

# Calcineurin regulates bone formation by the osteoblast

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Two of the most commonly used immunosuppressants, cyclosporine A and tacrolimus (FK506), inhibit the activity of a ubiquitously expressed  $\text{Ca}^{2+}$ /calmodulin-sensitive phosphatase, calcineurin. Because both drugs also cause profound bone loss in humans and in animal models, we explored whether calcineurin played a role in regulating skeletal remodeling. We found that osteoblasts contained mRNA and protein for all isoforms of calcineurin A and B. TAT-assisted transduction of fusion protein TAT-calcineurin  $A\alpha$  into osteoblasts resulted in the enhanced expression of the osteoblast differentiation markers *Runx-2*, alkaline phosphatase, bone sialoprotein, and osteocalcin. This expression was associated with a dramatic enhancement of bone formation in intact calvarial cultures. Calcineurin  $A\alpha^{-/-}$  mice displayed severe osteoporosis, markedly reduced mineral apposition rates, and attenuated colony formation in 10-day *ex vivo* stromal cell cultures. The latter was associated with significant reductions in *Runx2*, bone sialoprotein, and osteocalcin expression, paralleled by similar decreases in response to FK506. Together, the gain- and loss-of-function experiments indicate that calcineurin regulates bone formation through an effect on osteoblast differentiation.

FK506 | osteoporosis | *runx-2*

Calcineurin, a Ser/Thr protein phosphatase sensitive to  $\text{Ca}^{2+}$  and calmodulin, plays a critical role in coupling  $\text{Ca}^{2+}$  signals to cellular responses (1, 2). The calcineurin heterodimer consists of catalytic and regulatory subunits, A and B (1). The three known isoforms of mammalian calcineurin A ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), which are products of different genes, exhibit  $\approx 86\%$  sequence homology, whereas the B subunits are highly conserved from yeast to man (3). Calcineurin is distributed widely in the brain and lymphocytes (4–7) and has established roles in T cell activation, vesicular trafficking, cell growth, apoptosis, neuron depotentialization, muscle development, and cardiac valve formation (reviewed in refs. 8 and 9).

Both calcineurin  $A\alpha$  and  $A\beta$  knockout mice show defective antigen-specific T cell responses *in vivo*, indicating that both isoforms are required for T cell function (10, 11). In contrast, calcineurin  $A\alpha$ , but not  $A\beta$ , is required specifically for kidney development (12). Additionally, mossy-fiber cytoskeletons of calcineurin  $A\alpha^{-/-}$  brains appear abnormal, suggesting that calcineurin  $A\alpha$  regulates the phosphorylation of the microtubule-associated protein  $\tau$  (13). More recently, calcineurins  $A\alpha$  and  $A\gamma$  have been implicated in the pathogenesis of Alzheimer's disease and schizophrenia, respectively (14, 15). Thus, whereas the tissue distribution and function of the three calcineurin isoforms appears to be overlapping (16), isoform-specific functions are just beginning to be identified.

The two commonly used immunosuppressants, cyclosporin A and tacrolimus (FK506), potently inhibit calcineurin's phosphatase activity by interacting with distinct domains of the calcineurin A subunit (17). The systemic administration of either drug to rats causes a dramatic osteoporosis (18, 19). In humans, acute, rapid, severe bone loss ensues with a fracture incidence

approaching 65% (20, 21). It has been speculated, but never proven in either animals or humans, that cyclosporine A or FK506 use the calcineurin pathway to exert their skeletal effects. Obviously, the likelihood of such a pathway will depend on whether osteoblasts and osteoclasts, the likely target cells for both agents, express calcineurin and whether calcineurin plays a central role in the formation and (or) function of these cells.

We and others have shown recently that calcineurin plays a critical role in regulating the genesis and function of osteoclasts (22–25). Calcineurin  $A\alpha$  overexpression inhibits bone resorption by mature osteoclasts (23). In contrast, there are several reports indicating that the downstream substrate of calcineurin, NFAT, is necessary for osteoclastogenesis (24, 25). Thus, stem cell precursors from NFATc1 $^{-/-}$  mice fail to form osteoclasts (24). It is, therefore, possible that calcineurin positively regulates osteoclast formation but blocks the function of mature cells. Nonetheless, the role of calcineurin in bone formation by the osteoblast has remained unrecognized.

In this study, we demonstrate that calcineurin is expressed in osteoblasts. For gain-of-function studies, we fused the calcineurin  $A\alpha$  to TAT, a 12-amino-acid Arg-rich sequence that allows the transduction of full-length proteins into cells. The high-efficiency cellular uptake of TAT-calcineurin  $A\alpha$  resulted in a profound increase in the expression of the osteoblast differentiation markers *Runx-2*, alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin. This increase was associated with enhanced bone formation in calvarial cultures. In contrast, the calcineurin  $A\alpha^{-/-}$  mouse displayed severe osteoporosis with markedly reduced osteoblastic bone formation. *Ex vivo* cultures of osteoblasts derived from this mouse showed reduced colony-forming unit-fibroblastoid (CFU-F) formation and marker expression. The study, therefore, directly implicates the phosphatase calcineurin in the control of osteoblastic bone formation. Suppression of bone formation by calcineurin loss/inhibition may be the mechanism through which calcineurin inhibitors exert their clinical effects on the skeleton.

## Materials and Methods

**Immune Detection and Imaging.** MC3T3.E1 osteoblasts were fixed in paraformaldehyde (4% vol/vol in PBS, pH 7.4) for 20 min at 20°C and washed with Hanks' balanced salt solution (GIBCO/BRL) before being exposed to antiserum PP2BA $\alpha$  (goat anti-calcineurin  $A\alpha$  antiserum; Santa Cruz Biotechnology) or non-immune goat IgG or antiserum PP2BA $\alpha$  plus a monoclonal anti-TAT antibody (Applied Biosystems/Advanced Biotechnologies, Columbia, MD) or anti-hemagglutinin (HA) antibody (for colocalization studies) (all in DMEM, 1:100). After 6 h, the

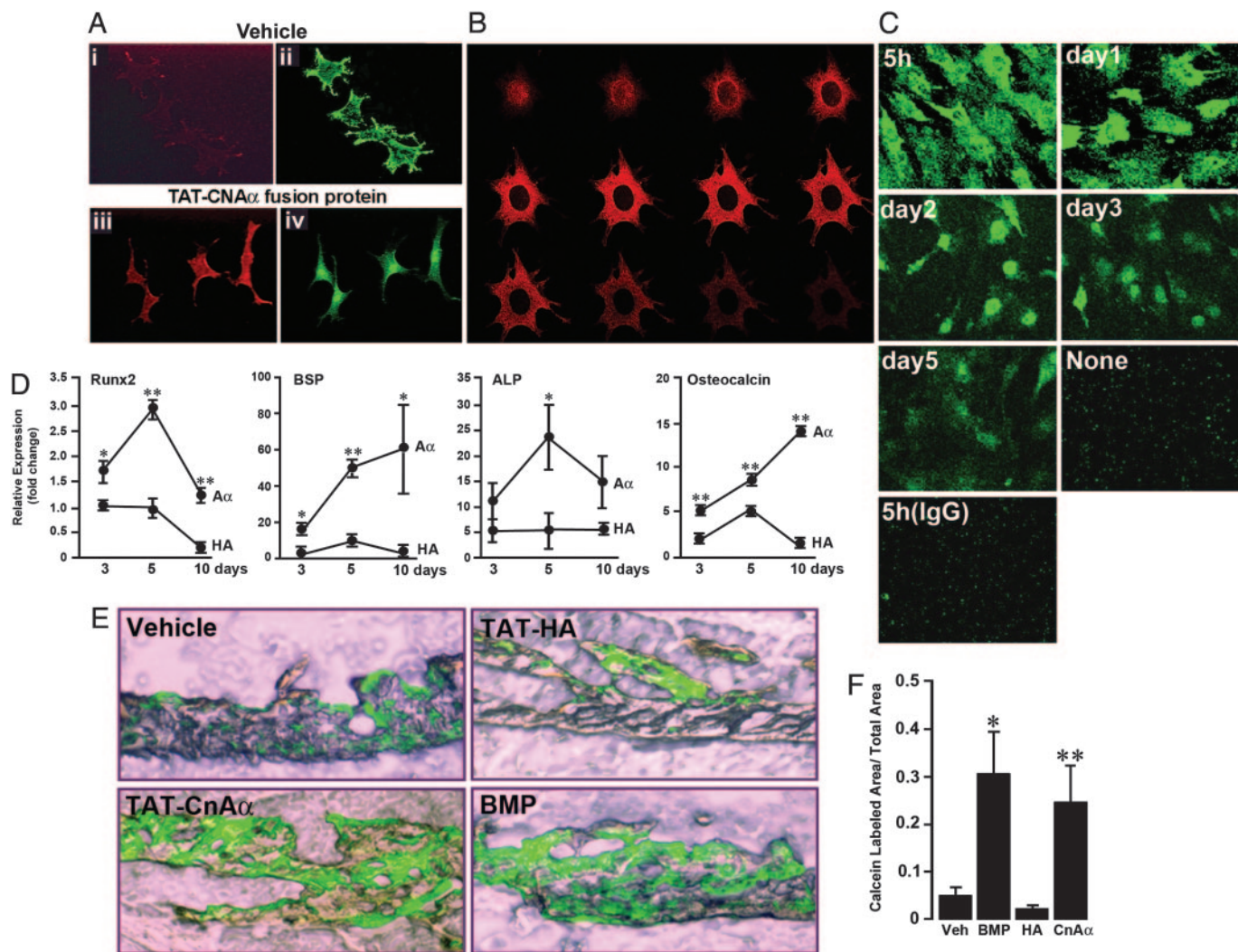
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Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; BSP, bone sialoprotein; CFU-F, colony-forming unit-fibroblastoid; HA, hemagglutinin; RyRs, ryanodine receptors.

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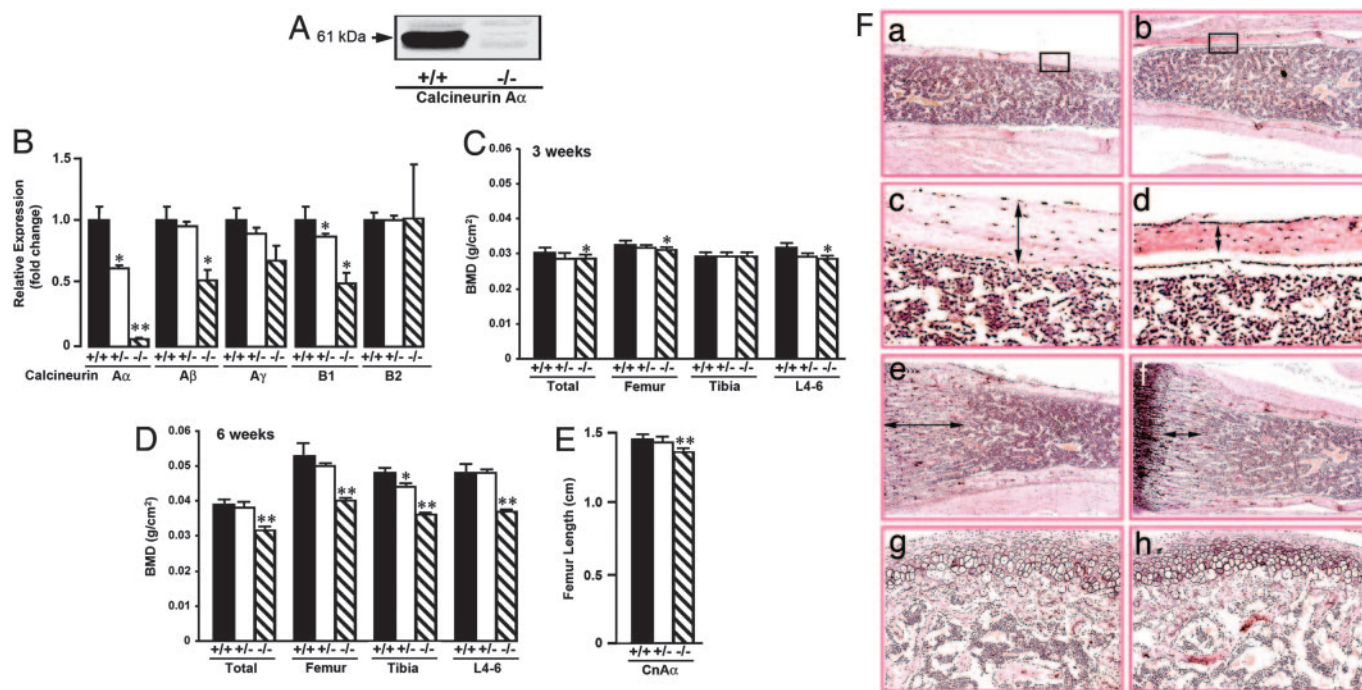
**Fig. 2.** Effect of overexpressing calcineurin A $\alpha$  as a TAT fusion protein on osteoblast differentiation and bone formation. (A) Immunohistochemical detection of calcineurin A $\alpha$  (CNA $\alpha$ ) and TAT, respectively, with anti-CNA $\alpha$  antiserum (PPB2A $\alpha$ , green) and anti-TAT antibody (red) in MC3T3.E1 osteoblasts that were transduced with TAT-CNA $\alpha$  fusion protein (200 nM) (Aiii and Aiv) or vehicle (Ai and Aii) for 10 min at 37°C. Note the similar distribution of red and green staining after transduction. (B) Confocal micrographs (1- $\mu$ m-thick coronal sections of the same cell) demonstrating the mainly cytosolic localization of TAT-CNA $\alpha$  fusion protein in intact cells by using an anti-TAT antibody (red). Note that the nucleus is spared, indicating that the TAT construct does not permeate into the nuclear compartment. (C) Immunolabeling for HA (green) in primary osteoblasts treated with TAT-HA (200 nM for 10 min), demonstrating the persistence of TAT-HA for at least 5 days. No green staining was seen without transduction or with the use of an irrelevant IgG. (D) Enhanced expression of osteoblast differentiation markers, *Runx-2* (early), BSP, ALP, and osteocalcin in MC3T3.E1 cells at 3, 5, and 10 days after transduction with TAT-CNA $\alpha$  (A $\alpha$ ) or TAT-HA, (control) (200 nM each for 10 min). The real-time PCR results are expressed as fold-change normalized to GAPDH and cell control (+SEM). Statistics by Student's *t* test; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ , comparisons with HA at every time point. (E and F) Sections of calvaria from newborn mice incubated in the presence of calcein with vehicle, TAT-HA (200 nM), TAT-CNA $\alpha$  (200 nM), or BMP-2, 200 ng/ml. Quantitative estimates of calcein-labeled surfaces expressed relative to total surface area (F), measured morphometrically by using FOVEA PRO (Reindeer Graphics). Mean  $\pm$  SEM from five fields from three or more sections were analyzed by Student's *t* test, comparing each treatment with vehicle/TAT-HA; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

the same section stained with an anti-calcineurin A $\alpha$  antibody, demonstrating specific immune labeling of osteoblasts. Omitting the primary antibody abolished immune labeling (Fig. 1Db).

**Calcineurin Overexpression Enhances Osteoblast Differentiation and Bone Formation.** MC3T3.E1 osteoblasts were incubated with TAT-calcineurin A $\alpha$  (10 min at 37°C) and stained, after glutaraldehyde fixation, with both anti-TAT (red) and anti-calcineurin A $\alpha$  (green) antibodies. Cells transduced with vehicle alone showed only anti-calcineurin A $\alpha$  (green) immune labeling, representing endogenous calcineurin A $\alpha$  (Fig. 2Aii). However, transduction with TAT-calcineurin A $\alpha$  resulted in a precise colocalization of the red and green immunostains within the same cells (Fig. 2Aiii and Aiv), consistent with the influx of exogenously applied fusion protein that

was detected by both antibodies. Confocal microscopy of the TAT-transduced fusion protein showed diffuse cytoplasmic staining with negligible localization to the nucleus across all coronal planes of the same cell (Fig. 2B), indicating that the TAT construct did not permeate the nucleus, an important control to rule out direct intranuclear actions of calcineurin. Finally, we showed that TAT-HA levels could be detected in the cytosol for the 5 days posttransduction, although levels diminished after day 2 (Fig. 2C).

Transduced MC3T3.E1 cells were grown under differentiating conditions in medium containing 10 mM ascorbic-2-phosphate for up to 10 days. We performed real-time RT-PCR to quantify markers of osteoblast differentiation *Runx-2*, ALP, BSP, and osteocalcin. Compared with TAT-HA, TAT-calcineurin A $\alpha$  produced a dramatic increase in the expression of *Runx-2*, ALP,



**Fig. 3.** Phenotypic characterization of the calcineurin  $A\alpha^{-/-}$  mice. (A) Western blot of a whole-bone extract showing the absence of the 61-kDa calcineurin  $A\alpha$  band in null mice. (B) Real-time PCR on RNA isolated from bone-marrow-derived osteoblasts to examine the expression of the calcineurin A and B isoforms (shown) in calcineurin  $A\alpha^{+/+}$ ,  $A\alpha^{+/-}$ , and  $A\alpha^{-/-}$  mice. (C and D) Severe osteoporosis in the calcineurin  $A\alpha^{-/-}$  mouse at 6 weeks. Comparisons of BMD ( $\text{g}/\text{cm}^2$ ) at femur, tibia, lumbar spine (L4–L6), and total body in 3- (C) and 6-week-old (D) calcineurin  $A\alpha^{+/+}$ ,  $A\alpha^{+/-}$ , and  $A\alpha^{-/-}$  mice by using a Lunar PIXImus small animal densitometer (Lunar, precision <1.5%). Student's *t* test was used to compare the BMDs of homozygotes and heterozygotes with wild-type littermates at each site. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . (E) Estimates of femur length (cm) in calcineurin  $A\alpha^{+/+}$ ,  $A\alpha^{+/-}$ , and  $A\alpha^{-/-}$  mice. Statistics by Student's *t* test; \*\*,  $P < 0.01$ . (F) Histological comparisons of cortical thickness (a and b at magnification  $\times 1$  and c and d at magnification  $\times 2$ ), overall trabecular volume (e and f), and growth plate (g and h) between 6-week-old calcineurin  $A\alpha^{-/-}$  and  $A\alpha^{+/+}$  mice. There was  $\approx 40\%$  reduction in diaphyseal cortical thickness (Table 1) and a mild reduction in trabecular bone (not quantifiable precisely at this age) in calcineurin  $A\alpha^{-/-}$  mice. The respective articular surfaces showed no apparent difference (data not shown).

BSP, and osteocalcin (normalized to GAPDH, a housekeeping gene) (Fig. 2D). That these effects were not reproduced by a control peptide, TAT-HA, indicated that the effects were not due to TAT introduction *per se*.

In separate experiments, we found that TAT-calcineurin  $A\alpha$  substantially enhanced calcein labeling of calvarial bones similarly to a known bone-forming molecule, BMP-2. Again, TAT-HA was without significant effect compared with vehicle treatment (Fig. 2E and F). The results show directly that calcineurin  $A\alpha$  promotes bone formation and are consistent with the diminished bone formation seen in the calcineurin  $A\alpha$  knockout mouse (see below).

**Calcineurin  $A\alpha^{-/-}$  Mice Show Diminished Bone Formation and Severe Osteoporosis.** Calcineurin  $A\alpha^{-/-}$  mice were kindly provided by D. O'Conner and J. Seidman (Harvard Medical School, Boston) (10). We confirmed by Western blotting that whole-bone extracts from the calcineurin  $A\alpha^{-/-}$  mouse did not express the  $\approx 61$ -kDa band, consistent with deletion of the calcineurin  $A\alpha$  gene (Fig. 3A). This finding was also confirmed in real-time quantitative PCR studies on RNA derived from bone marrow osteoblast precursors. The  $A\alpha$  heterozygotes were haploinsufficient, whereas the homozygotes had no detectable protein (Fig. 3B). Calcineurin  $A\beta$  and B1 showed modest decrements in expression, whereas calcineurin  $A\gamma$  and B2 showed no significant change (Fig. 3B), indicating that the phenotype resulted primarily from  $A\alpha$  deficiency, with minor contributions, if any, from the other catalytic and regulatory isoforms.

Lunar PIXImus small-animal densitometry showed significant reductions in BMD at the lumbar spine (L4–L6), femur, and

tibia, and total body in  $A\alpha^{-/-}$  mice compared with both  $A\alpha^{+/+}$  and  $A\alpha^{+/-}$  mice at 6 weeks and reduced BMD at the lumbar spine as early as 3 weeks (Fig. 3C and D). No significant BMD differences were noted at any site between the wild type and heterozygotes, although there was a general trend toward lower BMDs in the  $A\alpha^{+/-}$  mice compared with wild-type littermates. The mice were slightly runted, there was a <10% difference in femur lengths between  $A\alpha^{+/+}$  and  $A\alpha^{-/-}$  mice, and the heterozygotes showed no difference compared with wild-type mice (Fig. 3E).

The low bone density in the calcineurin  $A\alpha^{-/-}$  mouse was consistent with a dramatic  $\approx 40\%$  reduction in cortical thickness compared with wild-type littermates (Table 1). Fig. 3F confirms tibial cortical thinning (a–d) together with mild reductions in trabecular bone (e and f). No differences in the growth plate were noted (g and h). Tetracycline-labeling studies showed that the interlabel distance and mineral apposition rates were reduced significantly in the calcineurin  $A\alpha^{-/-}$  mouse, indicating a lower rate of osteoblastic bone formation (Table 1). In contrast, tartrate-resistant acid phosphatase (TRAP)-positive resorption surfaces, osteoid area, and bone area were not reduced significantly in calcineurin  $A\alpha^{-/-}$  mice compared with wild-type littermates (Table 1). Thus, there does not appear to be a significant osteoclast defect, at least *in vivo*.

**Calcineurin  $A\alpha$  Deletion or Inhibition Reduces Osteoblast Differentiation.** To study the cellular basis of the reduced osteoblastic bone formation *in vivo*, we cultured calvarial osteoblasts *ex vivo* for 10 days, measured CFU-F formation, and used real-time PCR to compare the expression of the differentiation markers *Runx2*,

**Table 1. Histomorphometric measurements**

Bone and parameter	Units	A $\alpha$ <sup>+/+</sup>	A $\alpha$ <sup>-/-</sup>
Tibia, diaphysis			
Cortex	$\mu\text{m}$	105 $\pm$ 8	62 $\pm$ 6*
Bone diameter	$\mu\text{m}$	610 $\pm$ 15	550 $\pm$ 50
Vertebra, L3			
Mineralizing surface	% of surface	39 $\pm$ 2.0	42.6 $\pm$ 1.5
Resorbed surface	% of surface	25 $\pm$ 7	21 $\pm$ 6
Interlabel distance	$\mu\text{m}$	20 $\pm$ 7	7 $\pm$ 3 <sup>†</sup>
Mineral apposition rate	$\mu\text{m/day}$	2.2 $\pm$ 0.8	0.8 $\pm$ 0.3 <sup>†</sup>
Osteoid area	mm <sup>2</sup>	0.40 $\pm$ 0.08	0.38 $\pm$ 0.08
Bone area	mm <sup>2</sup>	3.62 $\pm$ 0.14	3.22 $\pm$ 0.50

Selected histological measurements and calculated parameters in tibial cortex and L3 vertebral trabecular bone of 6-week-old tetracycline-labeled wild-type and calcineurin A $\alpha$ <sup>-/-</sup> mice. Measurements are from unstained, trichrome, or von Kossa-stained undecalcified sections and hematoxylin and eosin-stained decalcified sections by digital histomorphometry as described in ref. 26. Nonstandard units are as defined by Parfitt *et al.* (32). Mean  $\pm$  SD for cross sections from each of four animals per group. \*,  $P < 0.01$ ; †,  $P < 0.05$ ; relative to wild type by Student's *t* test.

BSP, and osteocalcin. Fig. 4A shows an  $\approx 40\%$  reduction in ALP-positive CFU-F formation in A $\alpha$ <sup>-/-</sup> mice and in wild-type mice treated with FK506 compared with respective controls (Fig. 4A). Fig. 4B shows a significant decrease in *Runx 2* expression and a profound inhibition of BSP and osteocalcin expression in A $\alpha$ <sup>-/-</sup> CFU-Fs, indicating that calcineurin A $\alpha$  gene deletion results in reduced osteoblast differentiation. Calvarial cells from wild-type mice were also treated with FK506 (0.01, 0.1, and 10  $\mu\text{g/ml}$ ), with the expectation that calcineurin A $\alpha$  gene deletion and calcineurin enzyme inhibition would have similar effects. There was a concentration-dependent decrease in *Runx2*, BSP, and osteocalcin expression in response to FK506 (Fig. 4 C–E).

## Discussion

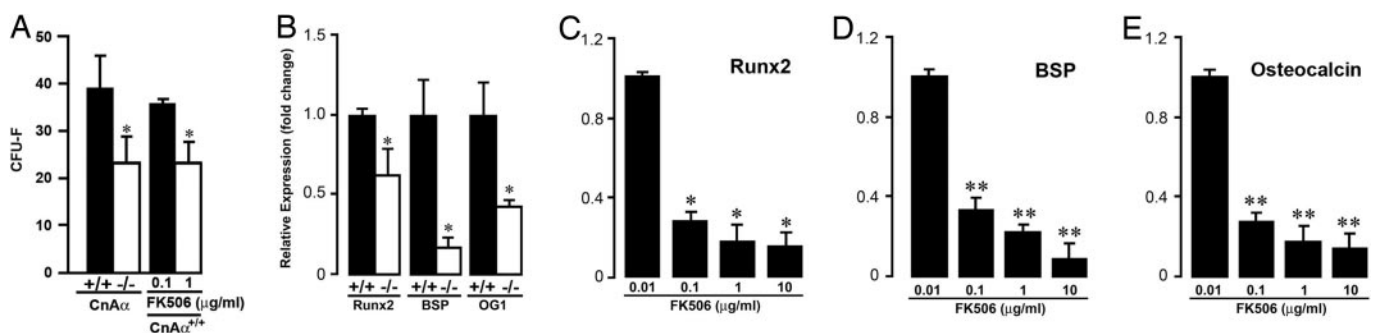
Together, the gain- and loss-of-function experiments indicate that calcineurin A $\alpha$  enhances osteoblast differentiation to positively regulate bone formation. All calcineurin isoforms are expressed in the osteoblast. Calcineurin A $\alpha$  promotes cell differentiation and bone formation when introduced into osteoblasts by using a high-efficiency TAT transduction system. The deletion or inactivation of calcineurin inhibits osteoblast differentiation. This attenuated osteoblast differentiation translates into markedly reduced bone-formation rates and profound osteoporosis in A $\alpha$ <sup>-/-</sup> mice. In these mice, there is no major decrement in the expression of other calcineurin A isoforms, suggesting that the phenotype resulted mainly from a loss of the A $\alpha$  isoform.

It is well known that FK506 complexes with the ubiquitously expressed FK-binding protein. This complex competitively binds to and inhibits calcineurin activation by Ca<sup>2+</sup> and calmodulin (34, 35). With such specificity, one would expect that the skeletal effects of calcineurin inhibition by FK506 and calcineurin gene deletion are identical. In other words, reduced osteoblast differentiation and bone formation should be the mechanism through which calcineurin inhibitors cause bone loss (18–21). Although this may be the case, the osteoporosis resulting from calcineurin inhibitors is characterized by a high-turnover state in which both bone resorption and through-coupling bone formation are elevated (18, 19). Several mechanisms could underlie the osteoclastic activation seen with calcineurin inhibitors. Because calcineurin overexpression directly attenuates osteoclast activity (23), calcineurin inhibitors would be expected to stimulate bone resorption. Alternatively, FK506 could enhance resorption independently of calcineurin or via T cells (36). It is becoming clear that T cells can mediate the bone loss after estrogen withdrawal (37). Thus, T cell deficiency abrogates the bone loss due to both estrogen withdrawal and immunosuppressant therapy (36, 37).

In contrast to acute calcineurin inhibition, relatively chronic calcineurin deficiency in A $\alpha$ <sup>-/-</sup> mice does not result in an *in vivo* osteoclastic phenotype. This compensation may arise from the dual actions of calcineurin: its facilitation of osteoclastogenesis (24, 25) versus its propensity to reduce the resorptive activity of mature cells (23). Thus, the deficiency of calcineurin should stimulate resorption but, paradoxically, reduce osteoclast formation, with no net change in histomorphometrically determined tartrate-resistant acid phosphatase (TRAP)-labeled surfaces (overall resorption). In the absence of elevated resorption and consistent with the action of calcineurin on osteoblast differentiation and function, the osteoporosis in A $\alpha$ <sup>-/-</sup> mice arises from a defect in bone formation. Nonetheless, it is difficult, without the development of cell-specific knockout mice, to speculate on which action, osteoclastic or osteoblastic, is most relevant to calcineurin's role in regulating skeletal homeostasis.

Expression of the master gene for osteoblast differentiation *Runx-2* is modestly decreased in the A $\alpha$ <sup>-/-</sup> null mouse but is more dramatically attenuated by FK506. *Runx-2*'s relatively modest suppression in the A $\alpha$ <sup>-/-</sup> mice likely results from compensation by the other isoforms A $\beta$  and A $\gamma$ ; FK506, on the other hand, suppresses the activity of all calcineurin isoforms and, thus, inhibits *Runx2* profoundly. Consistent with this finding, TAT-calcineurin A $\alpha$  stimulates *Runx-2* expression. It is unclear, however, whether *Runx-2* mediates the enhanced osteoblastic differentiation or whether its up-regulation is a secondary effect.

Indeed, it is possible that effects of calcineurin on osteoblast differentiation are exerted through the traditional NFAT sig-



**Fig. 4.** Effect of either calcineurin A $\alpha$  gene deletion or the inhibition of calcineurin enzyme activity by FK506 on the number of CFU-F (A) and the expression of osteoblast genes (B–E), namely *Runx2*, BSP, and osteocalcin in mouse primary osteoblasts after 10 days of culture in the presence of ascorbate-5-phosphate (1 mM). Student's *t* test was used to compare with wild-type littermates or zero-dose. \*,  $P < 0.06$ ; \*\*,  $P < 0.01$ .

nal pathway. In lymphocytes, for example, activation of calcineurin results in the NFAT-dependent activation of several genes, including the GM-CSF, interleukins 2, 3, 4, and 5, CD40, and the *Fas* genes (38). From our studies on myoblastic cells, we can infer additional dephosphorylation targets for calcineurin in the osteoblast, notably  $\text{I}\kappa\text{B}\beta$  (39). It is also possible that critical genes for  $\text{Ca}^{2+}$ -release channels, including  $\text{IP}_3$  receptors and ryanodine receptors (RyRs) that are abundantly expressed in the osteoblast, are potential targets for osteoblastic calcineurin. The overexpression of calcineurin  $\text{A}\alpha$  in pleuripotent C2C12 cells results in dramatic increases in the expression of RyR-1 (40). It is notable that C2C12 cells can form myocytes, osteoblasts, or adipocytes under appropriate differentiating conditions (41). Oxidative stress resulting from mitochondrial DNA deletion is also associated with the enhanced expression of both RyR-1 and calcineurin  $\text{A}\alpha$  (39). Thus, there is a direct relationship between the expression of calcineurin  $\text{A}\alpha$  and target RyR genes. In addition, inhibitors such as FK506 decouple the molecular interaction between RyRs and calcineurin (42, 43).

An interesting clinical paradigm has emerged that significantly enhances the importance of our discovery of a key role for calcineurin in osteoblastic bone formation. The *DSCR1* gene, a potent inhibitor of calcineurin activity located on human chromosome 21 is overexpressed in Down's syndrome as a result of trisomy (44). It has been proposed that such overexpression

results in defects in the development of the brain, immune system, heart, and skeleton in children with Down's syndrome (44). A peptide fragment of the *DSCR1* gene has been identified as a calcineurin competitor (45). Nonetheless, restoration of the *DSCR1* gene to disomy in the trisomy-16 mouse model of Down's syndrome has been found not to rescue the cardiac or craniofacial abnormalities (46), calling a role for calcineurin into question.

Finally, even though the phenotypes of calcineurin-inhibitor-treated and calcineurin  $\text{A}\alpha$  knockout animals differ somewhat, both cyclosporine and FK506 do reduce osteoblastic differentiation. Indeed, the drugs' ultimate effects in causing skeletal fractures may arise from poorly healing microcracks due to reduced bone formation. When the drugs are administered with glucocorticoids, these suppressive effects on bone formation may be even more pronounced. We anticipate that studies examining glucocorticoid effects in calcineurin-null mice will provide more definitive answers.

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