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mTOR inhibition rescues osteopenia in mice with systemic sclerosis

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Abbreviations used: ALP, alkaline phosphatase; ANC, all-nuclear cell; BMD, bone mineral density; BMMSC, BM mesenchymal stem cell; BV/TV, bone volume versus total volume; CFU-F, colony forming unit-fibroblast; ChIP, chromatin immunoprecipitation; CTX, cross-linked telopeptide; FBN1, fibrillin-1; HA/TCP, hydroxyapatite tricalcium phosphate; HFC, immunohistochemical; LPL, lipoprotein lipase; OCN, osteocalcin; OPG, osteoprotegerin; PI3K, phosphoinositide 3-kinase; PPARγ2, peroxisome proliferator-activated receptor γ2; rJANKL, soluble receptor activator of nuclear factor kβ ligand; SSc, systemic sclerosis; STAT6, signal transducer and activator of transcription-6; TKO, triple conditional KO; TRAP, tartrate-resistant acid phosphate.

Fibrillin–1 (FBN1) deficiency-induced systemic sclerosis is attributed to elevation of interleukin–4 (IL4) and TGF–β, but the mechanism underlying FBN1 deficiency–associated osteopenia is not fully understood. We show that bone marrow mesenchymal stem cells (BMMSCs) from FBN1-deficient (Fbn1+/−) mice exhibit decreased osteogenic differentiation and increased adipogenic differentiation. Mechanistically, this lineage alteration is regulated by IL4/IL4Rα–mediated activation of mTOR signaling to down–regulate RUNX2 and up–regulate PPARγ2, respectively, via P70 ribosomal S6 protein kinase (P70S6K).

Additionally, we reveal that activation of TGF–β/SMAD3/SP1 signaling results in enhancement of SP1 binding to the IL4Rα promoter to synergistically activate mTOR pathway in Fbn1+/− BMMSCs. Blockage of mTOR signaling by osteoblastic-specific knockout or rapamycin treatment rescues osteopenia phenotype in Fbn1+/− mice by improving osteogenic differentiation of BMMSCs. Collectively, this study identifies a previously unrecognized role of the FBN1/TGF–β/IL4Rα/mTOR cascade in BMMSC lineage selection and provides experimental evidence that rapamycin treatment may provide an anabolic therapy for osteopenia in Fbn1+/− mice.

Fibrillin–1 (FBN1), a major structural component of microfibrils in extracellular matrix plays an important role in organ development and tissue homeostasis (Sakai et al., 1986; Zhang et al., 1995). Recent studies show that LTBP–1 (latent transforming growth factor–β binding protein–1) interacts with FBN1 through its pro-domains (Charbonneau et al., 2004; Ramirez and Sakai, 2010). Thus, Fbn1 deficiency increases the level of activated TGF–β in the intercellular microenvironment. Clinically, Fbn1 gene mutation is linked to several human diseases, including systemic sclerosis (SSc)/scleroderma, Marfan’s syndrome, ectopic lentis, and the dominant form of Well-Marchesani syndrome (Lee et al., 1991; Charbonneau et al., 2004). These diseases are usually characterized by connective tissue fibrosis and skeletal disorders, but it is unclear whether Fbn1 deficiency contributes to bone disorders in these diseases.

As an established SSc mouse model, Fbn1 partial intragenic duplication mutant tight-skin mice (B6.Cg-Fbn1Tsk+/J; Fbn1+/−) show significantly reduced femoral bone mineral density (BMD) and altered trabecular microarchitecture (Barisic-Dujmovic et al., 2007). Loss of function of Fbn1 induced a bone disorder involving elongated bone length and reduced bone density, similar to Marfan syndrome (Dietz et al., 1991, 1994; Lee et al., 1991; Quarto et al., 2012).

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**Figure 1.** *Fbn1*-deficient (*Fbn1<sup>+/−</sup>*) mice exhibit osteopenia phenotype. (A–C) MicroCT (A), BMD (B), and BV/TV (C) analysis of trabecular bone (TB) area in WT and *Fbn1<sup>+/−</sup>* mouse distal femurs. (D) H&E staining of TB volume (yellow square) in distal femurs of *Fbn1<sup>+/−</sup>* mice (*n* = 15) and WT littermates (*n* = 10). Bar, 1 mm. (E) IHC staining of ALP<sup>+</sup> osteoblasts in *Fbn1<sup>+/−</sup>* mice and WT littermates. (F) TRAP staining of osteoclasts in distal femur trabecular bone (TB) and BM in WT and *Fbn1<sup>+/−</sup>* mice. Arrowheads, TRAP<sup>+</sup> osteoclasts (purple cells). Bar, 25 µm. (G–I) ELISA analysis of serum sRANKL (G), OPG (H), and CTX (I) in *Fbn1<sup>+/−</sup>* mice and WT littermates. (J) Toluidine blue staining showed the number of CFU-F from WT and *Fbn1<sup>+/−</sup>* BMMSCs. (K and N) Alizarin red staining of *Fbn1<sup>+/−</sup>* (K) and *Fbn1* siRNA knockdown (N) BMMSCs showed the capacity to form mineralized nodules when cultured under osteoinductive conditions. (L and O) Western blot analysis of *Fbn1<sup>+/−</sup>* (L) and *Fbn1* siRNA knockdown (O) BMMSCs showed expressed levels of FBN1 and the osteogenic genes RUNX2, ALP, and OCN. β-Actin was used as a protein loading control. (M and P) Subcutaneous implantation of BMMSCs in immunocompromised mice showed that bone (B), BM, and connective tissue (CT) were generated around HA/TCP (HA) at 8 wk after implantation. Bars, 50 µm. A semiquantitative analysis showed the amount of bone formation in BMMSC implants. (Q) Calcein double labeling of the metaphyseal trabecular bone in the distal femora showed the bone turnover rate in WT and *Fbn1<sup>+/−</sup>* mice. Bar, 25 µm. All experimental data verified in at least three independent experiments. Error bars represent the SD from the mean values. ***, P < 0.005; NS, not significant.
suggesting that dysregulation of TGF-β signaling by Fbn1 mutation is the major factor underlying this bone deficiency (Judge et al., 2004; Dietz et al., 2005; Lemaire et al., 2006). Fbn1+/−/ mice represent an autoimmune connective tissue disorder characterized by type 2 helper T cell (T_{H}2 cell) infiltration and vascular damage (Gabrielli et al., 2009). IL4, a key T_{H}2 cytokine plays a critical role in the regulation of fibrotic tissue deposition through the signal transducer and activator of transcription-6 (STAT6) pathway (Wynn, 2004). Although the mechanism that results in an elevated level of IL4 in Fbn1−/− mice is unknown, down-regulation of the IL4 gene in Fbn1−/− mice can rescue the pathogenesis in fibrotic diseases, suggesting that IL4 signaling is associated with fibrotic phenotype in SSC (Kodera et al., 2002). However, it is unknown whether IL4 signaling contributes to the osteoporotic phenotype in SSC mice.

BM mesenchymal stem cells (BMMSCs) constitute a population of self-renewal and multipotent cells that can differentiate into osteoblasts, adipocytes, fibroblasts, chondrocytes and nonmesenchymal cell types (Friedenstein et al., 1974; Prockop, 1997). BMMSCs are a promising cell source for bone regeneration and immunoregulatory therapies by interacting with several subsets of immune cells (Le Blanc et al., 2004; Uccelli et al., 2007; Ren et al., 2008; Sun et al., 2009; Uccelli and Mancardi, 2010; Akiyama et al., 2012). In response to stimulation from multiple environmental factors, BMMSCs can differentiate into different lineage cells, which are regulated at both transcriptional and translational levels (Shi et al., 2002; Shi and Gronthos, 2003). In the present study, we show that Fbn1 regulates BMMSC osteogenic/adipogenic lineage selection via IL4Rα/mTOR (the mammalian target of rapamycin) signaling. Blockage of the mTOR cascade by rapamycin, an anticancer and immune suppressive drug, ameliorates the osteopenia phenotype in Fbn1−/− SSC mice.

RESULTS
Fbn1 deficiency alters BMMSC lineage differentiation
Because Fbn1 gene mutation leads to significant loss of bone volume and increase in BM adipocytes in B6.Cg-Fbn1^{+/-} J (Fbn1+/−) mice (Barsic-Dujmovic et al., 2007), we hypothesize that Fbn1 deficiency may reduce osteogenic differentiation of BMMSCs and elevate their adipogenic differentiation. To test this hypothesis, we confirmed that Fbn1 deficiency resulted in an osteopenia phenotype in Fbn1+/− mice. MicroCT and histological analysis showed that BMD, bone volume versus total volume (BV/TV), and distal femoral trabecular bone structure of Fbn1+/− mice were markedly decreased compared with the WT littermates (Fig. 1, A–D). Histomorphometric analysis revealed that the numbers of both osteoblasts and osteoclasts in the femur of Fbn1+/− mice were significantly reduced in comparison with the WT group by alkaline phosphatase (ALP) immunohistochemical (IHC) staining and tartrate-resistant acid phosphate (TRAP) staining, respectively (Fig. 1, E and F). The level of soluble receptor activator of nuclear factor κB ligand (sRANKL), but not osteoprotegerin (OPG), was significantly reduced (Fig. 1, G and H). In addition, we examined the in vivo function of osteoclasts and found that the serum type I collagen cross-linked telopeptide (CTX) level was significantly decreased in Fbn1+/− mice (Fig. 1 I). These data imply that the loss of bone volume in Fbn1+/− mice may be mainly associated with an insufficient bone formation. To examine whether Fbn1 deficiency affects the stem cell properties of BMMSCs, we isolated BMMSCs (Fig. 2, A–C) to show that the number of colony forming unit-fibroblasts (CFU-Fs) was significantly reduced and the expression level of FBN1 was decreased in Fbn1−/− BMMSCs compared with the WT control (Fig. 1, J and L). The osteogenic differentiation capacity of Fbn1−/− BMMSCs was decreased, as indicated by reduced mineralized nodule formation and expression of the osteogenic regulators runt-related transcription factor 2 (RUNX2), ALP, and osteocalcin (OCN; Fig. 1, K and L). Using an established in vivo BMMSC implantation assay, in which 4 × 10^6 BMMSCs with carrier hydroxyapatite tricalcium phosphate (HA/TCP) particles were subcutaneously implanted into immunocompromised mice, we showed that BMMSCs derived from Fbn1−/− mice generated less bone structure than the WT group at 8 wk after implantation (Fig. 1 M). To further confirm the role of Fbn1 in BMMSC differentiation, we used siRNA to silence Fbn1 gene expression in BMMSCs (Fig. 2 D) and found a significantly reduced osteogenic differentiation, as assessed by alizarin red staining to show reduced mineralized nodule formation, immunoblotting analysis to show decreased expression of the osteogenic markers RUNX2, ALP, and OCN, and in vivo BMMSC implantation assay to show reduction in new bone formation (Fig. 1, N–P). To further confirm reduced osteogenesis in Fbn1+/− mice, we performed a calcein labeling assay to show that the bone turnover rate was decreased in Fbn1+/− mice (Fig. 1 Q). In contrast, Oil red O staining showed the number of adipocytes in Fbn1+/− BM was markedly increased when compared with the WT littermates (Fig. 2 E). To confirm that Fbn1 deficiency results in elevated adipogenesis, Fbn1+/− BMMSCs were found to exhibit a significantly elevated number of Oil red O−positive cells and up-regulation of the adipogenic regulators peroxisome proliferator-activated receptor γ2 (PPARγ2) and lipoprotein lipase (LPL) under adipogenic culture conditions (Fig. 2, F and G). Furthermore, we showed that Fbn1-silenced BMMSCs by siRNA had a significantly increased number of Oil red O−positive cells and up-regulation of PPARγ2 and LPL (Fig. 2, H and I). These data suggest that Fbn1 governs osteogenic/adipogenic lineage differentiation in BMMSCs.

Fbn1 modulates BMMSC lineage differentiation via IL4Rα/mTOR signaling
Because previous studies showed that Fbn1 mutation induced T_{H}2 infiltration with a high level of IL4 (Gabrielli et al., 2009), we used IHC staining and ELISA to confirm a higher number of IL4-positive cells in Fbn1+/− mouse BM and an elevated IL4 level in the peripheral blood (Fig. 3, A and B). In addition, we confirmed that Fbn1+/− mice showed significantly increased levels of T_{H}2 cells (Fig. 3, C–F). Quantitative PCR and ELISA showed that WT and Fbn1+/− BMMSCs expressed similar
showed that Fbn1 deficiency up-regulated IL4Rα downstream signaling phosphoinositide 3-kinase (P13K)–p110, phosphorylated Akt (p-Akt), and phosphorylated mTOR (p-mTOR) in Fbn1+/− BMMSCs (Fig. 3 J). To verify that IL4Rα/mTOR signaling contributes to BMMSC osteo/adipo-lineage selection, we examined whether knockdown of the IL4Rα/mTOR cascade could rescue the altered lineage differentiation in Fbn1+/− BMMSCs. Having confirmed that shRNAs could low levels of IL4 (Fig. 3, G and H). Next, we examined whether activation of IL4 downstream signaling contributed to reduced osteogenesis and elevated adipogenesis in Fbn1+/− mice. We used immunofluorescence staining to confirm that BMSCs coexpressed the MSC marker CD73 with IL4Rα (Fig. 3 I). Compared with control BMSCs, Fbn1+/− BMSCs showed up-regulated expression of IL4Rα (Fig. 3 J), which was further confirmed by qPCR analysis (Fig. 3 K). Western blotting also
knock down Il4ra and mTOR (Fig. 3 L) and that rapamycin treatment could inhibit mTOR signaling in a dose-dependent manner (Fig. 3 M), we showed that Il4ra and mTOR shRNAs, as well as rapamycin treatment, significantly improved osteogenic differentiation of Fbn1+/- BMMSCs, as indicated by alizarin red staining to show increased mineralized nodule formation. Western blotting to show elevated expression of the RUNX2, ALP, and OCN, and in vivo BMMSC implantation assay to show increased new bone formation at 8 wk after implantation (Fig. 4, A–C). Conversely, the elevated adipogenic differentiation in Fbn1+/- BMMSCs was significantly reduced in the shRNAs or rapamycin treatment groups, as indicated by a decreased number of Oil red O–positive cells and down-regulation of the PPARγ2 and LPL (Fig. 4 D). To avoid off-target effect, we used IL4Rα neutralizing antibody to block IL4 signaling and found decreased expression of p-mTOR and elevated expression of RUNX2 in Fbn1+/- BMMSCs when compared with control group (Fig. 4 E). We next revealed that mTOR regulated osteogenic differentiation of Fbn1+/- BMMSCs through its downstream signaling...
phosphorylated P70 ribosomal S6 protein kinase (P70S6K). Western blot analysis showed that p-P70S6K expression level was increased in Fbn1+/− BMSCs (Fig. 4 F). When p-mTOR expression was knocked down by IL4Rα and mTOR shRNAs, or rapamycin treatment, in Fbn1+/− BMSCs, a corresponding down-regulation of p-mTOR and p-P70S6K was revealed by Western blot analysis (Fig. 3 N). Although knockdown of P70S6K expression by siRNA in Fbn1+/− BMSCs resulted in up-regulation of RUNX2 expression (Fig. 4 G), the expression levels of mTOR was unaffected (Fig. 4 H). To avoid off-target effects, a second P70S6K siRNA was used to show elevated expression of RUNX2 (Fig. 4 I). In addition, the osteogenic differentiation was markedly increased in P70S6K knockdown Fbn1+/− BMSCs, as indicated by elevated mineralized nodule formation (Fig. 4 J) and expression of the RUNX2, ALP, and OCN (Fig. 4 K). In contrast, the number of Oil red O−positive cells (Fig. 4 L) and the expression levels of the adipogenic genes were significantly down-regulated (Fig. 4 M).
O–positive cells and the expression of PPAR\(\gamma\)2 and LPL (Fig. 5, G and H). These data suggest that activation of the IL4/IL4R\(\alpha\)/mTOR/P70S6K cascade contributes to osteogenic deficiency of \(Fbn1^{+/-}\)BMMSCs and that blockade of mTOR signaling may be a promising therapeutic approach for rescuing osteogenic deficiency in \(Fbn1^{+/-}\)mice.

Because mTOR signaling involves two complexes, namely mTOR complex 1 (TORC1) and complex 2 (TORC2), we next examined which mTOR complex plays a functional role in regulating \(Fbn1^{+/-}\)BMMSC differentiation. We found that \(Fbn1^{+/-}\)BMMSCs expressed elevated levels of mTOR/Raptor/TORC1 and mTOR/Rictor/TORC2 when compared with the control group (Fig. 5 I). When using siRNA to knockdown either TORC1 or TORC2, the expression of the osteogenic marker Runx2 was up-regulated in \(Fbn1^{+/-}\)BMMSCs after siRNA knockdown of either Rictor or Raptor (Fig. 5 J). Western blotting analysis showed that knockdown of Rictor resulted in down-regulation of both Raptor and Rictor, whereas knockdown of Raptor resulted in only down-regulation of Rictor. (L) To avoid off-target effects, second TORC1 or TORC2 siRNAs were used to show elevated expression of RUNX2 in BMMSCs. (M) Western blot showed that knockdown of Rictor, but not Raptor, resulted in down-regulation of p-Akt. All experimental data verified in at least three independent experiments. Error bars represent the SD from the mean values. ***, \(P < 0.005\).

Figure 5. IL4/mTOR signaling governs lineage commitment of BMMSCs. (A) Western blot showed that IL4 treatment resulted in elevated expression of p-mTOR in an dose-dependent manner in WT and \(Fbn1^{+/-}\)BMMSCs. (B) Western blot showed IL4 treatment resulted in elevated p-mTOR expression starting at 6 h after treatment and lasting until 72 h after treatment in WT BMMSCs. (C) Western blot showed the expression of mTOR signaling, including PI3K-p110, p-Akt, p-mTOR, and p-P70S6K in IL4-treated BMMSCs. (D) Alizarin red staining showed BMMSC-mediated mineralized nodule formation after mTOR activation. (E) Western blot indicated the expression of osteogenic genes RUNX2, ALP, and OCN in BMMSCs after mTOR activation. (F) H&E staining showed the capacity to form bone in mTOR-activated BMMSCs when implanted into immuno-compromised mice. Bar, 50 µm. A semiquantitative analysis showed the amount of bone formation in implants. (G) Oil red O staining showed the number of adipocytes in mTOR-activated BMMSCs. Bar, 50 µm. (H) Western blot showed the expression of adipogenic genes PPAR\(\gamma\)2 and LPL in mTOR-activated BMMSCs. (I) Western blot showed the expression levels of p-Rictor and p-Raptor in \(Fbn1^{+/-}\)BMMSCs. (J) Western blot showed the expression of osteogenic marker RUNX2 in \(Fbn1^{+/-}\)BMMSCs after siRNA knockdown of either Rictor or Raptor. (K) Western blotting analysis showed that knockdown of Rictor resulted in down-regulation of both Rictor and Rictor, whereas knockdown of Raptor resulted in only down-regulation of Rictor. (L) To avoid off-target effects, second TORC1 or TORC2 siRNAs were used to show elevated expression of RUNX2 in BMMSCs. (M) Western blot showed that knockdown of Rictor, but not Raptor, resulted in down-regulation of p-Akt. All experimental data verified in at least three independent experiments. Error bars represent the SD from the mean values. ***, \(P < 0.005\).
siRNAs to show they are able to elevate expression of RUNX2 in Fbn1+/− BMMSCs (Fig. 5 L). Because Akt is a downstream target of TORC2, we examined the expression levels of p-Akt after the knockdown of either TORC1 or TORC2 and confirmed that TORC2 acted upstream of TORC1 through the Akt cascade in Fbn1+/− BMMSCs (Fig. 5 M).

**IL4Rα is activated by the TGF-β pathway in Fbn1−/−-deficient BMMSCs**

It was reported that FBN1 interacts with LTBP-1 to bind TGF-β (Charbonneau et al., 2004; Ramirez and Sakai, 2010). As such, Fbn1 deficiency causes an increased release of TGF-β to the skin and lung (Gabrielli et al., 2009; Holm et al., 2011). We examined the level of TGF-β in Fbn1+/− mouse BM and Fbn1+/− BMMSC culture medium and found that both BM and medium contained elevated levels of TGF-β, as assessed by ELISA assay (Fig. 6 A). Next, we used Western blotting to confirm that TGF-β downstream signaling p-SMAD3 and SMAD-associated transcription factor SP1 (Docagne et al., 2004; Jungert et al., 2006) were significantly increased in Fbn1+/− BMMSCs compared with the control BMMSCs (Fig. 6 B). To further confirm that Fbn1 deficiency-mediated IL4Rα expression occurs through TGF-β release, we treated BMMSCs with recombinant TGF-β1 or Fbn1 siRNA, followed by TGF-β neutralizing antibody blocking. Western blot analysis revealed that both TGF-β1 and Fbn1 siRNA treatment increased IL4Rα levels, which could be blocked by TGF-β neutralizing antibody treatment (Fig. 6, C−F). To further confirm that TGF-β signaling activates IL4Rα expression, we used siRNA to block canonical TGF-β pathway and then examine the expression level of IL4Rα in Fbn1+/− BMMSCs. Western blot analysis showed that IL4Rα was significantly decreased in Fbn1+/− BMMSCs after Smad2/3 siRNA treatment (Fig. 6 G), indicating that TGF-β/SMAD3 signaling may directly regulate IL4Rα expression. To determine whether increased expression of IL4Rα in Fbn1+/− BMMSCs is attributed to the TGF-β/SMAD3 signaling, we generated an Il4rα promoter reporter construct in which the defined region of the Il4rα promoter and flanking region were placed upstream of a reporter gene encoding firefly luciferase. We used BIOBASE biological databases to search the Il4rα promoter sequence and found two SP1 and one STAT6 candidate sites, which were closely matching with the SMAD3-associated SP1 and IL4Rα downstream STAT6 transcription factors consensus targets (Fig. 6 H). Next demonstrated that Il4rα promoter activity was markedly induced in both Fbn1+/− and TGF-β−treated BMMSCs. When Fbn1+/− BMMSCs were transfected with reporter vector, the luciferase assay showed a significantly increased Il4rα promoter activity.
activity. Introduction of SP1 mutated reporter vector markedly diminished the expression of the IL4Rα-luciferase reporter in both Fbn1-/- and TGF-β–treated BMMSCs, indicating the direct initiation of IL4Rα expression by TGF-β–SMAD3–SP1 cascades (Fig. 6 H). We next determined whether SP1 regulates IL4Rα promoter in BMMSCs. Using chromatin immunoprecipitation (ChIP), the SP1 binding consensus sequence within the promoter region was examined to confirm its ability to activity. Introduction of SP1 mutated reporter vector markedly diminished the expression of the IL4Rα-luciferase reporter in both Fbn1-/- and TGF-β–treated BMMSCs, indicating the direct initiation of IL4Rα expression by TGF-β–SMAD3–SP1
recruit SP1. As expected, SP1-bound DNA at the candidate site was significantly enriched in TGF-β-treated BMMSCs and Fbn1<sup>+/−</sup> BMMSCs (Fig. 6 I), suggesting that TGF-β may act as an initiator to elevate IL4Rα expression. In parallel, we used a ChIP-qPCR assay and found that STAT6 directly bound to the predicted promoter region of IL4α and that STAT6 recruitment was enriched at the IL4α promoter in Fbn1<sup>+/−</sup> or TGF-β-treated BMMSCs (Fig. 6 I). Western blot analysis further confirmed that an IL4Rα-JAK1-STAT6 cascade was activated in Fbn1<sup>+/−</sup> BMMSCs, as shown by up-regulation of p-JAK1 and p-STAT6 expression (Fig. 6 J). These results prompted us to examine whether STAT6 induced IL4Rα expression at the transcriptional level using a reporter assay. Control BMMSCs, TGF-β-treated BMMSCs, and Fbn1<sup>+/−</sup> BMMSCs were transfected with a reporter vector or STAT6 candidate site mutated reporter vector, followed by luciferase assay. TGF-β-treated and Fbn1<sup>+/−</sup> BMMSCs showed increased promoter activity in comparison to control BMMSCs. Introduction of STAT6 mutated reporter vector markedly diminished the expression of the Il4rα-luciferase reporter in Fbn1<sup>+/−</sup> BMMSCs, but not TGF-β-treated BMMSCs. Thus, TGF-β may act as an initiator to elevate IL4Rα expression, and TGF-β-activated IL4Rα is independent from IL4Rα-STAT6 cascades (Fig. 6 H). These results prompted us to
examine whether TGF-β and IL4 synergistically activate the mTOR signaling in BMMSCs. We found that TGF-β, but not IL4, treatment elevated the expression levels of IL4Rα in BMMSCs by Western blot analysis (Fig. 6, K and L). Although TGF-β and IL4 treatments each elevated expression levels of p-mTOR in BMMSCs, combinatorial treatment of TGF-β and IL4 resulted in a marked increase in p-mTOR expression (Fig. 6, K and L). These results indicate that the Fbn1 deficiency-induced TGF-β–SMAD3–SP1/IL4Rα cascade synergistically enhances mTOR signaling with STAT6 signaling in Fbn1Δ/Δ BMMSCs at the transcriptional level, which leads to lineage alteration of Fbn1Δ/Δ BMMSC.

**Conditional knockout of IL4Rα/mTOR in BMMSCs/osteoblasts or rapamycin treatment ameliorates osteopenia phenotype in Fbn1Δ/Δ mice**

Because IL4Rα/mTOR alters BMMSC lineage commitment, depletion of mTOR expression in BMMSC lineage may serve as a key approach to recover Fbn1 deficiency-induced osteopenia. To achieve such a tissue-specific KO, we first generated Prx1-Cre; Il4rΔ/+; Fbn1Δ/Δ triple conditional KO (TKO) mice to KO IL4Rα in Fbn1Δ/Δ BMMSCs (Fig. 7 A). Il4rΔ/+ littermates were used as normal control, and Il4rΔ/Δ; Fbn1Δ/Δ (DKO) littermates were used as osteopenia models (Fig. 7 A). The efficacy of Cre-mediated deletion of floxed alleles was shown by qPCR analysis (Fig. 7 B). MicroCT analysis showed that TKO mice have significantly increased BMD compared with DKO mice, indicating elevated bone formation capacity compared with Ad-GFP-DKO-BMMSCs (Fig. 7 G). In contrast, Ad-Cre-DKO-BMMSCs showed a significant decrease in adipogenic differentiation compared with Ad-GFP-DKO-BMMSCs, as shown by a decreased number of Oil red O–positive cells and down-regulation of the adipogenic genes PPARγ2 and LPL (Fig. 7, I and J).

It has been shown that SP7–expressing osteoblastic progenitors have the ability to control osteoblastic/adipogenic lineage switch (Zhang et al., 2011; Song et al., 2012). To examine whether knocking down mTOR signaling in the preosteoblastic lineage can rescue the osteopenia phenotype in Fbn1Δ/Δ mice, we generated a tissue-specific KO, SP7-Cre; MtorΔ/Δ; Fbn1Δ/Δ (TKO) conditional Mtor KO mice (Fig. 9 A). Floxed Mtor littermates (MtorΔ/+) were used as normal control and MtorΔ/Δ; Fbn1Δ/Δ (DKO) littermates were used as osteopenia models (Fig. 9 A). The efficacy of Cre-mediated deletion of floxed allele was shown by qPCR analysis (Fig. 9 B). MicroCT analysis and histological analyses showed that TKO mice have significantly increased BMD and trabecular bone volume compared with DKO mice (Fig. 9 C), suggesting that Mtor conditional KO in the preosteoblastic lineage rescued osteopenia in Fbn1-deficient mice. The number of adipocytes in TKO mouse BM was significantly reduced when compared with the DKO mice, as assessed by Oil red O staining (Fig. 9 C). These data prompted us to use a drug, specifically rapamycin, to attempt to ameliorate the disease phenotype in Fbn1Δ/Δ mice.

Next, we examined whether rapamycin treatment rescued the osteopenia phenotype in Fbn1Δ/Δ mice. Rapamycin was intraperitoneally administered to Fbn1Δ/Δ mice at 8 or 6 wk of age for 14 or 28 consecutive days, respectively. The samples were harvested at 10 wk of age for further evaluation (Fig. 10 A). Histological and microCT analysis showed that rapamycin treatment elevated BMD and trabecular bone volume in Fbn1Δ/Δ mice (Fig. 10 B). The number of adipocytes in BM of rapamycin-treated Fbn1Δ/Δ mice was significantly reduced when compared with untreated Fbn1Δ/Δ mouse BM, as assessed by Oil red O staining (Fig. 10 B). BMMSCs isolated from littermates were used as osteopenia models (Fig. 8 A). The efficacy of Cre-mediated deletion of floxed allele was shown by qPCR analysis (Fig. 8 B). MicroCT analysis showed that Mtor conditional KO rescued BMD in Fbn1Δ/Δ deficiency–induced osteopenia (Fig. 8 C). Histological analysis showed that trabecular bone volume in TKO mice was increased compared with the DKO mice (Fig. 8 D). The numbers of adipocytes in TKO mouse BM were significantly reduced when compared with the DKO mice, as assessed by Oil red O staining (Fig. 8 D). To investigate the role of mTOR in the differentiation of Fbn1Δ/Δ deficient BMMSCs, we isolated BMMSCs from DKO mice and knocked down Mtor using an adenovirus expressing Cre with GFP or an adenovirus expressing GFP only as a control. Ad-Cre-DKO-BMMSCs showed significantly increased osteogenic differentiation, as assessed by alizarin red staining in the differentiation of Fbn1Δ/Δ BMMSCs, as shown by a decreased number of Oil red O–positive cells and down-regulation of the osteogenic genesRUNX2, ALP, and OCN (Fig. 8 F); and in vivo implantation indicating elevated bone formation capacity compared with the Ad-GFP-DKO-BMMSCs (Fig. 8 G). In contrast, Ad-Cre-DKO-BMMSCs showed a significant decrease in adipogenic differentiation compared with Ad-GFP-DKO-BMMSCs, as shown by a decreased number of Oil red O–positive cells and down-regulation of the adipogenic genes PPARγ2 and LPL (Fig. 8, H and I).

We next generated Prx1-Cre; MtorΔ/Δ; Fbn1Δ/Δ TKO mice to KO mTOR in Fbn1Δ/Δ BMMSCs (Fig. 8 A). MtorΔ/Δ littermates were used as normal control, and MtorΔ/Δ; Fbn1Δ/Δ (DKO) littermates were used as normal control, and MtorΔ/Δ; Fbn1Δ/Δ (DKO)
rapamycin-treated Fbn11/− mice showed significantly increased CFU-F number compared with the untreated group (Fig. 10 C). Osteogenic differentiation of BMMSCs from rapamycin-treated Fbn11/− mice was markedly improved when compared with the untreated Fbn11/− BMMSCs, as analyzed by alizarin red staining to show elevated mineralized nodule formation and Western blot analysis to show decreased expression of p-mTOR and p-P70S6K and increased expression of the RUNX2, ALP, and OCN (Fig. 10, D and E). In contrast, BMMSCs isolated from rapamycin-treated Fbn11/− mice showed a significant decrease in adipogenic differentiation compared with the untreated Fbn11/− BMMSCs, as shown by decreased number of Oil red O–positive cells and down-regulation of the PPARγ2 and LPL (Fig. 10, F and G).

DISCUSSION

FBN1 is essential for the formation of elastic fibers or microfibrils that provide strength and flexibility to connective tissue. Normally, FBN1 is abundant in the connective tissue of the aorta, ligature of the human eye lens, bones, and lungs. Mutation of the fibrillin gene causes SSc and Marfan’s syndrome, in which bone and connective tissue disorders are observed. The bone defects in SSc patients were considered to be an insignificant clinical symptom; however, recent emerging evidence shows that bone resorption may be a common clinical manifestation (Atteritano et al., 2013; Kilic et al., 2013; Omair et al., 2013). In the present study, we identified a marked osteopenia phenotype in Fbn1 mutant-induced SSc mice. Recently, it was suggested that FBN1 and FBN2 might differentially regulate endogenous BMP and TGF-β activity in osteoblasts to affect bone growth and development (Nistala et al., 2010). In this study, we reveal that Fbn1 deficiency results in an increased level of TGF-β in BM, which, in turn, initiates a cascade in which IL4Rα is up-regulated in BMMSCs via SMAD3/SP1 binding to Il4r promoter, leading, as a consequence, to the activation of mTOR–P70S6K signaling to directly suppress osteogenesis via RUNX2 and promote adipogenesis via PPARγ2, respectively. Osteogenic differentiation deficiency in Fbn11/− BMMSCs may contribute to an osteoporotic phenotype, such as low BMD or reduced trabecular bone structure, in Fbn11/− SSc mice. However, inhibition of mTOR signaling using
rapamycin treatment appears to be a promising approach to rescue osteoporotic disorders in the SSc mice.

It is known that mTOR signaling positively regulates expression of the adipogenic gene PPARγ2, which is critical for adipogenesis of BMSCs (Kim and Chen, 2004; Yu et al., 2008; Zhang et al., 2009). However, controversial findings were reported regarding mTOR signaling in osteogenesis in that mTOR may be required for osteoblast proliferation, which is involved in several signaling pathways through IL6 (Kozawa et al., 2001; Takai et al., 2007; Takai et al., 2008). In contrast, rapamycin may either inhibit (Shoba and Lee, 2003) or stimulate (Lee et al., 2010b; Martin et al., 2010) osteogenesis, depending on cell type or differentiation stages. Recently, it has been reported that bone matrix secretes IGF-1 (insulin-like growth factor-1) to recruit normal BMSCs for bone remodeling by activating mTOR signaling (Xian et al., 2012), indicating that mTOR may contribute to the activation of different sets of biological regulatory controls under certain conditions. In this study, we reveal that elevated IL4Rα expression leads to the activation of mTOR-P70S6K signaling in Fbn1+/− BMSCs, which directly inhibits the osteogenic gene RUNX2 and suppresses bone regeneration in vitro and in vivo. In contrast, rapamycin represses
IL4R/mTOR signaling at the early BMMSC (SP7-Cre) and osteoblastic progenitor stages (SP7-Cre). Previous studies showed that SP7-expressing osteoblastic progenitors have the ability to control the osteoblastic/adipogenic lineage switch (Zhang et al., 2011; Song et al., 2012) and that SP7 can be regulated by small microRNA (Zhang et al., 2011). Our data showed that SP7-Cre-mediated KO of IL4Rα/mTOR cascades can rescue the BMMSC lineage alteration and osteogenesis phenotype in Fbn1+/− mice. These data may encourage us to use a drug such as rapamycin to ameliorate the disease phenotype in Fbn1+/− mice. Rapamycin, a specific mTOR inhibitor, is a novel nonsteroidal anticancer and immunosuppressive drug (Abraham and Wiederecht, 1996; Wang et al., 2003; Shaw et al., 2004). Several preclinical studies showed that rapamycin is effective in treating autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis (Yoon, 2009; Islander et al., 2011). These diseases are usually associated with bone disorders (Weinstein, 2010). Although efficacy of rapamycin treatment in SSc patients must be assessed in clinical studies, phase I clinical trials showed optimal safety for its use in treating scleroderma patients (Su et al., 2009). Added experimental evidence showed that rapamycin treatment resulted in a significant improvement of skin phenotype with regulation of immune index, such as reducing the levels of IL4 and IL17 in Fbn1+/− mice (Yoshizaki et al., 2010). These data suggest that the underlying mechanism of rapamycin-mediated treatment may be associated with immunomodulation, leading to the down-regulation of TGF-β cells (Lee et al., 2010a). Up-regulation of osteogenesis and down-regulation of adipogenesis in rapamycin-treated Fbn1+/− mice via inhibition of mTOR signaling in BMMSCs may help to rebuild functional osteoblasts to improve niche microenvironment for immune cells and rebuild homeostasis for the immune system. Because of the extensive functional roles of rapamycin in immune, anticancer, and osteoporosis therapies, it is necessary to explore detailed mechanisms by which rapamycin may target several signaling pathways simultaneously.

**MATERIALS AND METHODS**

**Animals.** Female C57BL/6J, B6.Cg-Fbn1+/−/J, B6.Cg-Tg(Prx1-Cre)1Cjt/J, B6.Cg-Tg (Sp7-TTA, tero-EGFP/cre)1Amc/J, and B6.129S4-Mtor+/-IjcSc/J mice were purchased from The Jackson Laboratory and maintained in C57BL/6J background in at least 10 backcrosses. Age-matched female littermates were used in all experiments. Female immunocompromised nude mice (Beige m/mu XIDIII) were purchased from Harlan. IL4Rα null (B4αa−/−) and floxed (B4αafl/+; Herbert et al., 2004) mice were gifts from F. Brombacher (University of Cape Town, Cape Town, South Africa). To generate tissue-specific Cre-mediated KO models, Cre-, floxed-, and Fbn1+/− mice were intercrossed, and age-matched female littermates were used as WT controls. All animal experiments were performed under the institutionally approved protocols for the use of animal research (University of Southern California protocol numbers 11141, 11953, and 11327).

**MicroCT analysis.** Femurs were harvested and analyzed by Inveon microCT system (Siemens AG). Cross-sectional volumetric BMD was measured at right femur mid-diaphysis with a density phantom. Using 3-dimensional images, a region of interest in secondary spongiosa was manually drawn near the endocortical surface, and BV/TV was assessed as a cancellous bone morphometric parameter.
In vivo BMMSC implantation assay. Approximately 4.0 × 10^6 BMMSCs were mixed with HA/TCP ceramic powders (40 mg; Zimmer Inc.) and subcu-
taneously implanted into 8-wk-old immunocompromised mice (Gromoths et al., 2000). At 8 wk after implantation, the transplants were harvested, fixed in 4% PFA, and decalcified with 5% EDTA, pH 7.4, followed by paraffin embedding. The 6-µm paraffin sections were stained with H&E chemical staining. Total BV/TV was quantified by ImageJ software (National Institutes of Health).

In vivo Oil red O staining. To assess the adipose tissue in trabecular areas, femurs were fixed in 4% paraformaldehyde and decalcified with 5% EDTA, pH 7.4, followed by cryosection. Sections were stained with Oil Red O, and positive areas were quantified under microscopy and shown as a percentage of total areas.

In vivo rapamycin treatment. Rapamycin (LC Laboratories) was in the vehicle containing 0.2% sodium carboxymethylcellulose (Sigma-Aldrich) and 0.25% polysorbate 80 (Sigma-Aldrich). Rapamycin with vehicle was in-traperitoneally administered to Fbn1-deficient mice at a dose of 1.5 mg/kg/d for 14 and 28 d, respectively (Blazier et al., 1994). The disease group and control mice were treated with vehicle only. Treatment was started at age 6 or 8 wk, and all groups of mice were healthy.

Histology. To assess trabecular bone and BM areas, femurs were fixed in 4% paraformaldehyde (Sigma-Aldrich) and then decalcified with 5% EDTA, pH 7.4, followed by paraffin embedding. Paraffin sections (6 µm) were stained with hematoxylin and eosin (H&E) and analyzed by ImageJ software. To perform immunohistochemistry staining, the paraffin-embedded sections were blocked with serum matched to secondary antibodies, incubated with the ALP or IL4 specific antibodies (1:400; Santa Cruz Biotechnology, Inc.) at 4°C for overnight, and then stained using VECTASTAIN UNIVERSAL elite ABC kit and ImmPACT VIP Peroxidase Substrate kit (Vector Labora-
tories) according to the manufacturer’s instructions. To quantify osteoclast activity, mature osteoclasts were determined by TRAP-positive cells on the bone surface. Deparaffinized sections were refluxed with a mixture of 50% ethanol and 50% acetic acid for 10 min. TRAP staining solutions (1.6% naph-
thol AS-BI phosphate in N, N-dimethylformamide, 0.14% fast red-violet LB diazonium salt, 0.097% tartaric acid, and 0.04% MgCl2 in 0.2 M sodium ac-
etate buffer at pH 5.0) were freshly made. The sections were incubated in the solution for 10 min at 37°C under shield and counterstained with tol-
uidine blue. All reagents for TRAP staining were purchased from Sigma-
Aldrich. To label the mineralization fronts, the mice were given subcutaneous injections of calcine (15 mg/kg body weight; Sigma-Aldrich) in 2% sodium bicarbonate solution 10 and 3 d before sacrifice. Bone dynamic histomor-
phometric analyses for MAR, MS/BS, and BFR/BS were performed ac-
cording to the standardized nomenclature for bone histomorphometry (Parfitt et al., 1987).

ELISA. Peripheral blood serum and cell culture medium were collected, and sRANKL, OPG, IL4, and TGF-β protein levels were analyzed using mouse ELISA Ready-SET-GO kits (eBioscience), according to the manu-
facturer’s instructions. CTX levels were analyzed with a mouse C-telopeptide of type I collagen ELISA kit (Novartisbios), according to the manufacturer’s instructions. Human IL4 and TGF-β protein levels were analyzed using ELISA Ready-SET-GO kits (eBioscience), according to the manufacturer’s instructions. Human sRANKL protein levels were analyzed using sRANKL Super X ELISA kit (Antigenix America Inc.) and OPG levels were analyzed by OPG instant ELISA kit (eBioscience), according to the manufacturer’s instructions. For measurement of anti-dsDNA antibodies and ANA, protein levels were analyzed using anti-dsDNA ELISA kit (Assay Laboratories), according to the manufacturer’s instructions. For measurement of anti-dsDNA antibodies and ANA, periph-
ernuclear cells were isolated from BMMSCs using magnetic beads (Miltenyi Biotec). For measurement of anti-dsDNA antibodies and ANA, protein levels were analyzed using anti-dsDNA ELISA kit (Assay Laboratories), according to the manufacturer’s instructions. For measurement of anti-dsDNA antibodies and ANA, periph-
eral blood serum samples were collected from all experimental mice and ana-
lized by commercially available ELISA kits (Alpha Diagnostics) according to the manufacturer’s instructions.

Isolation of mouse BMMSCs. Single suspension of BM-derived all-nuclear cells (ANCs; 15 × 10^6) from femurs and tibias were seeded into 100 mm culture dishes (Corning) and cultured at 37°C with 5% CO2. After 2 d, nonadherent cells were removed, and attached cells were cultured for 16 d in α-MEM (Invitrogen) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). These plastic attached single colonies were pas-
saged with frequent medium changes to eliminate potential hematopoietic cells contamination (Soleimani and Nadri, 2009; Zhu et al., 2010). Flow cy-
mometric analysis was performed to show a surface epitope profile of isolated BMMSCs (Fig. 2 A). To further confirm that the single colony-derived plastic adherent BMMSCs were not contaminated with hematopoietic cells, we depleted T cells in BMMSC culture using CD3 antibody treatment. In brief, 1 ml CD3 antibody was added to 10^6 BMMSCs and the mixture was incu-
bated at 4°C for 30 min. The cells were centrifuged for 5 min to remove unbound antibody, resuspended in 1 ml diluted (1:6) rabbit complement (Pel-
Frezz Biologicals) and incubated at 37°C for 30 min. Subsequently, CD3 antibody/complement treatment was repeated once (Gilmore et al., 1986). In addition, we sorted BMMSCs from BM cells according to their MSC-specific surface markers using flow cytometry, as reported previously (Hoolihan et al., 2012). BM cells were selected by lineage markers (Lin−) and then sorted by PDGFRα and Sca-1 (PaxS). Alizarin Red staining and Western blot analysis showed that BMMSCs derived from plastic collected single colonies, CD3 antibody/complement depletion, and flow cytometric sorting had similar osteogenic abilities (Fig. 2, B and C). For CFU-F assay, 10^5 ANCs from BM were seeded in T25 cell culture flasks (Corning). After 16 d, the cultures were washed by PBS and stained with 1% toluidine blue solution with 2% paraformaldehyde. The cell clusters with more than 50 cells were counted as colonies under microscopy.

Osteogenic differentiation assay. BMMSCs were cultured under osteo-
egenic culture condition, containing 2 mM β-glycerophosphate (Sigma-Aldrich), 100 μM l-ascorbic acid 2-phosphate (Wako), and 10 mM dexamethasone (Sigma-Aldrich) in the growth medium. After 3 wk induction, 1% Alizarin Red S (Sigma-Aldrich) staining was performed to detect matrix mineraliza-
tion, and the stained areas were quantified by ImageJ software and shown as a percentage of the total area.

Western immunoblotting. Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Fisher Scientific) with protease and phosphatase inhibitors (Roche), and proteins were quantified using protein concentra-
tion assay (Bio-Rad Laboratories). 20 μg of proteins were separated by SDS-
PAGE and transferred to 0.2 μm nitrocellulose membranes (Millipore). The membranes were blocked with 5% nonfat dry milk and 0.1% Tween-20 for 1 h, followed by incubation overnight with the primary antibodies diluted in blocking solution according to manufacturer’s instructions. Antibodies to mouse ALP, LPL, PPARγ2, IIL4Ra, P3K-p110, phospho-Smad2/3, and SP1 were purchased from Santa Cruz Biotechnology, Inc. Antibodies to human RUNX2, ALP, and OCN were purchased from Santa Cruz Biotechn-
ology, Inc. Antibodies to phospho-mTOR (Ser2448), mTOR, phospho-Akt (Ser473), Akt, phospho-P70S6K (T389; S371), phosphor-Raptor, phosphor-
Raptor, and Smad2/3 were obtained from Cell Signaling Technology. Antibodies to mouse RUNX2, FBN1, OCN, and β-actin were purchased from Abcam, GeneTex, Millipore, and Sigma-Aldrich. The membranes were then incubated for 1 h in HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) diluted at 1:10,000 in blocking solution. Immunoreactive proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and BioMax film (Kodak). The intensity of bands was measured by using ImageJ software and normalized to β-actin.

Adipogenic differentiation. For adipogenic induction, 500 nM 1,25-dihydro-
pyl-3,5-di-oxypregn-20-ene-3Z,5Z-16α,21-diol (Sigma-Aldrich), 10 μM 1,25-dihydro-
pyl-3,5-di-oxypregn-20-ene-3Z,5Z-16α,21-diol (Sigma-Aldrich), and 100 nM t-ascorbic acid phosphate were added into the growth medium. After 7 d, the cultured cells were stained with Oil red O (Sigma-Aldrich), and positive cells were quantified under microscopy and shown as a number to total cells.

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RNAsi and chemical reagent treatments. BMSCs (0.5 x 10^6) were seeded to a 6-well culture plate and treated with Pxn1 shRNA (Santa Cruz Biotechnology, Inc.), P70s6k shRNA (Santa Cruz Biotechnology, Inc.), Il4rα shRNA (Santa Cruz Biotechnology, Inc.), or Msr shRNA (Addgene), according to the manufacturers’ instructions. After transfection, cells were either used for protein extraction for Western immunoblotting or for differentiation induction. For chemical reagent treatments, serum-starved MSCs were treated with 10–100 ng/ml IL-4 (R&D Systems), 50 nM rapamycin (LC Laboratories), or 1 µg/ml TGF-β neutralized antibody (R&D Systems). For Western immunoblotting, MSCs were cultured under growth medium with drugs for 24 h, and protein was extracted by using M-PER mammalian protein extraction reagent. For differentiation induction, MSCs were cultured under inductive conditions in the presence of drugs (added every 3 d) for 3 wk, followed by staining and gene expression analysis.

Flow cytometric analysis of T helper 2 cells. To detect T helper 2 percentage of CD4+ cells in BM and spleenocyte (Ansel et al., 2004), BM or spleocyte-derived single-suspension ANCs were incubated with anti-CD4-PerCP (BioLegend), followed by staining with anti–Foxp3–γ-APC (BioLegend) and anti-IL-4-PE (eBioscience), anti–IL-5-PE (eBioscience), or anti–IL-13-Fluor 660 (eBioscience) using an intracellular antigen staining kit (eBioscience). To further identify T helper 2 cells infiltration, ANCs were incubated with anti-CD4-PerCP, followed by staining with combined T helper 2 cytokines, such as anti–IL-4-PE/anti–IL-13-Fluor 660 and anti-IL-5-PE/anti–IL-13-Fluor 660 antibodies, using an intracellular antigen staining kit. Because CC-chemokine receptor 3 (CCR3) has been reported as a marker for T helper 2 cells (Sallusto et al., 1997), we detected CD4+CCR3+, CD4+CCR3+IL4+, and CD4+CCR3+IL13+ subpopulations using anti-CCR3-FITC antibody (BioLegend). Cells were analyzed by FACS Calibur with CellQuest software (BD).

Immunofluorescence microscopy. BMSCs were cultured on 4-well chamber slides (Nunc; 2 x 10^4/well) and then fixed with 4% paraformaldehyde. The Chamber slides are incubated with primary anti CD373 antibody (1:400; BD) and anti-IL-4Rα (1:400; BD) at 4°C for overnight and then treated with Rhodamine-conjugated secondary antibody (1:400; Southern Biotechnology, Inc.) or 1 µg/ml TGF-β neutralized antibody (R&D Systems). For immunoprecipitations, 1:100 dilution of SP1 antibody was used to capture protein–DNA complexes, and isotype-matched IgG was used as negative control. All resulting precipitated DNA samples were quantified with real-time PCR and expressed as the percentage of input DNA.

Statistics. All experimental group sizes were chosen to ensure adequate statistical power despite the highly variable nature of the studies performed. No animal excluded, and animals were randomly assigned groups for the studies. All experiments were not performed in a blinded fashion. Data were assessed for normal distribution and similar variance between groups. Comparisons between two groups were analyzed by independent unpaired two-tailed Student’s t test, and the comparisons between more than two groups were analyzed by one-way ANOVA with the Bonferroni adjustment. The p-values <0.05 were considered statistically significant.

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