the interstimulus interval, the same grating was presented but remained stationary in order to avoid a sudden luminance change, which is known to create a transient cortical response exhibiting poor orientation tuning. We verified by single-unit recordings that standing gratings did not evoke cortical activity.
13. We do not rule out, however, the possibility that a few distinct states were mixed in the PCS spatial pattern when this procedure was applied, because single cortical neurons are known to be selective for more than one visual attribute of the stimulus (for example, orientation, direction, and spatial frequency).
14. This functional map for orientation preference shown in Fig. 1D is defined as a single condition orientation map. Thus far such maps could not be obtained due to the large noise previously associated with voltage-sensitive dye imaging. Only the recent technical advances allowed us to obtain reproducible single condition maps (*7*). To minimize contributions from the intrinsic signals, the data were filtered above 0.6 Hz and the maps were computed with frames from 100 to 150 ms after stimulus onset, much earlier than the onset of the slow intrinsic signal. Normally, maps are revealed by differential imaging only [T. Bonhoeffer and A. Grinvald, *J. Neurosci.* **13**, 4157 (1993)]. The single condition map was validated by comparing it to the differential orientation maps obtained by using two stimuli of orthogonal orientations (Fig. 1B). In addition, the map was confirmed by imaging based on intrinsic signals. The latter two types of maps can be obtained with a much better signal-so-noise ratio. Therefore, we are confident that the PCS shown here corresponds to the relevant functional architecture.
15. The evident variability in both single-unit and population responses could have various sources, including the impact of ongoing activity on evoked activity (*2*) and relatively large noise in these single-trial images. The values of the correlation coefficients warrant a discussion. The fact that correlation coefficients never reach high values (above 0.6) could manifest the mixing of several cortical states in one PCS during the averaging, or it could result from the residual noise in the recording of instantaneous activity patterns. The values of the correlation coefficients should be compared with the highest value that can be obtained for two maps derived under the same experimental conditions. The correlation between two maps produced by the same stimuli on two independent sets of trials was 0.81.
16. We converted the correlation coefficient between the population activity and the neuron's PCS into a predicted instantaneous firing, as will be explained later (Fig. 3). We then generated a Poisson spike train using this predicted instantaneous rate.
17. The average correlation coefficient for all five pairs of spontaneous and evoked activity sessions was 0.73 (range 0.65 to 0.81) from five different cats.
18. We expect that spontaneous activity of a cortical neuron can be predicted by this procedure whenever the neuron has response properties that are common to many other neurons. In other cases where the tuning properties are not that robust, or the neuron is a member of neuronal assemblies possessing different spatial structures, other types of analysis, such as clustering techniques, may be required.
19. In Fig. 2 we used the neuron's PCS to predict the spike trains. In Fig. 1, C and D, we showed that the PCS and the map of the functional architecture are very similar. Therefore, here we performed the same analysis using both the functional map and the PCS. The results were very similar. In Fig. 3 we show the results based on the functional map.
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Stimulation of Bone Formation in Vitro and in Rodents by Statins

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Osteoporosis and other diseases of bone loss are a major public health problem. Here it is shown that the statins, drugs widely used for lowering serum cholesterol, also enhance new bone formation in vitro and in rodents. This effect was associated with increased expression of the bone morphogenetic protein–2 (*BMP-2*) gene in bone cells. Lovastatin and simvastatin increased bone formation when injected subcutaneously over the calvaria of mice and increased cancellous bone volume when orally administered to rats. Thus, in appropriate doses, statins may have therapeutic applications for the treatment of osteoporosis.

Diseases of bone loss are a major public health problem for women in all Western communities. It is estimated that 30 million Americans are at risk for osteoporosis, the most common of these diseases, and there are probably 100 million people similarly at risk worldwide (*1*). These numbers are growing as the elderly population increases. Despite recent successes with drugs that inhibit bone resorption, there is a clear need for nontoxic anabolic agents that will substantially increase bone formation in people who have already suffered substantial bone loss. There are no such drugs currently approved for this indication.

In a search for agents that enhance osteoblast differentiation and bone formation, we looked for small molecules that activated the promoter of the bone morphogenetic protein–2 (*BMP-2*) gene. We chose this assay because osteoblast differentiation is enhanced by members of the BMP family, including BMP-2 (*2*), whereas other bone growth factors such as transforming growth factor–β and the fibroblast growth factors (FGFs) stimulate osteoblast proliferation but inhibit osteoblast differentiation (*3*). To test the effects of compounds on *BMP-2* gene expression, we used the firefly luciferase reporter gene driven by the mouse BMP-2 promoter (−2736/+114 base pairs). The gene was transfected into an immortalized murine osteoblast cell line, which was derived from a transgenic mouse in which simian virus–40 (SV40) large T antigen was directed to cells in the osteoblast lineage (*4*), and the effects on the promoter were assessed by luciferase activity in the cell lysates.

We examined more than 30,000 compounds from a natural products collection and identified the statin lovastatin as the only natural product in this collection that specifically increased luciferase activity in these cells. The statins are commonly prescribed drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co-A) reductase and decrease hepatic cholesterol biosynthesis, thereby reducing serum cholesterol concentrations and lowering the risk of heart attack (*5, 6*). We also examined the effects of related statins simvastatin, mevastatin, and fluvastatin in this assay. Each of these compounds was maximally effective at 5 μM and had no effects at concentrations lower than 1 μM. The increase in luciferase activity was blocked by the immediate downstream metabolite of HMG Co-A reductase, mevalonate (*7*), which suggests that the effects on bone formation were causally linked to inhibition of this enzyme [although mevalonate may

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have cellular effects independent of the cholesterol biosynthesis pathway (*8*)]. Cultured murine (2T3) or human (MG-63) bone cells exposed to statins showed enhanced expression of *BMP-2* mRNA, as assessed by Northern (RNA) blot analysis (Fig. 1). This effect appeared to be specific, because the statins did not alter expression from the BMP-4 promoter (Fig. 1) or from promoters derived from the gene encoding interleukin-6, the gene encoding parathyroid hormone (PTH)–related peptide, or from SV40 and cytomegalovirus. A sandwich enzyme-linked immunosorbent assay for BMP-2 revealed increased protein production in MG-63 cells incubated with simvastatin (a 2.7-fold increase with 2.5 μM simvastatin).

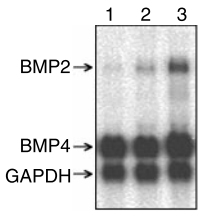


Fig. 1. Northern blot analyses of effects of simvastatin on human MG-63 osteoblasts. BMP-2 mRNA expression, but not BMP-4 mRNA expression, is enhanced by simvastatin. Control media (lane 1) or simvastatin was added to MG-63 osteoblasts to a final concentration of 2 μM (lane 2) and 5 μM (lane 3), and the cells were then cultured for 48 hours. Blots were developed simultaneously with human BMP-2, BMP-4, and glucose-6 phosphate dehydrogenase (GAPDH). The ratio of BMP-2 to GAPDH after culture with simvastatin at 2 μM and 5 μM concentrations was 1.5 and 2.8, respectively. The ratio of BMP-4 to GAPDH after culture with simvastatin at 2 μM and 5 μM concentrations was 0.9 and 1.1, respectively.

To investigate the biological effects of statins on bone, we added them to neonatal murine calvarial (skullcap) bones in organ culture (*9, 10*). Calvaria from 4-day-old pups of Swiss white mice (Harlan Sprague-Dawley) were explanted, dissected free of adjacent connective tissue, placed in tissue culture medium containing 0.1% bovine serum albumin (BSA), and incubated with test compounds for 3 to 7 days. The amount of new bone formation was then assessed morphologically as described in (*10*) and Table 1. Lovastatin, simvastatin, fluvastatin, and mevastatin each increased new bone formation by approximately two- to threefold, an increase comparable to that seen in this assay after treatment with BMP-2 and fibroblast growth factor–1 (FGF-1). There was also a striking increase in new bone and in osteoblast cell numbers at all stages of differentiation (Fig. 2, A and B, and Table 1).

We next injected lovastatin and simvastatin into the subcutaneous tissue overlying the murine calvaria in vivo (*11–14*). The bone cells of the calvaria are responsive to both bone-resorbing factors and osteoblast-stimulating factors (*10–15*). This technique requires reproducible placement of small volumes of factors or compounds adjacent to bone. It is minimally invasive, and the calvarial periosteum is not scraped or damaged. Male Swiss ICR (Institute for Cancer Research) white mice, 4 to 5 weeks of age, were injected three times per day for 5 days over the right side of the calvaria with either vehicle or the test compound. Each injection contained the compound dissolved in 50 μl of phosphate-buffered saline (PBS) with 2% dimethylsulfoxide and 0.1% BSA. Mice were killed on day 21, and calvaria were removed for histomorphometric analysis. We observed an almost 50% increase in new bone formation after only 5 days of treatment, again comparable to that seen with FGF-1 (Fig. 3, A and B) and BMP-2. However, FGF-1 also increases proliferation of cells in the overlying subcutaneous tissue, an effect not seen with BMP-2 or the statins (Fig. 3).

To determine whether the statins stimulate new bone formation when administered systemically, we tested their effect on trabecular

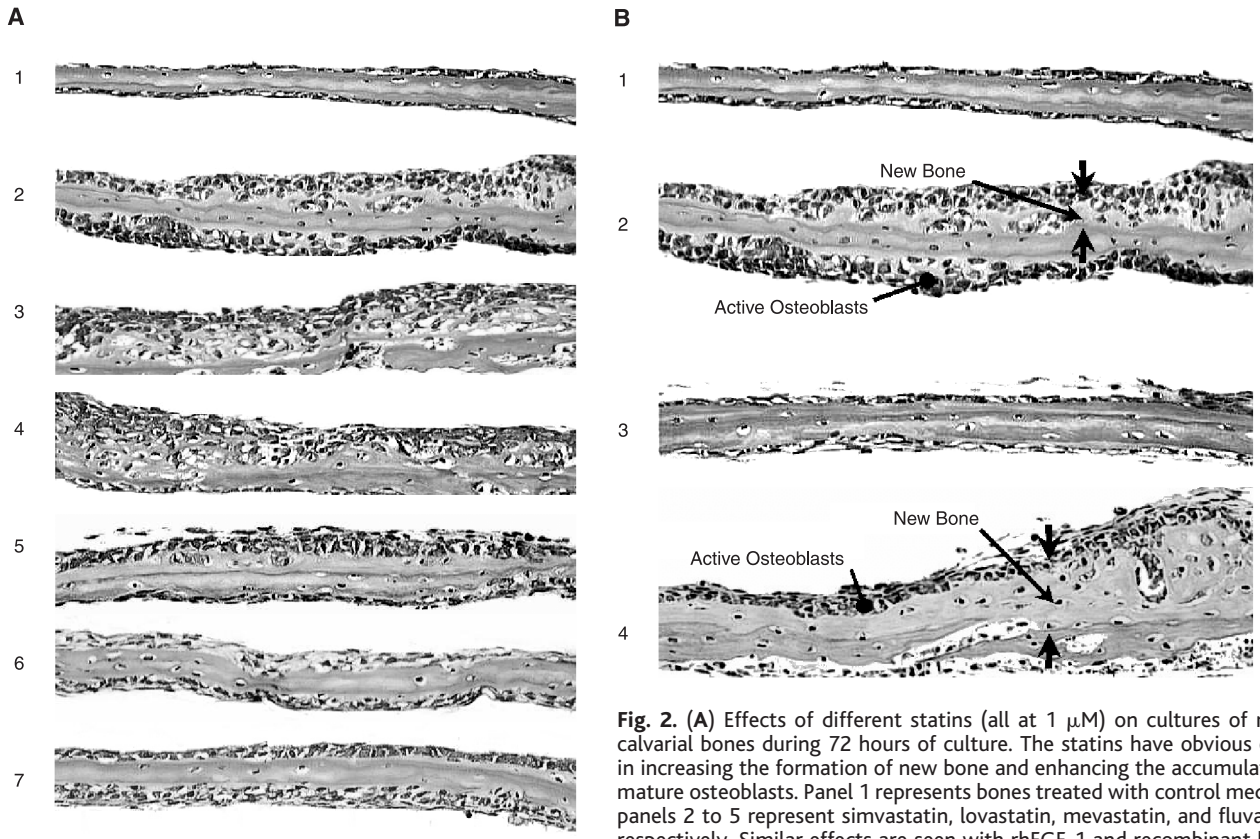
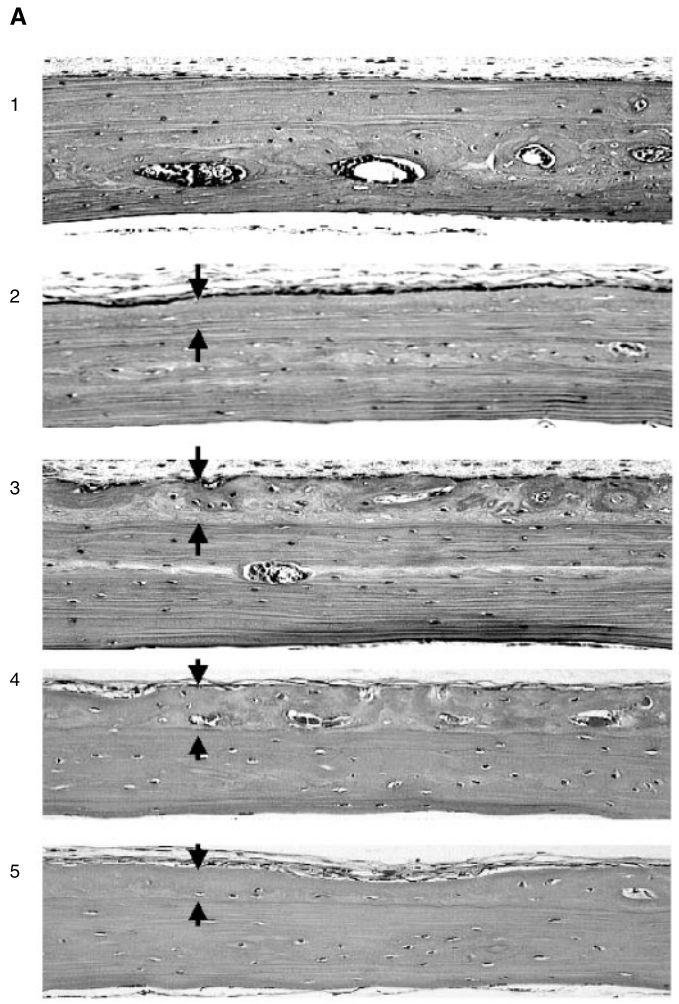


Fig. 2. (**A**) Effects of different statins (all at 1 μM) on cultures of murine calvarial bones during 72 hours of culture. The statins have obvious effects in increasing the formation of new bone and enhancing the accumulation of mature osteoblasts. Panel 1 represents bones treated with control media and panels 2 to 5 represent simvastatin, lovastatin, mevastatin, and fluvastatin, respectively. Similar effects are seen with rhFGF-1 and recombinant human BMP-2 (rhBMP-2) (40 ng/ml) (panel 6) and (100 ng/ml) (panel 7), which are positive controls in this experiment. (**B**) Effects of simvastatin (1 μM) added to cultures of explanted murine calvaria for 4 or 7 days. Panels 1 and 2 represent bones treated with control media or simvastatin, respectively, for 4 days. There is marked cellular proliferation and accumulation of mature osteoblasts adjacent to new bone in the bones treated with simvastatin. Panels 3 and 4 represent bones treated with control media or simvastatin, respectively, for 7 days. By this time, there is a more marked increase in the thickness of new bone.



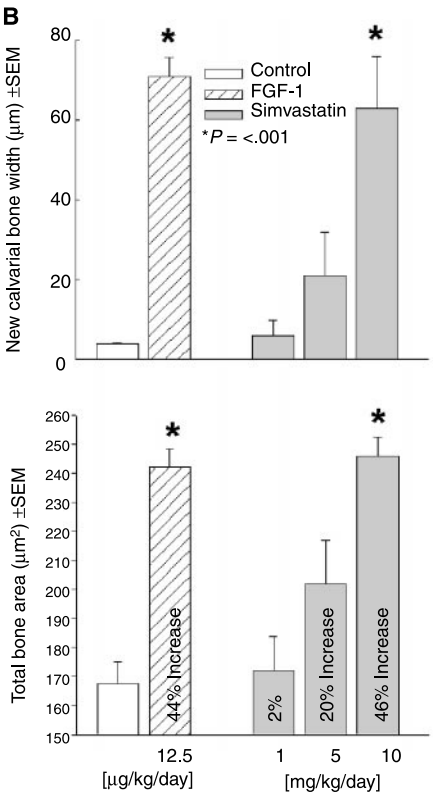


Fig. 3. (**A**) Effect of simvastatin on new bone formation after local subcutaneous injection over the murine calvaria. Mice were injected daily with vehicle alone (panel 1) or with simvastatin at 5 mg/kg/day (panel 2) or at 10 mg/kg/day (panel 3) for 5 days, and bones were then histologically examined at day 21. There is a marked increase in new bone width in statin-treated versus vehicle-treated mice. Panel 4 represents rhFGF-1 (12.5 μg/kg/day) and panel 5 represents rhBMP-2 (30 μg/kg/day) as positive controls. They cause effects similar to those of simvastatin. The arrows indicate the new bone that has been formed as a consequence of simvastatin treatment. (**B**) Quantitative effects of simvastatin on the width of calvarial bones after local injection into the scalp in (A). Total bone area of the right calvaria (the injected side) was measured from the site of muscle implantation to the suture. The width of the new calvarial bone formed was measured at four points (at 0.5-mm intervals), starting at the muscle implantation site. The mean was calculated for each calvaria measured. The effects are compared with those of FGF-1.

Table 1. Effects of simvastatin (experiment 1) and lovastatin (experiment 2) on neonatal murine calvarial bone organ cultures. Explanted bones were cultured for 72 hours, and four bones were in each treatment group. Values are means ± SEM.

Treatment	Simvastatin		Lovastatin	
	New bone area (per mm² × 10^{-3})	Cells (per 0.3 mm of bone)	New bone area (per mm² × 10^{-3})	Cells (per 0.3 mm of bone)
Control	3.4 ± 0.8	98 ± 7	4.5 ± 0.5	84 ± 3
BMP-2 (40 ng/ml)	6.5 ± 0.8*	145 ± 5*	7.9 ± 0.6*	110 ± 3*
Statin				
0.062 μM	5.3 ± 1.1	110 ± 9	4.4 ± 0.7	83 ± 5
0.125 μM	8.2 ± 0.7*	135 ± 4*	7.6 ± 1.0	87 ± 13
0.25 μM	14.5 ± 1.8*	167 ± 17*	10.1 ± 0.6*	102 ± 5*
0.5 μM	14.0 ± 1.1*	190 ± 26*	12.4 ± 2.6*	132 ± 7*

*Significantly greater than control ($P < 0.01$).

bone volume in female rats after oral administration. Because osteoporosis occurs most frequently in postmenopausal women, we administered statins to ovariectomized rats as well as rats with intact ovaries (Table 2). At the completion of the experiment, the rats were killed by anesthetic overdose, and the right tibia, the right femur, and the lumbar vertebrae were removed and fixed in formalin. The proximal end of the tibia and the lumbar vertebrae were embedded in paraffin or plastic, and sections were prepared for histomorphometric analysis. Bone formation rates (BFRs) were measured in paraffin-embedded sections. Before being killed, all animals were treated with a fluorochrome labeling regimen that resulted in the deposition of double-fluorochrome labels on active bone-forming surfaces. This regimen consisted of tetracycline [15 mg per kilogram of body weight (15 mg/kg) in 200 μl of distilled water] and calcein green [20 mg/kg in 200 μl of PBS (pH 7.2)]. These were administered by intraperitoneal injection before killing on days −14 and −4, respectively. We measured bone volume, osteoid volume (in plastic-embedded sections), osteoblast surface, osteoclast surface, and osteoclast number (*16*). Recombinant human FGF-1 (rhFGF-1) (experiment 1) and synthetic human PTH (amino acids 1 through 34 from the NH_2-terminal end) (experiment 3) were used as positive controls. The statins caused increases in trabecular bone volume of between 39 and 94% after treatment. This was clearly due to an anabolic (bone-forming) effect because there was a parallel increase in BFRs with the use of dynamic parameters (Table 2). There was a concomitant decrease in osteoclast numbers where these were also assessed.

Recently, it has been shown that certain bisphosphonates, which are inhibitors of bone resorption and are widely used as therapy for osteoporosis, also act on the cholesterol biosynthesis pathway (*17–19*) by targeting enzymes more distal in the mevalonic acid pathway than HMG Co-A reductase. It has been postulated that these enzymes are required for prenylation of small proteins such as Rho and Ras and that interference

Table 2. Effects of simvastatin on trabecular bone volume and bone formation rates. Simvastatin was given in doses of 5 to 50 mg/kg/day by oral gavage for 35 days to (i) 3-month-old virgin female rats (experiment 1), (ii) 3-month-old virgin female rats that had been ovariectomized within 7 days after the start of treatment (experiment 2), and (iii) 3-month-old virgin female rats that had been ovariectomized 2 months before treatment (experiment 3). In each experiment, the rats were weight matched and divided into treatment groups of 10. The rats were lightly anesthetized with isofluorane before ovariectomy. Animals were pair fed throughout the experimental period and body weights were determined weekly. Values in parentheses are percent change from vehicle-treated controls. BV/TV, bone volume/tissue volume; Ocl, osteoclasts; BFR, bone formation rate; OVX/veh, ovariectomized rats treated with vehicle; hPTH, human PTH; ND, not determined.

Treatment	Trabecular bone volume (% BV/TV)	BFR ($\mu m^3/\mu m^2$/day)	No. of Ocl/mm^2 of bone surface
	Experiment 1		
Control	13.4 ± 1.4		13.7 ± 1.2
Simvastatin (10 mg/kg/day)	18.6 ± 1.4* (+39)	ND	11.6 ± 1.4 (−15)
hFGF-1 (100 μg/kg/day)	21.4 ± 1.7* (+60)	ND	7.5 ± 1.3* (−45)
	Experiment 2		
OVX/veh	6.9 ± .87	0.6 ± 0.1	8 ± 0.2
Simvastatin (1 mg/kg/day)	8.6 ± .41 (+25)	ND	ND
Simvastatin (10 mg/kg/day)	13.4 ± 2* (+94)	1.2 ± .11 (100*)	7 ± 0.3 (−12.5)
	Experiment 3		
OVX/veh	4.6 ± 0.58	0.151 ± 0.01	1.2 ± 0.1
Simvastatin (5 mg/kg/day)	9 ± 0.8* (+96)	0.196 ± .021* (30)	0.9 ± 0.1 (−25)
Simvastatin (10 mg/kg/day)	8.6 ± 0.9* (+87)	0.229 ± .034* (52)	0.78 ± .06* (−33)
hPTH (80 μg/kg/day)	20 ± 1.9* (+348)	0.228 ± .025* (51)	0.84 ± 0.15 (−30)

*Significantly greater than control ($P < 0.01$).

with this process may lead to osteoclast apoptosis and cessation of bone resorption (*18, 20*). We cannot exclude the possibility that the statins both inhibit bone resorption and promote bone growth, and we did observe a concomitant decrease in osteoclast numbers (Table 2). However, this effect appeared minor in comparison to the effect on new bone formation and osteoblast maturation.

The statins used in our studies and currently on the market are not ideal for use as systemic bone-activation agents. These statins were selected for their capacity to lower serum cholesterol, which requires targeting to HMG Co-A reductase in hepatic cells. Thus, the concentration of statin in other tissues is much lower than in the liver. The most efficacious statins would be those that distribute themselves to the bone or bone marrow. A preliminary retrospective analysis of older women taking lipid-lowering agents suggests that statin use is accompanied by greater hip bone mineral density and lower risk of hip fractures (relative risk = 0.30) (*21*); however, the sample size (598 statin users) was too small to yield definitive information.

The most powerful anabolic agents for bone are the peptide growth factors intrinsic to the tissue. For example, systemically administered FGF-1 restores trabecular microarchitecture and increases bone volume (*15*). However, all of the peptide growth factors have disadvantages—they can be mitogenic to other bone cells and nonselective in their effects. In addition, the FGFs cause hypotension, which limits their potential use in elderly patients (*22*).

Our results suggest that statins, which are orally bioavailable and have been safely administered to patients for more than a decade, may merit further investigation as potential anabolic agents for bone. When the doses are extrapolated from humans to rats with respect to lipid lowering, the statins' effects on bone occur at doses similar to the lipid-lowering doses used in humans.

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Requirement for B Cell Linker Protein (BLNK) in B Cell Development

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Linker proteins function as molecular scaffolds to localize enzymes with substrates. In B cells, B cell linker protein (BLNK) links the B cell receptor (BCR)–activated Syk kinase to the phosphoinositide and mitogen-activated kinase pathways. To examine the in vivo role of BLNK, mice deficient in *BLNK* were generated. B cell development in $BLNK^{-/-}$ mice was blocked at the transition from $B220^+CD43^+$ progenitor B to $B220^+CD43^-$ precursor B cells. Only a small percentage of immunoglobulin M^{++} (IgM^{++}), but not mature $IgM^{lo}IgD^{hi}$, B cells were detected in the periphery. Hence, BLNK is an essential component of BCR signaling pathways and is required to promote B cell development.

Engagement of the BCR activates distinct families of cytoplasmic protein tyrosine kinases (PTKs) to phosphorylate enzymes that are required for the generation of second messengers (*1*). In turn, the coordinate generation of second messengers is important for normal B cell function because disruption of selected signaling pathways is associated with B cell anergy (*2*). Linker or adapter molecules play integral roles in linking the BCR-activated PTKs with these enzymes. One such linker molecule, BLNK (also known as SLP-65, BASH, and BCA), is phos-

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