Corrections

MEDICAL SCIENCES

Correction for "NF-κB inhibits osteogenic differentiation of mesenchymal stem cells by promoting β-catenin degradation," by Jia Chang, Fei Liu, Min Lee, Benjamin Wu, Kang Ting, Janette N. Zara, Chia Soo, Khalid Al Hezaimi, Weiping Zou, Xiaohong Chen, David J. Mooney, and Cun-Yu Wang, which appeared in issue 23, June 4, 2013, of *Proc Natl Acad Sci USA* (110:9469–9474; first published May 20, 2013; 10.1073/pnas.1300532110).

The authors note that Fig. 1 appeared incorrectly. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

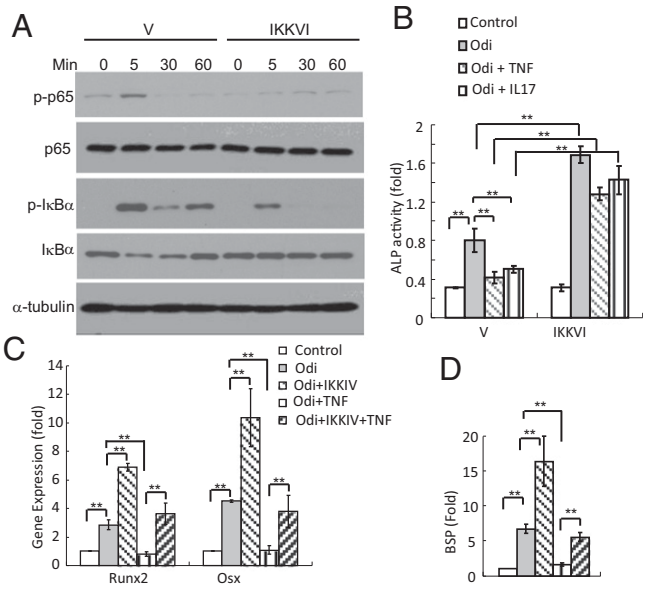

Fig. 1. The IKKβ small molecule inhibitor, IKKVI, promotes osteogenic differentiation by inhibiting NF-κB. (*A*) IKKVI inhibited IKK activities induced by TNF in mMSCs. Cells were pretreated with IKKVI or vehicle control for 30 min and then treated with TNF for the indicated times. The phosphorylation and degradation of IκBα and p65 phosphorylation were examined by Western blot. (*B*) IKKVI overcame TNF and IL-17 inhibition of ALP in mMSCs by inhibiting NF-κB. The results are the average value from three independent experiments and presented as mean ± SD. $^{**}P < 0.01$. Odi, osteogenic differentiation-inducing media. (*C*) IKKVI attenuated TNF inhibition of Runx2 and Osx by inhibiting NF-κB in mMSCs, as assessed by Real-time RT-PCR. $P < 0.01$. (*D*) IKKVI attenuated TNF inhibition of BSP induction by inhibiting NF-κB in mMSCs.

www.pnas.org/cgi/doi/10.1073/pnas.1313266110

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NEUROSCIENCE

Correction for "Aβ induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss," by Maria Talantova, Sara Sanz-Blasco, Xiaofei Zhang, Peng Xia, Mohd Waseem Akhtar, Shu-ichi Okamoto, Gustavo Dziewczapolski, Tomohiro Nakamura, Gang Cao, Alexander E. Pratt, Yeon-Joo Kang, Shichun Tu, Elena Molokanova, Scott R. McKercher, Samuel Andrew Hires, Hagit Sason, David G. Stouffer, Matthew W. Buczynski, James P. Solomon, Sarah Michael, Evan T. Powers, Jeffery W. Kelly, Amanda Roberts, Gary Tong, Traci Fang-Newmeyer, James Parker, Emily A. Holland, Dongxian Zhang, Nobuki Nakanishi, H.-S. Vincent Chen, Herman Wolosker, Yuqiang Wang, Loren H. Parsons, Rajesh Ambasudhan, Eliezer Masliah, Stephen F. Heinemann, Juan C. Piña-Crespo, and Stuart A. Lipton, which appeared in issue 27, July 2, 2013, of *Proc Natl Acad Sci USA* (110:E2518–E2527; first published June 17, 2013; 10.1073/pnas.1306832110).

The authors note that their conflict of interest statement was omitted during publication. The authors declare that "S.A.L. is the inventor on world-wide patents for the use of memantine and NitroMemantine for neurodegenerative disorders; Y.W. is also a named inventor on the patents for NitroMemantine. Per Harvard University guidelines, S.A.L. participates in a royalty-sharing agreement with his former institution Boston Children's Hospital/Harvard Medical School, which licensed the drug memantine (Namenda) to Forest Laboratories, Inc."

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STATISTICS

Correction for "Using distance correlation and SS-ANOVA to assess associations of familial relationships, lifestyle factors, diseases, and mortality," by Jing Kong, Barbara E. K. Klein, Ronald Klein, Kristine E. Lee, and Grace Wahba, which appeared in issue 50, December 11, 2012, of *Proc Natl Acad Sci USA* (109:20352–20357; first published November 21, 2012; 10.1073/pnas.1217269109).

The authors note that: "The phrase 'non-Euclidean pedigree dissimilarity'" on page 20355, right column, first paragraph, line 3, is not correct. As a result of the error, the text from page 20355, right column, line 1 to page 20365, right column, line 7, and Figs 3 and 4 are superfluous and should be omitted.

"The pedigree dissimilarity in the article is in fact Euclidean, a consequence of the fact that the matrix of kinship coefficients $\{\varphi_{ij}\}$ is positive definite, a fact that has been long since known. Thus, there is no reason to invoke the pedigree embedding by regularized kernel estimation (RKE), and the striking similarity between *Upper* and *Lower* of Fig. 3, and also between Figs. 2 and 4, is not surprising. In theory, they should be identical. The very minor differences can be explained by the small amount of regularization applied here in the RKE method. The rest of the paper, including results and discussion, is not affected. We thank Daniel Gianola and Gustavo de los Campos for pointing out the mistake."

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NF-κB inhibits osteogenic differentiation of mesenchymal stem cells by promoting β-catenin degradation

Jia Chang[a], Fei Liu[b], Min Lee[c], Benjamin Wu[c], Kang Ting[d], Janette N. Zara[e], Chia Soo[e], Khalid Al Hezaimi[f], Weiping Zou[g], Xiaohong Chen[h], David J. Mooney[i], and Cun-Yu Wang[a,1]

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Edited* by Shu Chien, University of California, San Diego, La Jolla, CA, and approved April 29, 2013 (received for review January 9, 2013)

Mesenchymal stem cell (MSC)-based transplantation is a promising therapeutic approach for bone regeneration and repair. In the realm of therapeutic bone regeneration, the defect or injured tissues are frequently inflamed with an abnormal expression of inflammatory mediators. Growing evidence suggests that proinflammatory cytokines inhibit osteogenic differentiation and bone formation. Thus, for successful MSC-mediated repair, it is important to overcome the inflammation-mediated inhibition of tissue regeneration. In this study, using genetic and chemical approaches, we found that proinflammatory cytokines TNF and IL-17 stimulated IκB kinase (IKK)–NF-κB and impaired osteogenic differentiation of MSCs. In contrast, the inhibition of IKK–NF-κB significantly enhanced MSC-mediated bone formation. Mechanistically, we found that IKK–NF-κB activation promoted β-catenin ubiquitination and degradation through induction of Smurf1 and Smurf2. To translate our basic findings to potential clinic applications, we showed that the IKK small molecule inhibitor, IKKVI, enhanced osteogenic differentiation of MSCs. More importantly, the delivery of IKKVI promoted MSC-mediated craniofacial bone regeneration and repair in vivo. Considering the well established role of NF-κB in inflammation and infection, our results suggest that targeting IKK–NF-κB may have dual benefits in enhancing bone regeneration and repair and inhibiting inflammation, and this concept may also have applicability in many other tissue regeneration situations.

Wnt | osteoimmunology | adult stem cells

Bone marrow mesenchymal stem cells (MSCs) are multipotent progenitor cells that can differentiate into osteoblasts, chondrocytes, and adipocytes (1–4). In addition to bone marrow, MSCs can also be isolated from numerous tissue sources including synovium, fat, muscle, umbilical cord, and oral tissues (1–5). MSC differentiation is precisely regulated and orchestrated by the mechanical and molecular signals from the extracellular environment (6–13). Therefore, to efficiently harness MSCs for therapeutic purposes, understanding the molecular mechanisms underlying MSC differentiation is of paramount importance. Due to their osteogenic capacity, MSCs are considered to be the most promising cell types for bone regeneration and repair. A large number of studies have shown that MSCs are able to form bone for the repair of defects in various animal models (1–5). However, most of these studies were performed under optimized surgical conditions with minimal tissue inflammation. In the realm of therapeutic bone regeneration, the defective or injured tissues are frequently inflamed with an abnormal expression of inflammatory mediators (14–17). Growing evidence suggests that proinflammatory cytokines inhibit osteogenic differentiation and bone formation (18–24). Thus, to achieve successful MSC-mediated repair, it will likely be necessary to overcome inflammation-mediated inhibition of tissue regeneration.

The transcription factor nuclear factor kappa B (NF-κB) is a master regulator of inflammation and host immune responses. NF-κB can be activated by proinflammatory cytokines such as TNF and interleukin-17 (IL-17), LPS, and viral DNA in cases of inflammatory diseases and tissue injuries (25–30). The IκB kinase (IKK) complex plays an essential role in NF-κB activation by phosphorylating and degrading IκBs (25–30). Recently, we found that IKK–NF-κB signaling in differentiated osteoblasts has an antianabolic effect on bone formation. Time- and stage-specific inhibition of IKK–NF-κB in differentiated osteoblasts significantly enhanced bone matrix formation and mineral density during postnatal bone growth (22, 23).

Proinflammatory cytokines such as TNF have been shown to inhibit MSC differentiation to osteoblasts in vitro (22, 23, 31). However, the underlying mechanisms by which they inhibit MSC differentiation are not fully understood. Moreover, although significant progress has been made in understanding how MSCs modulate functions of T cell and macrophages, little is known how local inflammation affects MSC-mediated bone regeneration and repair in vivo. In this study, we found that IKK–NF-κB was required for TNF inhibition of MSC osteogenic differentiation. IL-17, a cytokine produced by T helper 17 (Th17) cells, also potently inhibited MSC differentiation by activating IKK–NF-κB, indicating immune cells can impair MSC-mediated bone regeneration and repair in inflammation. Mechanistically, we found that IKK–NF-κB signaling promoted β-catenin ubiquitination and degradation through induction of Smurf1 and Smurf2. Importantly, the delivery of the small molecule IKK inhibitor in vivo significantly improved MSC-mediated regeneration and repair of calvarial bone.

Results

TNF and IL-17 Inhibit the Osteogenic Differentiation of MSCs by Activating IKK–NF-κB. To determine whether TNF activated the IKK–NF-κB signaling pathway in MSCs, we treated mouse MSCs (mMSCs) with TNF for 0, 5, 30, and 60 min. As shown in Fig. 1*A*,

Author contributions: F.L., B.W., K.T., C.S., K.A.H., W.Z., and C.-Y.W. designed research; J.C., F.L., M.L., J.N.Z., and X.C. performed research; M.L. and W.Z. contributed new reagents/analytic tools; J.C., B.W., K.T., J.N.Z., C.S., K.A.H., D.J.M., and C.-Y.W. analyzed data; and D.J.M. and C.-Y.W. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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MEDICAL SCIENCES

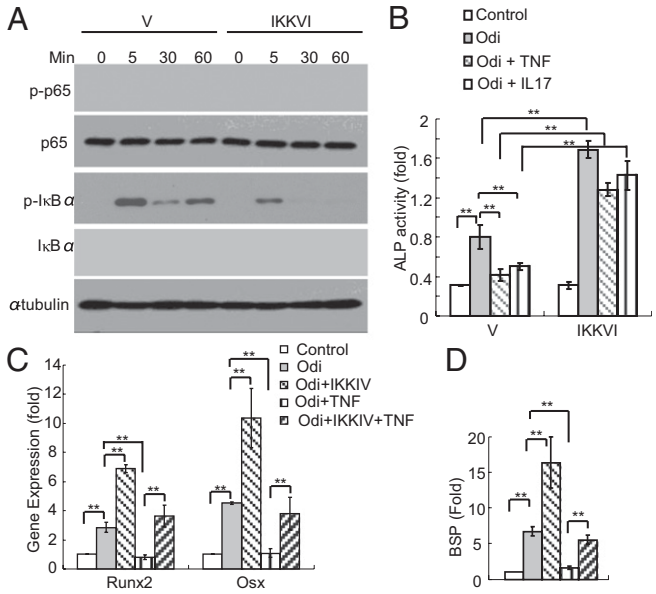

Fig. 1. The IKKβ small molecule inhibitor, IKKVI, promotes osteogenic differentiation by inhibiting NF-κB. (*A*) IKKVI inhibited IKK activities induced by TNF in mMSCs. Cells were pretreated with IKKVI or vehicle control for 30 min and then treated with TNF for the indicated times. The phosphorylation and degradation of IκBα and p65 phosphorylation were examined by Western blot. (*B*) IKKVI overcame TNF and IL-17 inhibition of ALP in mMSCs by inhibiting NF-κB. The results are the average value from three independent experiments and presented as mean ± SD $^{**}P < 0.01$. Odi, osteogenic differentiation-inducing media. (*C*) IKKVI attenuated TNF inhibition of Runx2 and Osx by inhibiting NF-κB in mMSCs, as assessed by real-time RT-PCR. $P < 0.01$. (*D*) IKKVI attenuated TNF inhibition of BSP induction by inhibiting NF-κB in mMSCs.

TNF rapidly activated IKK to induce the phosphorylation and degradation of IκBα in mMSCs. Because IκBα is a direct NF-κB target gene, we observed that the level of IκBα returned to normal levels after 60 min. Previously, we and others have shown that IKKβ directly phosphorylates the p65 transactivation domain on serine 536 (S536) (32). Western blot analysis showed TNF rapidly induced p65 phosphorylation on S536 as determined by anti–phospho-p65-S536 antibodies (Fig. 1*A*). To determine whether TNF affected MSC differentiation, MSCs were grown in osteogenic differentiation-inducing media (Odi) and treated with or without with TNF. As shown in Fig. 1*B*, TNF significantly inhibited alkaline phosphatase activity (ALP), an early differentiation marker. The expression of Runx2 and Osx, two master osteoblast-specific transcription factors, was also significantly inhibited by TNF as determined by real-time RT-PCR analysis (Fig. 1*C*). Consistently, TNF also inhibited the expression of bone sialoprotein (BSP) (Fig. 1*D*). Previously, we have shown that the IKKβ small molecule inhibitor, IKKVI, specifically blocked IKK and inhibited NF-κB activation (33). As shown in Fig. 1*A*, IKKVI was able to inhibit TNF-induced phosphorylation and degradation of IκBα as well as p65 phosphorylation in MSCs. ALP assays showed that IKKVI significantly attenuated TNF inhibition of ALP activity (Fig. 1*B*). Real-time RT-PCR revealed that IKKVI also significantly reduced TNF inhibition of runt-related transcription factor 2 (Runx2), osterix (Osx), and bone sialoprotein (BSP) expression (Fig. 1 *C* and *D*). MSCs have been found to inhibit Th17 cell function in immune diseases (34). We were interested in whether IL-17 produced in high levels by Th17 cells activated IKK–NF-κB in mMSCs. IL-17 also induced the phosphorylation of p65 and IκBα in a time-dependent fashion that was inhibited by IKKVI (Fig. S1*A*). Consistently, IL-17 also inhibited osteogenic differentiation of MSCs. IKKVI treatment abolished IL-17-mediated inhibition of ALP activities (Fig. 1*B*). IKKVI also potently reversed IL-17-mediated inhibition of Runx2, Osx, BSP, and osteocalcin (OCN) (Fig. S1 *B–D*). Although IL-17 inhibited osteogenic mineralization mediated by MSCs, IKKVI potently attenuated IL-17 inhibition of mineralization (Fig. S1*E*).

To further confirm our results, we used IKKβ conditional knockout mice because IKKβ null mutations are embryonically lethal (25, 35). We isolated mMSCs from IKKβflox/flox mice and subsequently infected these cells with adenoviruses expressing Cre recombinases. Western blot analysis showed that more than 90% of IKKβ was deleted in MSCs (IKKβKO MSCs) by Cre recombinases compared with wild-type (WT) MSCs (Fig. 2*A*). Consistently, we found that the deletion of IKKβ inhibited the expression of IL-6, a well known NF-κB target gene, in these cells when treated with TNF and IL-17 (Fig. S2 *A* and *B*). Although TNF inhibited the expression of Runx2, Osx, and BSP in WT mMSCs, similar TNF inhibitions were significantly reduced in IKKβKO MSCs (Fig. 2 *B–D*). Moreover, we found that the depletion of IKKβ also significantly attenuated IL-17 inhibition of Runx2, Osx, BSP, and OCN expression in IKKβKO mMSCs (Fig. S2 *C–F*). To determine whether the depletion of endogenous IKKβ promoted bone formation in vivo, both IKKβKO and WT mMSCs were mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) carriers and then transplanted into nude mice. Histological analysis revealed that IKKβKO MSCs formed a greater volume of bone tissues than WT MSCs 8 wk after transplantation (Fig. 2 *E* and *F*).

IKK–NF-κB Activation Promotes β-Catenin Degradation Through Induction of Smurf1 and Smurf2. To understand the molecular mechanisms by which IKK–NF-κB inhibited osteogenic differentiation of MSCs, we screened several signaling pathways and key molecules associated with MSC differentiation. Recently, Wnt/β-catenin signaling has been found to play an essential role in skeletal development and bone formation (36–45). Unexpectedly, we found that TNF and IL-17 treatment gradually reduced the cytosolic and nuclear levels of β-catenin in mMSCs (Fig. S3 *A* and *B*). Similarly, TNF and IL-17 induced β-catenin degradation

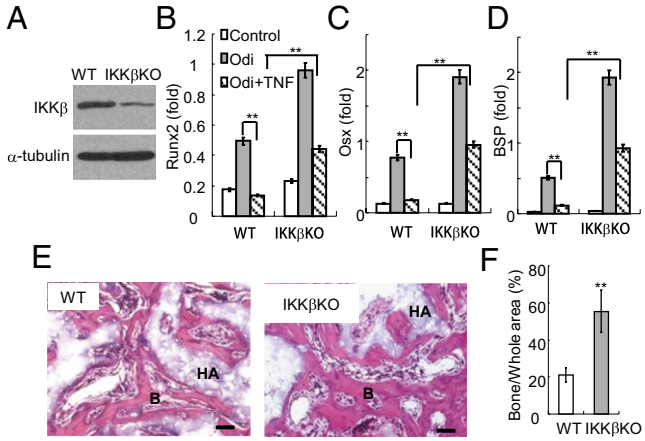

Fig. 2. The depletion of IKKβ promotes osteogenic differentiation of mMSCs and attenuates TNF inhibition of MSC differentiation. (*A*) Depletion of IKKβ in mMSCs. mMSCs from IKKβflox/flox mice were infected with adenoviruses expressing Cre recombinase or empty vector for 24 h. (*B*) Depletion of IKKβ enhanced Runx2 induction in MSCs. IKKβKO mMSCs or WT mMSCs were induced to differentiate in the presence or absence of TNF for 1 d. Runx2 mRNA was assessed by real-time RT-PCR. (*C*) Depletion of IKKβ enhanced Osx induction in mMSCs. (*D*) Depletion of IKKβ enhanced BSP induction. (*E* and *F*) Depletion of IKKβ in mMSCs promoted bone formation in vivo. B, new bone; HA, HA/TCP carrier. (Scale bar, 100 μm.) $^{**}P < 0.01$.

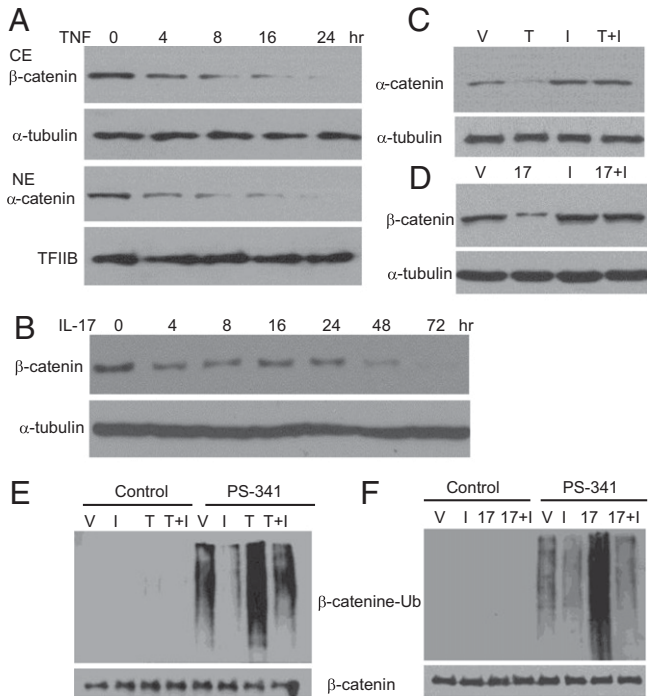

Fig. 3. The activation of NF-κB by TNF and IL-17 promotes β-catenin ubiquitination and degradation. (*A*) TNF induced β-catenin degradation in hMSCs. CE, cytosolic extracts; NE, nuclear extracts. (*B*) IL-17 promoted β-catenin degradation in hMSCs. Cells were treated with IL-17 for indicated times, and the cytosolic levels of β-catenin were examined by Western blot. (*C*) IKKVI inhibited TNF-induced β-catenin degradation in hMSCs. I, IKKVI; T, TNF; T+I, TNF + IKKVI; V, vehicle control. (*D*) IKKVI inhibited IL-17–induced β-catenin degradation in hMSCs. V, vehicle control; 17, IL-17; I, IKKVI; 17+I, IL-17 + IKKVI. (*E*) TNF induces β-catenin ubiquitination through activation of IKK in hMSCs. hMSCs were treated with TNF and IKKVI in the presence or absence of PS-341 as indicated for 4 h. β-catenin was immunoprecipitated with anti–β-catenin and probed with anti-ubiquitin monoclonal antibodies. The whole cell lysates were probed with anti–β-catenin as an internal control. I, IKKVI; T, TNF; T+I, TNF + IKKVI; V, vehicle control. (*F*) IL-17–induced β-catenin ubiquitination by activating IKK in hMSCs. I, IKKVI; V, vehicle control; 17, IL-17; 17+I, IL-17 + IKKVI.

in human MSCs (hMSCs) (Fig. 3 *A* and *B*). Consistently, we found that TNF also inhibited β-catenin–dependent transcription induced by Wnt-3a as determined by the Topflash reporter assay (Fig. S3*C*). To determine whether TNF and IL-17 promote β-catenin degradation through IKK–NF-κB, MSCs were pretreated with IKKVI followed by treatment with TNF and IL-17. Western blot analysis revealed that IKKVI potently blocked the degradation of β-catenin induced by both TNF and IL-17 (Fig. 3 *C* and *D*). β-Catenin degradation is mediated by the ubiquitin-proteasome pathway. We used the specific proteasome inhibitor PS-341 to block β-catenin degradation, allowing us to determine whether TNF and IL-17 might modulate β-catenin ubiquitination by activating NF-κB (46). Interestingly, IKKVI reduced basal ubiquitination of β-catenin. Although TNF potently induced β-catenin ubiquitination, IKKVI significantly inhibited TNF-induced β-catenin ubiquitination in MSCs (Fig. 3*E*). Similarly, IKKVI also inhibited IL-17–induced β-catenin ubiquitination in MSCs (Fig. 3*F*).

To understand how IKK–NF-κB promoted β-catenin degradation, we examined whether IKK–NF-κB regulated Smurf1 and Smurf2, because it has been shown that TNF induces Smurf1 and Smurf2 expression (47, 48). Real-time RT-PCR revealed that TNF induced both Smurf1 and Smurf2 expression in mMSCs. IKKVI treatment abolished TNF-induced expression of Smurf1 and Smurf2, indicating that induction of Smurf1 and Smurf2 was dependent on IKK–NF-κB (Fig. 4*A*). IL-17 could also induce Smurf2 in an IKK-dependent manner (Fig. 4*B*). However, we were unable to detect IL-17 induction of Smurf1 expression, which was probably due to the fact that IL-17 is a less potent activator of NF-κB than TNF. To determine whether NF-κB directly regulated Smurf1 and Smurf2, we performed chromatin immunoprecipitation (ChIP) assays. TNF induced p65 binding to both Smurf1 and Smurf2 promoters in mMSCs (Fig. 4 *C* and *D*). IKKVI significantly inhibited p65 binding to their promoter (Fig. 4 *E* and *F*). Similarly, TNF also directly induced p65 binding to the promoter of Smurf1 and Smurf2 in an IKK-dependent manner in hMSCs (Fig. S4 *A–D*).

Because IL-17 only induced Smurf2, we first determined whether Smurf2 played a role in IL-17–induced β-catenin degradation by the knockdown of Smurf2 using smart pool siRNA. Real-time RT-PCR showed that Smurf2 siRNA inhibited Smurf2 expression induced by IL-17 (Fig. 5*A*). Western blot analysis revealed that the knock-down of Smurf2 reversed IL-17–induced β-catenin degradation in mMSCs (Fig. 5*B*). Also, we found that the knock-down of Smurf2 abolished IL-17–induced β-catenin degradation in MSC differentiation (Fig. 5*C*). Consistently, the knock-down of Smurf2 significantly attenuated IL-17 inhibition of bone matrix gene expression as determined by real-time RT-PCR (Fig. 5 *D–F*), indicating that Smurf2 depletion restored mMSC differentiation. Similarly, we found that the knock-down of Smurf1 and Smurf2 potently inhibited TNF-induced β-catenin ubiquitination and degradation (Fig. S5 *A* and *B*) and attenuated TNF inhibition of mMSC differentiation (Fig. S5 *C* and *D*).

IKKVI Promoted Craniofacial Bone Regeneration and Repair. Craniofacial bone defects are frequently caused by bone injuries which may have elevated inflammatory responses. We tested whether IKKVI could improve craniofacial bone defect repair mediated by MSCs using a syngeneic rat model. A critical-size

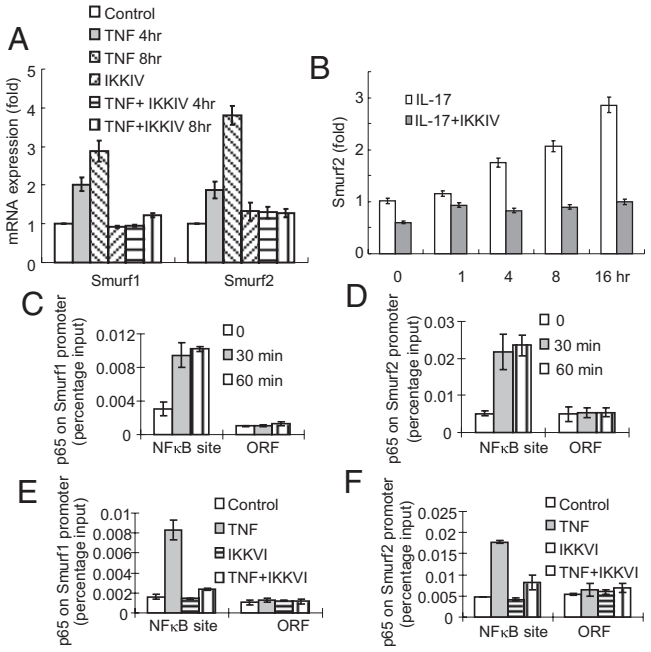

Fig. 4. NF-κB activation induces the expression of Smurf1 and Smurf2 in MSCs. (*A*) TNF induced the expression of Smurf1 and Smurf2 through IKK–NF-κB. (*B*) IL-17–induced Smurf2 expression through IKK–NF-κB. (*C* and *D*) TNF induced p65 binding to the promoter of Smurf1 and Smurf2 promoter in mMSCs. (*E* and *F*) IKKVI inhibited p65 binding to the promoter of Smurf1 and Smurf2 in mMSCs.

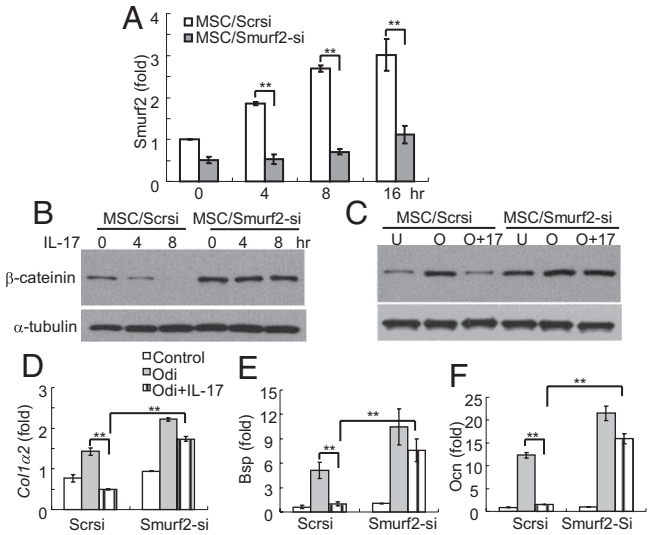

Fig. 5. The depletion of Smurf2 inhibits β-catenin degradation and maintains osteogenic differentiation of MSCs. (*A*) Smurf2 siRNA inhibited IL-17–induced Smurf2 expression in mMSCs. (*B*) Knock-down of Smurf2 inhibited β-catenin degradation induced by IL-17 in mMSCs. (*C*) Knock-down of Smurf2 inhibited IL-17–induced β-catenin degradation during osteogenic differentiation of MSCs. O, osteogenic inducing media; O+17, osteogenic inducing media + IL-17; U, untreated. (*D–F*) Knock-down of Smurf2 attenuated IL-17 inhibition of osteogenic differentiation of MSCs. Cell treatment was performed as described in (*C*). The expression of type I collagen α2 (*Col1a2*), *Bsp*, and *Ocn* was examined by real-time RT-PCR.

calvarial defect was generated in rats. Rat MSCs were loaded onto the apatite-coated 85/15 poly(D,L-lactic-coglycolic) acid (PLGA) scaffolds with or without IKKVI and subsequently placed on the defect. Ten weeks after the operation, microcomputed tomography (μCT) analysis showed that the calvarial defects implanted with the MSCs without IKKVI displayed significantly less repair than that with MSCs with IKKVI, and a clear bone defect was still obvious (Fig. 6*A*). Significantly more new bone formation was observed in the MSCs with IKKVI than without IKKVI. The MSCs with IKKVI group showed extensive new bone formation at the center and periphery of the calvarial defect, indicating osseous integration of the new bone with the defect periphery (Fig. 6*A*). Histological analysis revealed that only small bone nodules were generated in defects implanted with the MSCs without IKKVI, with fibrous tissue separating the new bone nodules from the margins of the calvarial defect. In contrast, the MSCs with IKKVI displayed extensive new bone formation that bridged the defect, with excellent osteointegration (Fig. 6*B*). Immunohistochemistry confirmed that newly generated bones expressed Ocn (Fig. 6*B*, second and fourth panels). Moreover, μCT analysis revealed that, in addition to increased bone volume, the bone mineral density (BMD) of new bone at the calvarial defect generated by IKKVI was significantly higher than that of the control ($P < 0.01$), suggesting that IKKVI strongly enhances mineralization of MSCs in large calvarial defects (Fig. 6 *C* and *D*).

Discussion

MSC-based transplantation is a promising therapeutic approach for bone regeneration and repair. However, to improve its osteogenic capacity under an inflamed microenvironment, it is critical to develop novel strategies for overcoming inflammation to achieve a successful regenerative therapy. In this study, we found that proinflammatory cytokines TNF and IL-17 stimulated IKK–NF-κB and impaired osteogenic differentiation of MSCs. The inhibition of IKK–NF-κB significantly enhanced MSC-mediated bone formation. To translate our basic findings to potential clinic application, we showed that the IKK small molecule inhibitor, IKKVI, enhanced osteogenic differentiation and bone formation of MSCs in vitro and in vivo. More importantly, IKKVI significantly promoted MSC-mediated bone regeneration and repair of a critical-size calvarial defect in a syngeneic rat model. Considering the well established role of NF-κB in inflammation and infection, our results suggest that targeting IKK–NF-κB may provide a novel approach to improve bone regeneration and repair under compromised conditions. Contrary to our findings, two earlier studies showing that NF-κB activation by TNF promoted osteogenic differentiation of MSCs (49, 50). We could not provide an explanation for this discrepancy. However, it should be pointed out that both studies did not test their findings in vivo. Consistent with our studies, Kaneki et al. (47) found that TNF inhibited bone formation in vivo. Very recently, Chen et al. (51) showed that DNA damage could inhibit osteogenic differentiation of MSCs and accelerated bone aging by

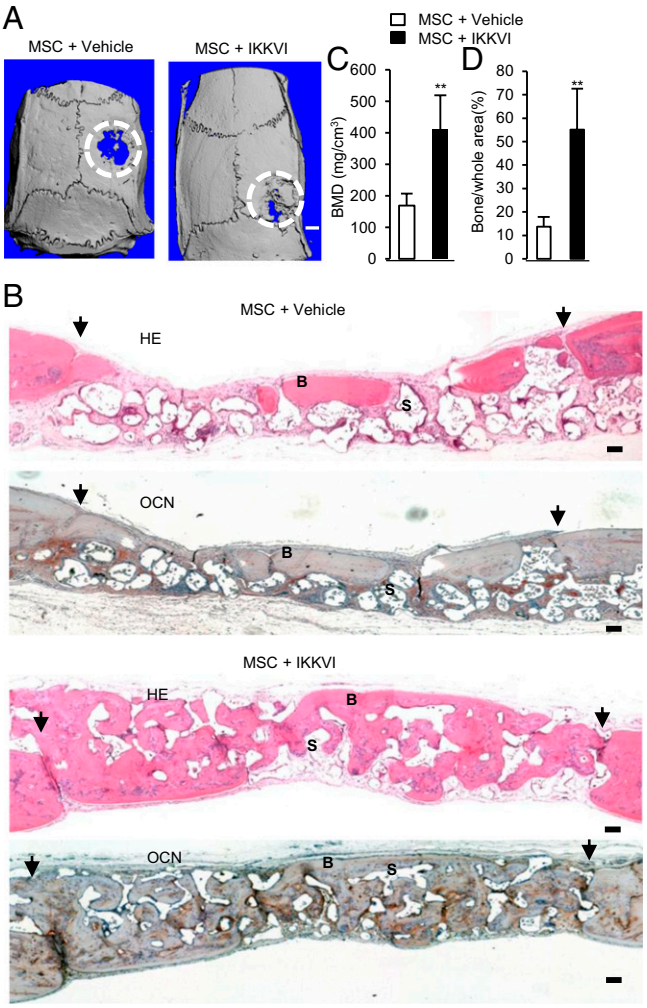

Fig. 6. IKKVI promoted the repair of calvarial bone defect mediated by MSCs in vivo. (*A*) IKKVI promoted the repair of calvarial bone defects as determined by μCT. Rat MSCs were loaded on apatite-coated PLGA scaffolds with or without IKKVI. The scaffolds were placed on the calvarial defects. 10 wk post operation, the calvarias were harvested and the defect repairs were examined by μCT. Dotted circle, defect area. (*B*) IKKVI promoted the repair of calvarial bone defect as determined by H&E staining and immunohistochemistry. B, new bone; S, apatite-coated PLGA scaffold. (Scale bar, 100 μm.) (*C*) IKKVI enhanced BMD as determined by μCT. (*D*) Qualitative measurement of bone formation by μCT. $^{**}P < 0.01$.

activating NF-κB in vitro and vivo, further supporting that NF-κB is an important target for bone diseases and tissue regeneration.

Our work has uncovered a crosstalk between Wnt and NF-κB signaling in MSCs. TNF has been found to stimulate Runx-2 degradation through Smurf1 and Smurf2 in addition to inhibition of Runx2 mRNA expression (19). However, the molecular mechanism by which TNF induces the expression of Smurf1 and Smurf2 was not clear. We found that the inhibition of IKK–NF-κB abolished TNF-induced Smurf1 and Smurf2 expression. Because our ChIP assays revealed that NF-κB directly bound to the promoters of Smurf1 and Smurf2, it was likely that IKK–NF-κB directly controlled the expression of Smurf1 and Smurf2 in MSCS. More importantly, we found that the induction of Smurf1 and Smurf2 by IKK–NF-κB promoted β-catenin degradation in MSCs. Th-17 cells have been found to play an important role in the pathogenesis of osteoimmune diseases (22, 32). In this study, we found that MSCs were responsive to IL-17 stimulation, indicating that Th-17 cells may inhibit MSC function and bone formation by producing IL-17. Interestingly, IL-17–induced Smurf2, but not Smurf1, in MSCs. Because both IL-17 and TNF activated IKK–NF-κB, the precise reason for this difference was not clear. Although IL-17 activated IKK–NF-κB, this activation was relatively slower than that of TNF. The promoter of Smurf1 might have a high threshold for removing corepressors and recruiting coactivators. Another possibility is that TNF, but not IL-17, may activate other signaling pathways that facilitate NF-κB to induce Smurf1 in MSCs. In general, NF-κB has been considered as a transcription factor that positively induces gene expression. Supporting this notion, many genes involved in inflammation and immune responses, such as IL-8 and IL-6, are up-regulated by NF-κB. Elevated levels of TNF and IL-17 have been detected in a variety of chronic inflammatory bone diseases including arthritis, osteoporosis, and periapical and periodontal diseases (14, 15, 21, 22, 24). By identifying NF-κB–dependent Smurf1 and Smurf2 induction, our results suggest that IKK–NF-κB may promote protein degradation by post-transcriptional mechanisms. Interestingly, Dickkopf-1 (DKK1) expression by chondrocytes in osteoarthritis was found to correlate with inflammatory cytokine levels (52). DKK1 suppressed nuclear β-catenin accumulation and promoted chondrocyte apoptosis. Because NF-κB is activated in inflammation, it is possible that NF-κB might also down-regulate β-catenin by inducing DKK1 in osteoarthritis.

Bone formation is compromised in a variety of chronic inflammatory and metabolic diseases, including arthritis, diabetes, and periodontitis (20–24). Our results suggest that elevated inflammatory mediators in these diseases may inhibit bone formation by activating IKK–NF-κB. Inhibition of IKK may help to improve bone regeneration and repair under chronic inflammatory conditions in those diseases. Moreover, in chronic inflammatory or acute injury conditions, the IKK inhibitor may help not only to control inflammation, but also to promote bone regeneration and repair. Based on our studies, targeting IKK–NF-κB may provide an efficacious treatment strategy for restraining inflammation while simultaneously promoting bone regeneration.

Materials and Methods

Cell Culture and Lentiviral Transduction. Human, mouse and rat MSCs were purchased from Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott and White and grown in DMEM (Invitrogen) supplemented with 15% (vol/vol) FBS. hMSCs were also purchased from Stemcell Technologies for some experiments and showed similar results. To generate IKKβKO mMSCs and control cells, mMSCs were isolated from the bone marrow of 2-mo-old IKKβflox/flox mice and infected with adenoviruses expressing Cre recombinases or green fluorescent proteins. The positive markers Sca-1 and CD29 and the negative markers CD45 and CD11b (Fig. S6) were used to isolate mMSCs from mouse bone marrow as described by others (7). SiRNA was purchased from Dharmacon and transfection was performed as described (6). The target sequences for shRNA were: mouse Smurf2, 5′-GACCAACAGCAACAGCAAG-3′; and 5′-GATAAAATT-TCGTGGAGAA-3′; luciferase, 5′-GTGCGTTGCTAGTACCAAC-3′; and mouse Smurf1 and Smurf2 SMARTpool siRNA.

ALP and Alizarin Red Staining. To induce osteogenic differentiation, MSCs were grown in differentiation-inducing media containing 100 μg/mL ascorbic acid, 2 mM β-glycerophosphate and 1 μM dexamethasone for indicated times. ALP staining was performed using a kit from Sigma-Aldrich as described (23). For detecting mineralization, cells were induced for 2–3 wk, fixed with 70% (vol/vol) ethyl alcohol (ETOH) and stained with 2% (vol/vol) Alizarin red (Sigma-Aldrich). To quantitatively measure calcium deposition, Alizarin Red was destained with 10% (vol/vol) cetylpyridinium chloride in 10 mM sodium phosphate for 30 min at room temperature. The concentration was determined by absorbance measurement at 562 nm on a multiplate reader using a standard calcium curve in the same solution. The final calcium level in each group was normalized with the total protein concentration prepared from a duplicate plate.

Western Blot Analysis, Real-Time RT-PCR, and ChIP Assays. Western blot analysis was performed as described (34). The primary antibodies were from the following sources: anti-IκBα polyclonal antibodies anti-ubiquitin monoclonal antibodies from Santa Cruz Biotechnology; anti–phospho-IκBα, anti–phospho-p65 and anti-p65 polyclonal antibodies from Cellular Signaling; anti–α-tubulin monoclonal antibodies from Sigma-Aldrich; and anti–β-catenin monoclonal antibodies from Oncogene Research Products. For real-time RT-PCR, total RNA was extracted according to the manufacturer's protocol. Two-microgram aliquots of RNAs were synthesized using random hexamers and reverse transcriptase according to the manufacturer's protocol (Invitrogen). The real-time PCR reactions were performed using the QuantiTect SYBR Green PCR kit (Qiagen) and the Icycler iQ Multicolor Real-time PCR Detection System. 18S rRNA was used for normalization. The primers were synthesized by Invitrogen, and their sequences are provided in *SI Materials and Methods*. ChIP assays were carried out using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's recommendation. The primer sequences were provided in *SI Materials and Methods*.

Scaffold Preparation and Craniofacial Defect Model. For transplantation of mouse IKKβKO MSCs and control cells, hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic particles (Zimmer) were used as a carrier. Approximately 1.0×10^6 of the cells were mixed with 40 mg of HA/TCP ceramic particles and then transplanted s.c. into the dorsal side of 6-wk-old nude mice (Taconic). Eight weeks after transplantation, the transplants were harvested and processed for H&E staining. For the craniofacial defect model, apatite-coated PLGA scaffolds were used. The scaffold preparation, animal surgery, histology and μCT analysis were described in *SI Materials and Methods* (53–55).

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