imply that the $Esl\text{-}1^{-/-}$ osteopenia phenotype is due to the combinatorial effects of both decreased bone mineralization and elevated bone resorption.

Loss of ESL-1 in OBs Inhibits Their Maturation and Enhances OB-Dependent Osteoclastogenesis. To further test whether ESL-1 plays a role in OB proliferation, primary OBs from $Esl\text{-}1^{-/-}$ or WT P1 calvaria were cultured and assessed for cell proliferation (Fig. 3*A*). The proliferation rate of $ESL\text{-}1^{-/-}$ OB is unchanged compared with the WT, consistent with the observation in the histomorphometric analysis (Fig. 2*A*). However, the decreased MAR in the $Esl\text{-}1^{-/-}$ vertebrae suggests that ESL-1 may play an important role in OB differentiation (Fig. 2*B*). To assess this, bone marrow stromal cells (BMSCs) were cultured in osteogenic medium containing ascorbic acid and β-glycerol phosphate. The cultures were assayed for early OB differentiation by alkaline phosphatase (ALP) staining at day 6 and for late OB differentiation/mineralization by Alizarin Red S staining at day 21, respectively. Our results showed that ALP-positive areas are not significantly changed in $Esl\text{-}1^{-/-}$ OBs (Fig. 3*B*). However, Alizarin Red S staining for mineralized matrix is dramatically decreased compared with WT cells (Fig. 3*C*), suggesting that ESL-1 is involved in the late differentiation and mineralization of OBs.

Elevated N.Oc/B.Pm and Oc.S/BS in $Esl\text{-}1^{-/-}$ bone suggest that ESL-1 may play a role in osteoclastogenesis. OC formation is influenced by intrinsic factors such as colony stimulating factor 1 receptor (CSF1R) or receptor activator of nuclear factor κB (RANK) in the OC progenitors, or by extrinsic stimulators such as macrophage colony-stimulating factor (M-CSF) or RANK ligand (RANKL) derived from OBs or other cell lineages within the bone microenvironment (20). First, to evaluate the intrinsic function of ESL-1 in osteoclastogenesis, bone marrow monocytes (BMMCs) derived from bone marrows of 4-wk-old WT or $Esl\text{-}1^{-/-}$ mice were treated with M-CSF and RANKL to induce OC differentiation. We found that $Esl\text{-}1^{-/-}$ BMMCs show no distinctive difference in osteoclastogenesis compared with WT controls (Fig. 3*D*),

Table 1. Quantification of μCT scanning reveals a severe osteoporosis in $Esl\text{-}1^{-/-}$ bones

Bone samples	Genotypes	BV/TV	BS/BV	Tb.N	Tb.Th	Tb.Sp	Cortical.Th
	WT	0.1095 ± 0.0294	56.9721 ± 4.6514	3.0659 ± 0.5731	0.0353 ± 0.0030	0.3006 ± 0.0652	0.1201 ± 0.0009
1-mo femurs	$Esl\text{-}1^{-/-}$	0.0508 ± 0.0103	68.0776 ± 0.9726	1.7237 ± 0.3313	0.0294 ± 0.0004	0.5706 ± 0.1387	0.0873 ± 0.0008
	P	0.0054	0.0017	0.0030	0.0051	0.0029	0.0004
	WT	0.1476 ± 0.0033	43.2653 ± 1.5988	2.8626 ± 0.2660	0.0483 ± 0.0007	0.3081 ± 0.0342	0.2143 ± 0.0191
3-mo femurs	$Esl\text{-}1^{-/-}$	0.0703 ± 0.0221	43.9756 ± 1.1180	1.5533 ± 0.5343	0.0448 ± 0.0013	0.6447 ± 0.2021	0.1657 ± 0.0085
	P	0.0039	0.5693	0.0191	0.0134	0.0466	0.015
	WT	0.2948 ± 0.0279	33.2170 ± 0.9125	4.9064 ± 0.5747	0.0602 ± 0.00017	0.1457 ± 0.0242	N/A
3-mo vertebrae L4	$Esl\text{-}1^{-/-}$	0.1481 ± 0.0068	42.7234 ± 1.7368	3.1603 ± 0.1113	0.0469 ± 0.0019	0.2698 ± 0.0109	N/A
	P	0.0009	0.0011	0.0067	0.0008	0.0013	N/A

The 1-mo-old and 3-mo-old femurs and the 3-mo-old L4 vertebrae were scanned and quantified by μCT. Quantified parameters, including BV/TV (bone volume/tissue volume), BS/TV (bone surface/tissue volume), Tb.N (trabecular bone number), Tb.Th (trabecular bone thickness), Tb.Sp (trabecular bone separation), and Cortical.Th (cortical bone thickness), indicate a distinctive osteoporotic phenotype in $Esl\text{-}1^{-/-}$ long bones and vertebrae.
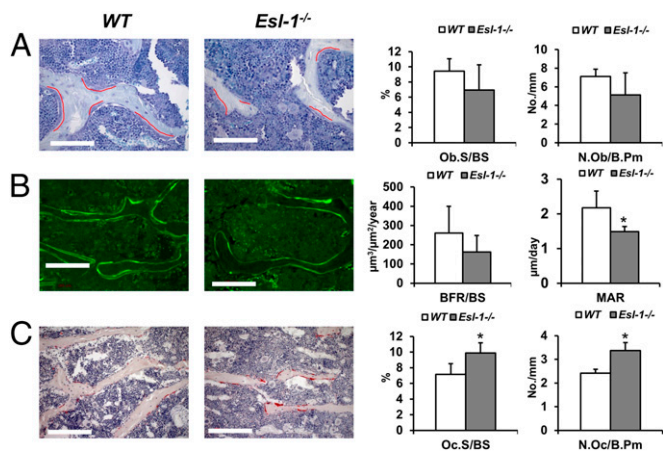
Fig. 2. Osteopenia in *Esl-1*$^{-/-}$ mice is the result of elevated bone resorption and decreased bone mineralization. Bone histomorphometric analysis of 3-mo-old *Esl-1*$^{-/-}$ vs. WT L4 vertebra. (*A*) The OB number and surface are not changed significantly. The OBs on bone surfaces are underscored with red curves. (Scale bar: 100 μm.) OB number per bone surface (Ob.S/BS) and OB number per bone perimeter (N.Ob/B.Pm) are quantified at *Right*. (*B*) Calcein double labeling with a 4-d interval. (Scale bar: 100 μm.) Bone dynamic indices are shown at *Right*. Bone formation rate/bone surface (BFR/BS) are trending toward but not significantly decreased, whereas mineral apposition rate (MAR) is significantly decreased in *Esl-1*$^{-/-}$ mice. (*C*) The OC numbers and surfaces are significantly increased in *Esl-1*$^{-/-}$ L4 vertebrae. (Scale bar: 50 μm.) OCs were identified by TRAP staining. OC surface per bone surface (Oc.S/BS) and OC number per bone perimeter (N.Oc/B.Pm) are quantified at *Right*. n = 5 per group. *P < 0.05.

suggesting that loss of ESL-1 does not potentiate the response of BMMCs to the stimulation of M-CSF and RANKL. Hence, the elevated OC formation in the *Esl-1*$^{-/-}$ bone may be due to the altered signaling context in the microenvironment of *Esl-1*$^{-/-}$ bone, likely through an OB–OC coupling mechanism. To assess this possibility, we cocultured WT vs. *Esl-1*$^{-/-}$ splenocytes (used as the OC progenitors) with *Esl-1*$^{-/-}$ vs. WT BMSCs, to detect whether loss of ESL-1 increases the potency of OB lineage cells in the induction of osteoclastogenesis, as well as whether loss of ESL-1 increases the response of OC progenitors in forming mature OCs. The results showed that, compared with WT BMSCs, the *Esl-1*$^{-/-}$ BMSCs are able to induce greater OC formation irrespective of the genotypes of the splenocytes. Meanwhile, WT and *Esl-1*$^{-/-}$ splenocytes show no difference in osteoclastogenesis when cocultured with the BMSCs of the same genotypes (Fig. 3*E*). These data suggest that the ESL-1 acts in the osteoblastic lineage cells, likely by modulating paracrine signals in the bone microenvironment, to indirectly regulate OC differentiation.

Changes of Bone Homeostasis-Related Genes and TGF-β Signaling in the *Esl-1*$^{-/-}$ Bone. To further understand the molecular basis underlying the *Esl-1*$^{-/-}$ low-bone mass phenotype, as well as what specific paracrine signal(s) act(s) downstream of *Esl-1* to mediate OB–OC coupling, we first compared the gene expression pattern between P1 WT vs. *Esl-1*$^{-/-}$ calvaria by quantitative RT-PCR (qPCR). We found that the early OB differentiation markers, such as Runt-related transcription factor 2 (*Runx2*), osterix (*Osx*), collagen 1a1 (*Col1a1*), alkaline phosphatase (*Alp*), and bone sialoglycoprotein (*Bsp*) are largely unchanged, while osteocalcin (*Ocn*), an OB late differentiation marker, is significantly decreased (Fig. 4*A*). These data suggest that loss of ESL-1 does not significantly affect early OB differentiation but inhibits OB terminal differentiation, consistent with the bone histomorphometry and primary cell culture data. Moreover, the OC differentiation markers including tartrate-resistant acid phosphatase (*Trap*) and matrix metalloproteinase 9 (*Mmp9*) are significantly increased,

consistent with the elevated Oc.S/BS identified in *Esl-1*$^{-/-}$ bone (Fig. 4*B*). Meanwhile, RANKL, the important stimulator of osteoclastogenesis, and osteoprotegerin (*Opg*), the antagonist of RANKL, are both elevated in *Esl-1*$^{-/-}$ calvaria. However, the RANKL/OPG ratio is increased, suggesting that overall the bone microenvironment in the *Esl-1*$^{-/-}$ bone promotes OC formation (Fig. 4*B*).

Next, to understand whether ESL-1 regulates bioavailability of TGF-β in bone using a mechanism similar to that of cartilage, we first compared the expression of TGF-β downstream targets in P1 *Esl-1*$^{-/-}$ vs. WT mouse calvaria. TGF-β downstream targets including *p21* (cyclin-dependent kinase inhibitor 1), plasminogen activator inhibitor-1 (*Pai-1*), *Pthrp*, matrix metallopeptidase 13 (*Mmp13*), and connective tissue growth factor (*Ctgf*) are significantly increased in the *Esl-1*$^{-/-}$ calvaria (Fig. 4*C*). The gene expression of TGF-β ligands (TGF-β1,2,3), TGF-β receptors 1, 2, and Smad2,3 (Mothers against decapentaplegic homolog 2,3) are not significantly changed in *Esl-1*$^{-/-}$ calvaria (Fig. 4*D*), suggesting that enhanced TGF-β signaling in *Esl-1*$^{-/-}$ bone may be due to a posttranscriptional mechanism, such as elevation in intracellular maturation of pro–TGF-β as is observed in *Esl-1*$^{-/-}$ cartilage. This notion is confirmed by Western blots showing a markedly increased mature TGF-β1 in *Esl-1*$^{-/-}$ calvarial protein in comparison with the WT controls (Fig. 4*E*), similar to our previous observation in the *Esl-1*$^{-/-}$ cartilage.

TGF-β Antibody Ameliorates Defective OB Differentiation and OC Induction. These above observations suggest that the elevated active TGF-β in the bone microenvironment may be the primary causal factor for the osteopenia in *Esl-1*$^{-/-}$ mice. To test this, we first assessed to what extent blocking TGF-β availability using 1D11, a pan–TGF-β antibody, can ameliorate the defect of OB differentiation in ex vivo cultures (Fig. 5 *A* and *B*). Our data show that, in the WT BMSCs, 1D11 treatment significantly accelerates mineral deposition in the culture at an early time point (3.07-fold increase at day 8), but the effect appears to be blunted with prolonged treatment (1.19-fold increase but not significant at day 16), possibly because the number of unmineralized cells become depleted
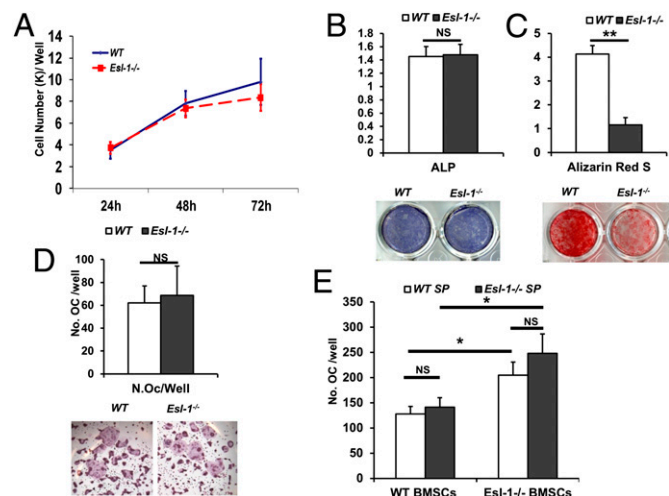

Fig. 3. Loss of ESL-1 in osteoblastic lineage cells attenuates late OB differentiation and enhances OB-dependent osteoclastogenesis. (*A–C*) Function of ESL-1 in OB proliferation and differentiation. Loss of ESL-1 does not affect primary calvarial OB proliferation (*A*) or early OB differentiation of BMSCs cultured in osteogenic medium as indicated by ALP staining (day 6) (*B*), but significantly attenuates late OB differentiation and mineralization as indicated by Alizarin Red S staining (day 21) (*C*). (*D*) BMMCs derived from WT vs. *Esl-1*$^{-/-}$ mice show comparable osteoclastogenesis upon treatment with M-CSF and RANKL. (*E*) In BMSC/splenocyte cocultures, *Esl-1*$^{-/-}$ BMSCs more strongly induce OC formation than WT BMSCs, irrespective of the genotype of splenocytes. SP, splenocytes. n = 3 per group; *P < 0.05, **P < 0.01; NS, nonsignificance.
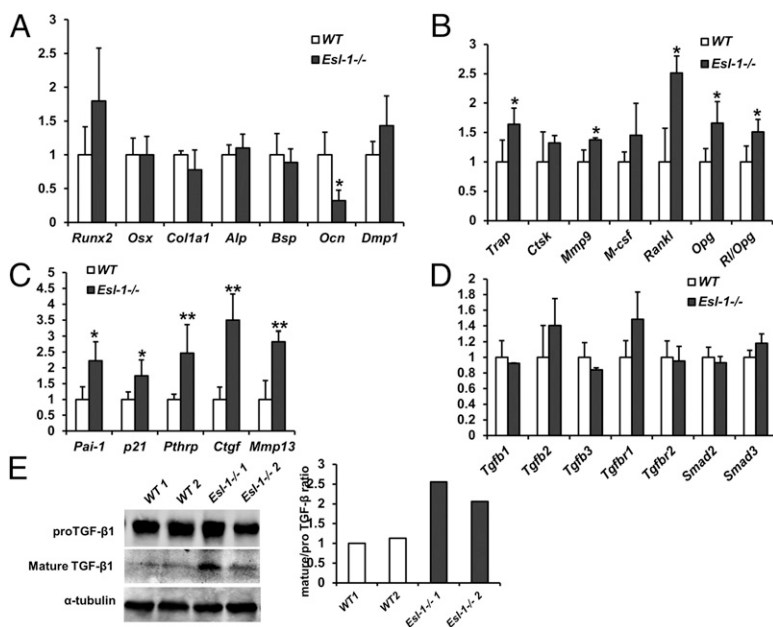
Fig. 4. Expression profile of bone homeostatic or signaling related genes in Esl-1$^{-/-}$ vs. WT calvaria. (A–D) qPCR analysis of gene expression ($n = 5$ per group; *$P < 0.05$, **$P < 0.01$; NS, nonsignificance; y axis: the fold change): gene expression of OB differentiation markers (A); expression of OC differentiation markers and key regulators of osteoclastogenesis (B); expression of TGF-β downstream target genes (C); expression of TGF-β signaling components (D). (E) Mature TGF-β is increased in the Esl-1$^{-/-}$ calvaria as detected by Western blot. The normalized ratios of mature/pro–TGF-β are quantified at Right.

at the later time point. In contrast, 1D11 treatment shows a stronger and significant effect in accelerating mineralization of Esl-1$^{-/-}$ BMSCs at both time points (4.41-fold increases at day 8, and 1.75-fold at day 16), at which times the delayed Esl-1$^{-/-}$ OB mineralization is largely rescued and shows no significant difference to treated WT BMSCs (Fig. 5A).

Next, to evaluate to what extent increased bioactive TGF-β derived from Esl-1$^{-/-}$ OBs is responsible for the overactive bone resorption, we used 1D11 to treat OB–OC cocultures. The results showed that 1D11 can significantly decrease/rescue the osteoclastogenesis induced by Esl-1$^{-/-}$ BMSCs. Meanwhile, 1D11 treatment also shows a trend toward an attenuation of osteoclastogenesis induced by WT BMSCs. These data support that the elevated TGF-β derived from OB plays an important role in mediating the dysregulated OB–OC coupling in the osteopenia in Esl-1$^{-/-}$ mice (Fig. 5B).

Anti–TGF-β Antibody Effectively Improves Esl-1$^{-/-}$ Low Bone Mass. To further confirm whether elevated TGF-β bioavailability in vivo is the major cause of the Esl-1$^{-/-}$ osteopenia, and to evaluate the therapeutic effect of 1D11 (a pan–TGF-β monoclonal antibody) on osteopenia caused by excessive TGF-β activity, we attempted to correct Esl-1$^{-/-}$ low bone mass with systemic treatment of 1D11. Consistent with a previous report by Edwards et al. (4), our data show that 1D11 treatment in WT mice for 4 wk resulted in a significant accrual of trabecular bone in L4 vertebra (Fig. 5C), as evidenced by a 37.8% increase in BV/TV (26.7% of the placebo group vs. 35.4% of the 1D11-treated group). In addition, an anabolic effect of 1D11 is also evident with finding of increased Tb.Th and Tb.N in conjunction with decreased Tb.Sp in the 1D11-treated WT mice, compared with placebo-treated WT mice (Fig. 5D). More importantly, 1D11 treatment exhibits an even more dramatic anabolic effect on Esl-1$^{-/-}$ bones: the BV/TV of L4 vertebra is significantly increased by 105% (12.5% of the placebo group vs. 25.7% of the 1D11-treated group), close to that of the placebo-treated WT group (BV/TV, 26.7%). The other bone parameters, such as BS/TV, Tb.N, Tb.Th, and Tb.Sp are also corrected to the levels of placebo-treated WT mice (Fig. 5D). Overall, 1D11 treatment vastly improves the low bone mass of the Esl-1$^{-/-}$ mice, suggesting that the excessive bioactive TGF-β in the bone microenvironment is a major contributor to the osteopenia in Esl-1$^{-/-}$ mice.

Discussion

Our data suggest that ESL-1, a Golgi-localized protein, is a crucial regulator in bone homeostasis. Loss of ESL-1 results in a severe osteopenia in mice with decreased bone formation and elevated bone resorption, due to increased TGF-β signaling in the bone. This is in accordance with our previous finding of ESL-1 as a negative regulator of TGF-β bioavailability in cartilage (19). Distinct from most other cells, in which LLC is the major form of secreted TGF-β, bone cells predominantly secrete pro–TGF-β and SLC (maturated pro–TGF-β) with high efficiency (21, 22). This suggests that SLC represents the readily available pool of TGF-β in bone, and that the SLC initially embedded in bone matrix can greatly influence the dose of bioactive TGF-β in the bone microenvironment upon bone resorption. In Esl-1$^{-/-}$ bone tissue, we detected an increased TGF-β maturation that may potentiate the bone matrix to release greater amounts of bioactive TGF-β during bone resorption. Moreover, significantly elevated expression of multiple TGF-β downstream targets in Esl-1$^{-/-}$ calvaria support this notion. TGF-β maturation is catalyzed by furin, which is a housekeeping protease, processing a broad spectrum of substrates such as VEGF and PTHrP, etc. (23, 24). We previously showed that ESL-1 binding to pro–TGF-β leads to blockade of furin on TGF-β cleavage (19). This mechanism may provide a more specific and effective control on TGF-β maturation than other mechanisms that directly act on furin production and activity.

TGF-β signaling in the bone microenvironment affects OBs and OCs, and regulates skeletal homeostasis in a context-dependent manner. The complexity of its regulation is well represented in a number of genetic models. For examples, TGF-β1$^{-/-}$ mice show impaired migration of mesenchymal stem cells to the newly formed bone surface during bone remodeling, thereby exhibiting a late-onset low bone mass (10). Accordingly, gain of TGF-β1 activation and secretion in Camurati–Engelmann disease causes high bone mass (9). Conversely, OB specific overexpression of mature TGF-β2 results in osteoporosis with elevated bone resorption and undermineralization of bone matrix (7). More interestingly, haploinsufficiency of Smad3, a main downstream effector of canonical TGF-β signaling, leads to high bone mass, whereas the Smad3$^{-/-}$ mice show low bone mass (25). This seemingly contradictory observation emphasizes that TGF-β is an important coupling factor of bone remodeling whose bioavailability/dose needs to be finely regulated at multiple levels to meet the dynamic requirement of bone remodeling at different phases. Here, our data support that ESL-1 is a crucial regulator
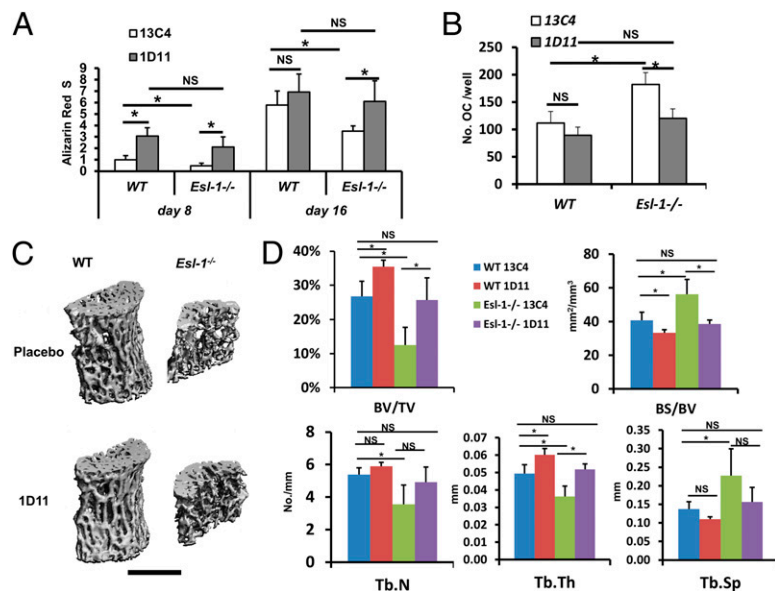
Fig. 5. Negating bioactive TGF-β effectively corrects the defects in *Esl-1*$^{-/-}$ primary cultures and osteopenia phenotypes. (*A*) 1D11 rescues delayed mineralization of *Esl-1*$^{-/-}$ OBs. (*B*) 1D11 rescues overactive osteoclastogenesis induced by *Esl-1*$^{-/-}$ BMSCs. (*C*) µCT images of the L4 vertebra at 8-wk-old male WT vs. *Esl-1*$^{-/-}$ mice treated with either placebo (13C4) or 1D11 for 4 wk before collection. (Scale bar: 1 mm.) (*D*) Quantified bone parameters, including BV/TV, Tb.N, Tb.Th, and Tb.Sp, indicate an effective correction of the *Esl-1*$^{-/-}$ osteopenia by 1D11 treatment. *n* = 4 per group; *$P < 0.05$; NS, nonsignificance.

acting at the level of TGF-β maturation before TGF-β being embedded in the bone matrix, thus preventing overactivation of TGF-β signaling during bone resorption. The importance of a regulatory mechanism for TGF-β maturation has been previously demonstrated in the control of blood pressure homeostasis as well as cartilage development (19, 26). However, it has not been appreciated in bone homeostasis and pathogenesis. Here, our finding suggests that, along with other mechanisms controlling TGF-β bioavailability during bone remodeling, including TGF-β expression, secretion, and activation from latency, the modulation of TGF-β maturation is also a pivotal and regulatable step for proper bone homeostasis.

Published data from in vivo or in vitro studies support that TGF-β decreases bone mineralization, whereas blockade of TGF-β enhances mineralization (7, 27). Previous studies also show that TGF-β signaling delays osteocalcin expression as well as OB terminal differentiation through suppressing Runx2 function (28). TGF-β also regulates expression of matrix proteins and MMPs, which play roles in matrix organization and mineralization. *Esl-1*$^{-/-}$ bone shows changes in TGF-β downstream targets, including decreased osteocalcin, and elevated CTGF and MMP13, etc. (Fig. 4 *A* and *C*), which may partly contribute to the delayed mineralization in the *Esl-1*$^{-/-}$ bone. Elevated CTGF in bone causes osteopenia by suppression of OB activity in mineralization and bone formation (29). Loss of MMP13 increases trabecular bone mass by attenuating matrix resorption during endochondral ossification (30).

That TGF-β stimulates RANKL production has been revealed in vivo. For example, excessive TGF-β bioavailability in the bone of Fibrillin 2 knockout (*Fbn2*$^{-/-}$) mice significantly elevates OB-derived RANKL level and leads to osteoporosis (31). In our previous studies, we identified that PTHrP, a TGF-β downstream target, is significantly up-regulated in *Esl-1*$^{-/-}$ cartilage and accounts for the shortening of the growth plate (19). Interestingly, we found that PTHrP is also significantly elevated along with other TGF-β downstream targets in the bone. PTHrP plays a dual role during bone remodeling. The OB-specific PTHrP deficiency leads to osteoporosis with decreased OB survival, but the OC formation is also impaired (32). A prolonged and continuous infusion of PTHrP stimulates bone resorption and bone loss (33). Similarly, humoral hypercalcemia of malignancy (HHM) occurs in patients with tumor cells expressing a large amount of PTHrP into the circulation causing overactive osteoclastogenesis and osteoporosis (34). The augmented PTHrP production we observed in the *Esl-1*$^{-/-}$ bone may partly resemble the prolonged and continuous PTHrP administration or HHM.

Blockade of TGF-β signaling in vivo by 1D11 or SD-208 treatment (an inhibitor of the TGF-β type 1 receptor) has been shown to exert significant anabolic effects on bone accrual (4, 5). In our 1D11 treatment groups, the BV/TV in *Esl-1*$^{-/-}$ vertebrae is elevated to a greater extent than that in WT controls (105% vs. 37.8% increase), implying that TGF-β signaling is a major downstream target of ESL-1 during bone homeostasis. Meanwhile, although the 1D11-treated *Esl-1*$^{-/-}$ BV/TV is corrected to a comparable level of the untreated WT BV/TV (25.6% vs. 26.7%), it is still significantly lower than 1D11-treated WT BV/TV (25.6% vs. 35.4%). This is likely due to the substantially different BV/TV baseline between *Esl-1*$^{-/-}$ to WT bone, intrinsic limitations of the treatment protocol (dosage or duration), or the undefined TGF-β–independent ESL-1 function(s) in bone homeostasis. Nevertheless, these data suggest that 1D11 can be potentially used for treating low bone mass caused by altered TGF-β signaling either directly or indirectly, such as that in Marfan syndrome (35, 36) or osteolytic bone metastasis of cancers (27).

Because ESL-1 and TGF-β are also expressed in the OC and its progenitors, it is possible that ESL-1 may play a similar role in regulating TGF-β maturation in these cells during bone homeostasis. However, due to the relatively smaller population of the monocyte/OC lineage compared with the OB lineage in the bone microenvironment, their contribution to the overall TGF-β bioavailability may be limited. Moreover, loss of ESL-1 in OC precursors apparently does not affect osteoclastogenesis (Fig. 3 *D* and *E*), suggesting that the intrinsic function of ESL-1 in monocytic cells may not be directly involved in OC differentiation. To
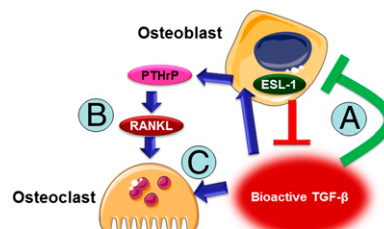

Fig. 6. A schematic of ESL-1 function in bone remodeling. ESL-1 acts in OBs to suppress production of bioactive TGF-β in the bone microenvironment, which inhibits OB mineralization (A), increases PTHrP and RANKL production from OB to activate osteoclastogenesis (B), and facilitates OC differentiation (C).

definitively elucidate the relative contribution of OB or OC-derived ESL-1 in regulating bone homeostasis, a cell-specific ESL-1 conditional knockout allele needs to be established and studied. Moreover, our three-point bending test on *Esl-1*$^{-/-}$ femurs show largely similar results to TGF-β bone specific transgenic mice, with the exception of an elevated elastic modulus (25). This difference may be caused by different test methods used, i.e., whole-bone fracture test vs. nanoindentation and/or wet bone vs. dry bone. However, it is also possible that ESL-1 may regulate matrix composition in a TGF-β–independent manner, which deserves further study.

In summary, ESL-1 plays a crucial role in bone remolding by modulating TGF-β maturation in cells of OB lineage, which ultimately influences TGF-β bioavailability in the bone microenvironment and affects the differentiation of OBs and OCs as well as their coupling (as illustrated in Fig. 6). This study reveals that TGF-β maturation is a critical and regulatable step during bone homeostasis. Targeting TGF-β maturation may provide a new perspective to develop therapies for skeletal or other disorders related to dysregulated TGF-β signaling.

Materials and Methods

Animal Model. *Esl-1*$^{-/-}$ mice were generated as previously described (19), and maintained and used following the Animal Care and Use Committee (IACUC) at the Baylor College of Medicine.

Bone Tissue Preparation, μCT Analysis, and Bone Histomorphometry. Mice were injected with calcein (1.5 mg/mouse) twice at 6 d and 2 d before killing. Femurs and lumbar vertebrae were collected and scanned by μCT (μCT-40; Scanco) for quantification of trabecular and cortical bone parameters. The scanned bones were subsequently embedded in resin for sectioning. Toluidine blue staining, TRAP staining, and calcein double labeling are used for visualization of OBs, OCs, and the dynamics of bone accrual, respectively; histomorphometry data were analyzed using a Bioquant Osteo system.

Treatment with 1D11 Antibody. The 4-wk-old male *Esl-1*$^{-/-}$ and WT mice on C57/B6 background were injected with 1D11 antibody at 10 μg/g body weight for 4 wk, three times each week. The placebo controls were injected at the same condition with 13C4 antibody (nonspecific mouse IgG). The lumbar vertebrae were collected for μCT analysis. For primary OB culture or OB–OC coculture, 1D11 or 13C4 were added into the corresponding culture medium at 10 μg/mL.

Statistics. Student *t* tests were used to determine whether the difference between two groups of data are normally distributed or not. *P* values less than 0.05 are considered statistically significant.

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