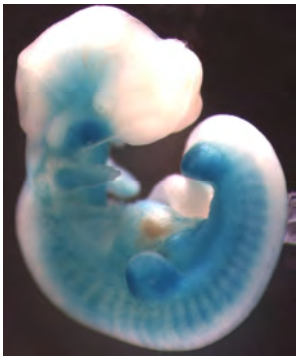
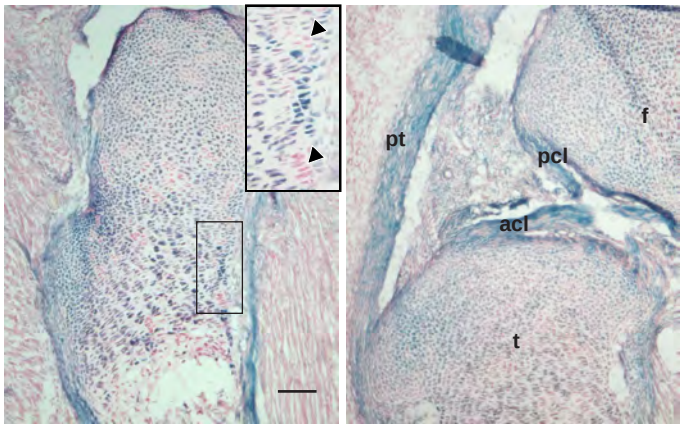
Supplemental figure 1.

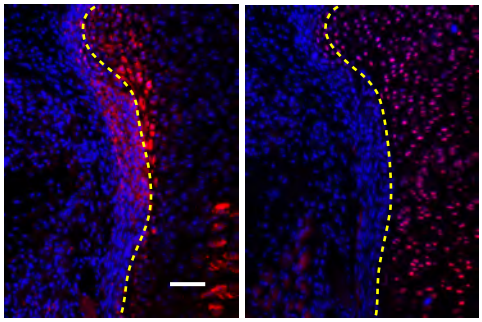


Supplemental figure 2.



Proximal femur Knee joint

Supplemental figure 3.

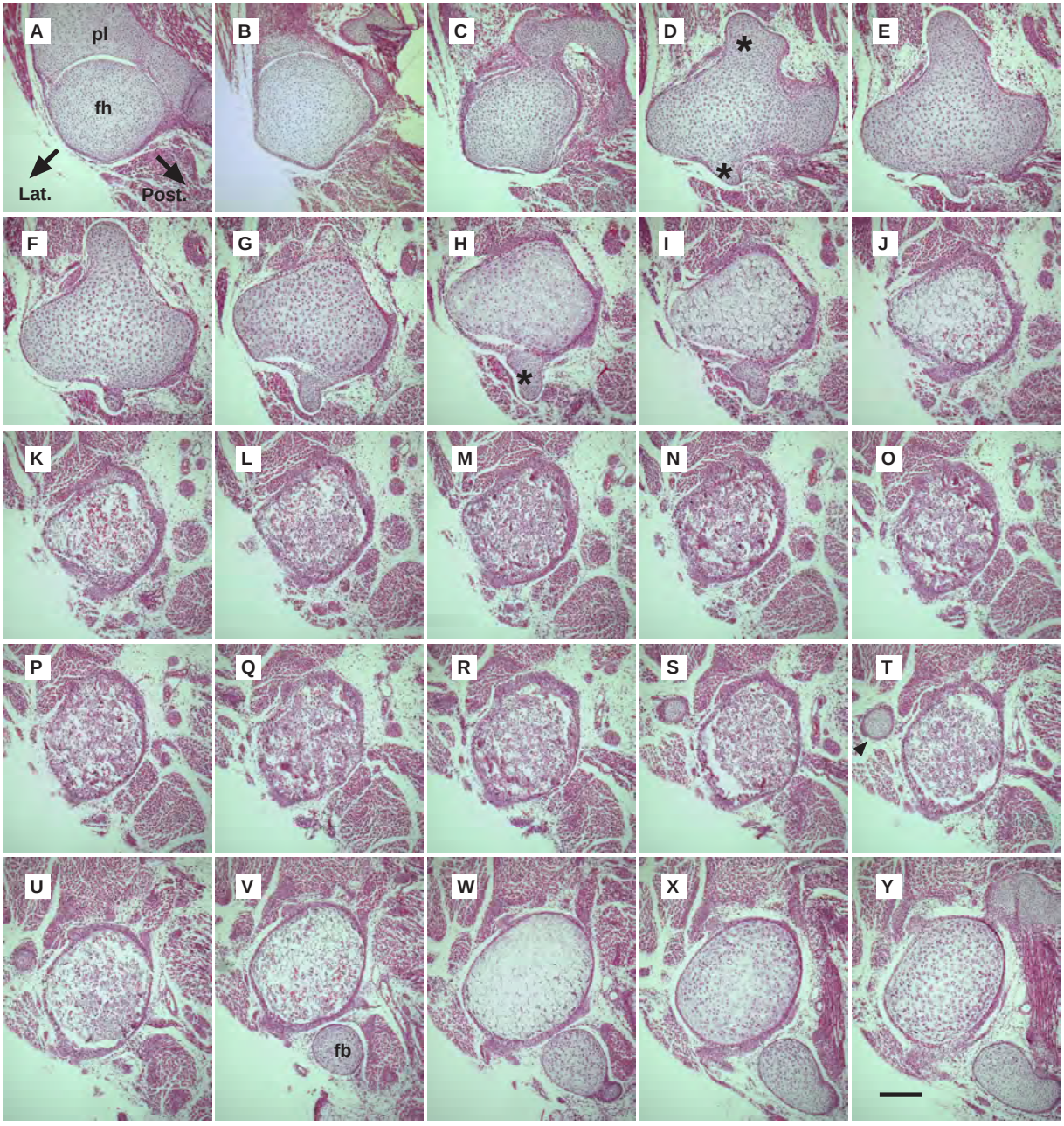


ALK5 SOX9

Supplemental Fig. S1. Dermo1-Cre-mediated recombination patterns by β-galactosidase staining in *Dermo1*$^{Cre/wt}$*;ROSA26* reporter mice. Targeted introduction of Cre into the *Dermo1* locus mediates loxP recombination in the mesenchymal tissue for future bone. Whole-mount detection of β-galactosidase activity in E10.5 embryos. Mesenchyme-specific Cre expression was observed in the limb bud, craniofacial mesenchyme, and body wall.

Supplemental Fig. S2. Histological analysis for expression patterns of Dermo1-Cre in ROSA26 reporter mice. Cryosections of E17.5 hindlimbs stained with β-galactosidase. Chondrocytes, perichondrial cells, ligaments, and synovial cells stained positively for β-galactosidase. The boxed area is enlarged in the inset. Note that some chondrocytes, escaped from Cre-mediated recombination, were negative for X-gal staining. Arrowheads, chondrocytes negative for X-gal staining. Abbreviations: f, femur; t, tibia; pt, patella tendon; acl, anterior cruciate ligament; pcl, posterior cruciate ligament. Bar, 100μm.

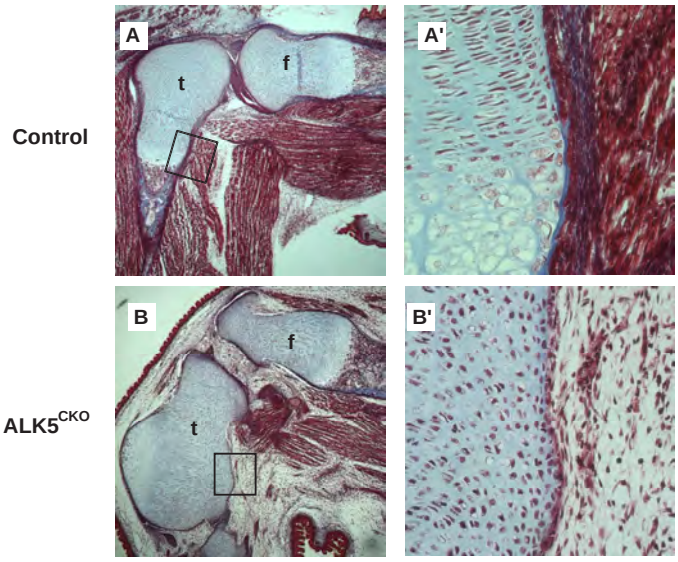
Supplemental Fig. S3. Immunoflourescent detection of ALK5 and Sox9 in the growth plate and perichondrium of normal E17.5 femurs at the Ranvier's ossification groove. Note that chondrocytes located in the periphery of cartilage adjacent to the perichondrium expressed ALK5, as well as Sox9. Bar, 50μm.



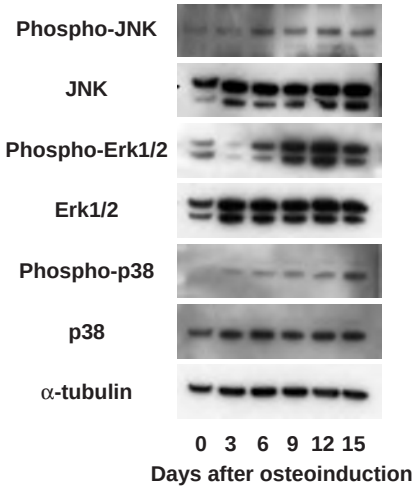
Supplemental figure 4.

Supplemental Fig. S4. H-E staining of serial transverse sections of femurs from the proximal to the distal in ALK5^{CKO} embryos at E18.5. Paraffin slices (5μm) at approximately 60μm intervals were subjected to H-E staining. Ectopic cartilaginous tissues protruded from cartilage at the Ranvier's ossification groove and bone bark (perichondrial bone ring) and perichondrium surrounding the ectopic protrusion was very thin. Asterisks and arrowheads indicate cartilaginous protrusions and solitary ectopic cartilage, respectively. Lat, lateral; Post, posterior; pl, pelvis: fh, femoral head: fb, fibula. Bar, 200μm.

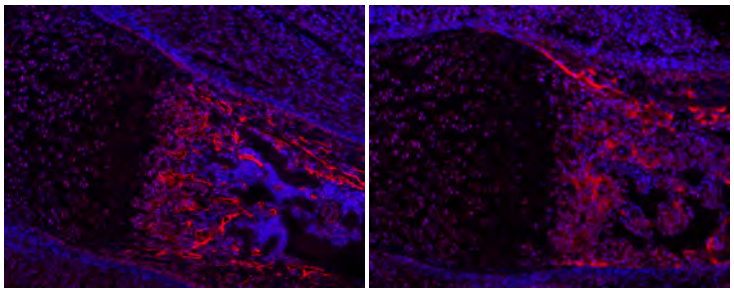
Supplemental figure 5.



Supplemental figure 6.



Supplemental figure 7.

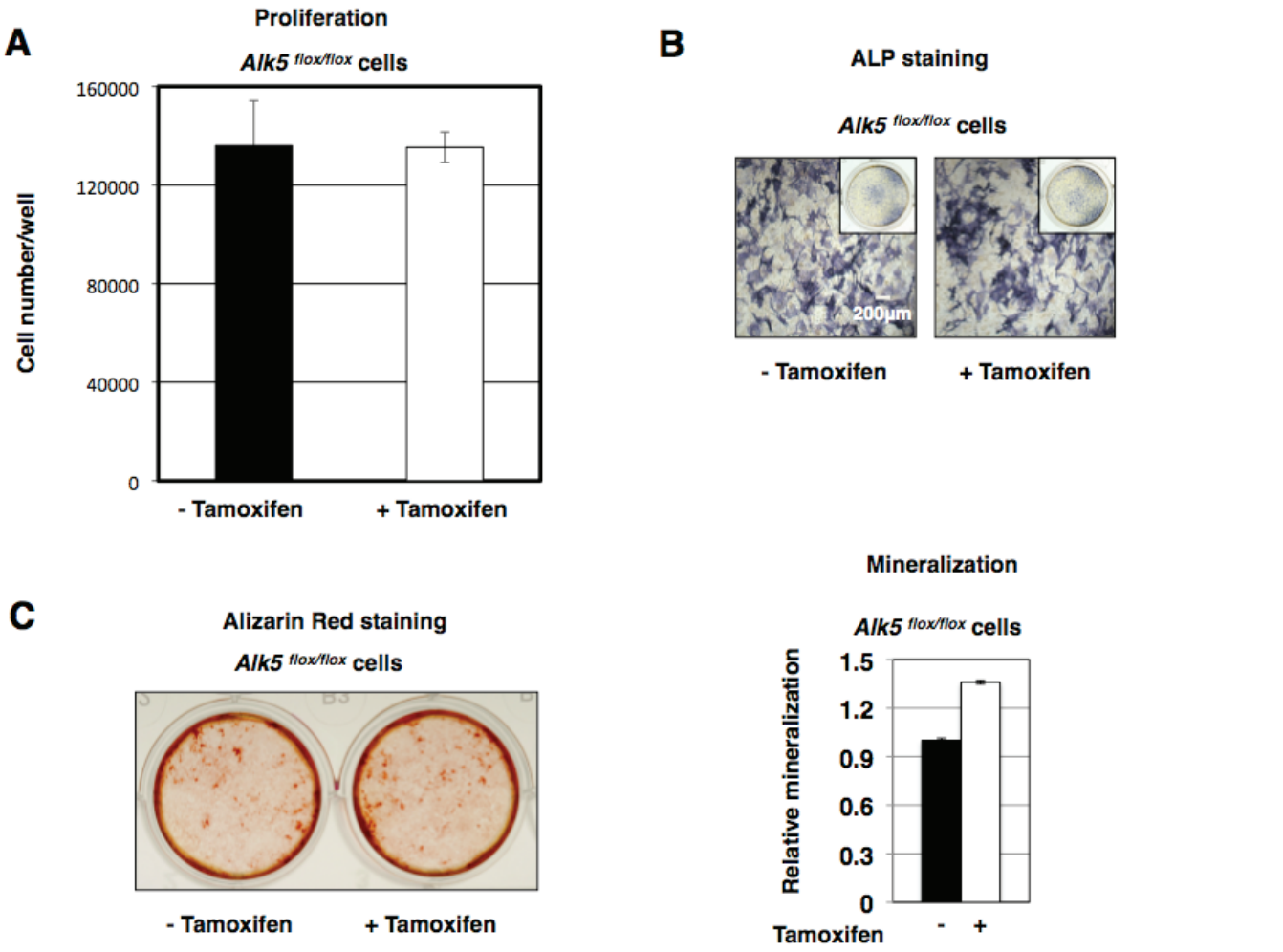


Supplemental Fig. S5. Sagittal sections of E18.5 hindlimbs of control (A) and ALK5^{CKO} embryos (B) were examined with Masson's trichrome staining. Higher magnifications of the boxed area in A and B are shown in A’ and B’, respectively. Formation of the perichondrium was impaired in ALK5^{CKO} embryos.

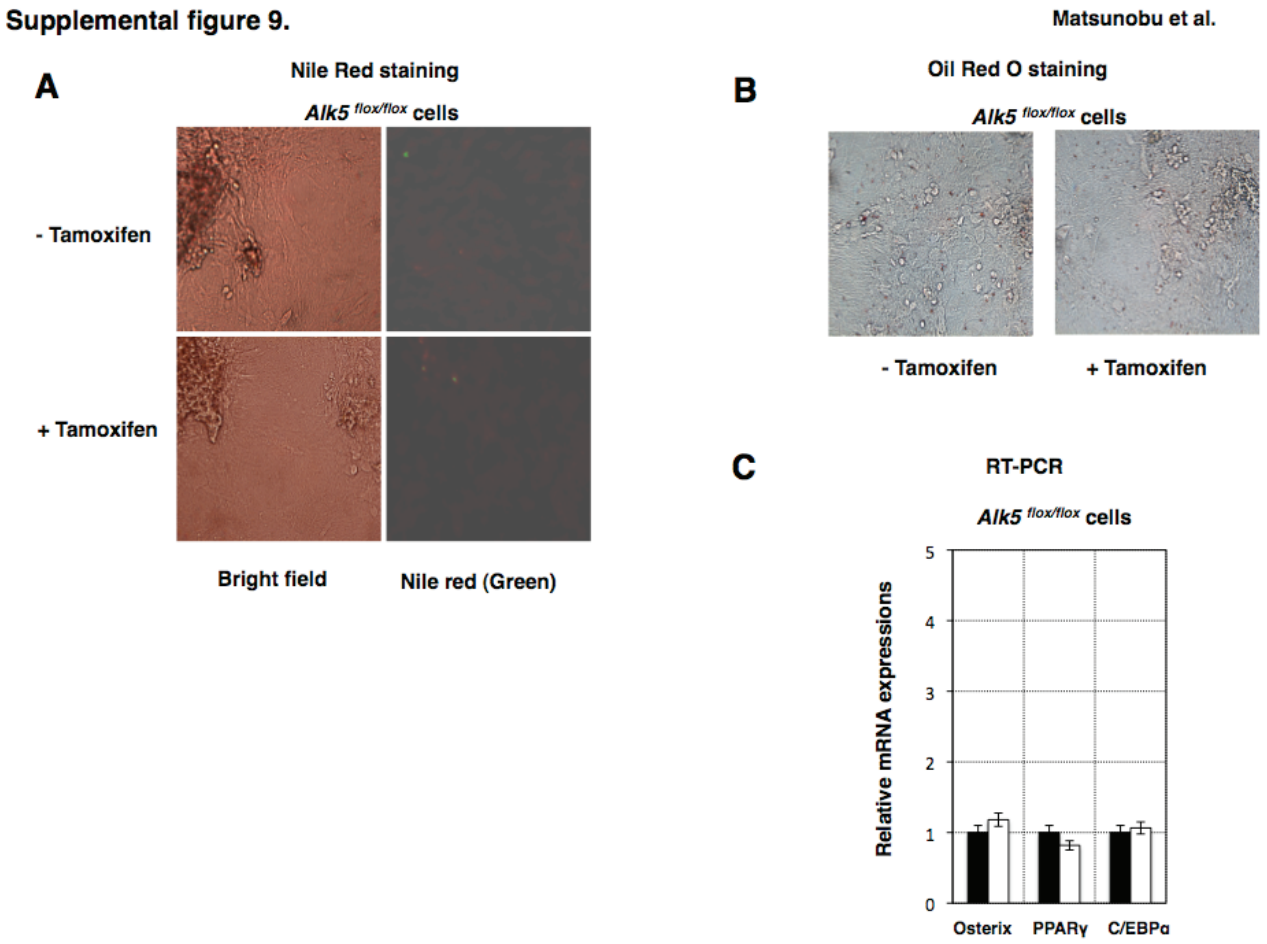
Supplemental Fig. S6. Activation of JNK, Erk1/2, and p38 during normal primary calvarial cell differentiation. Cultures of primary calvarial cells from control mice were incubated in osteoinduction medium and cell lysates were prepared at the indicated times and analyzed by Western blot. JNK, Erk1/2, and p38 were activated with maturity of calvarial cells.

Supplemental Fig. S7. Immunoflourescent detection of phospho-smad1/5/8 in the growth plate and perichondrium of E18.5 control and ALK5^{CKO} femurs. There was no difference in the phosphorylation level of Smad1/5/8 between control and ALK5^{CKO} femurs.

Supplemental figure 8. Matsunobu et al.

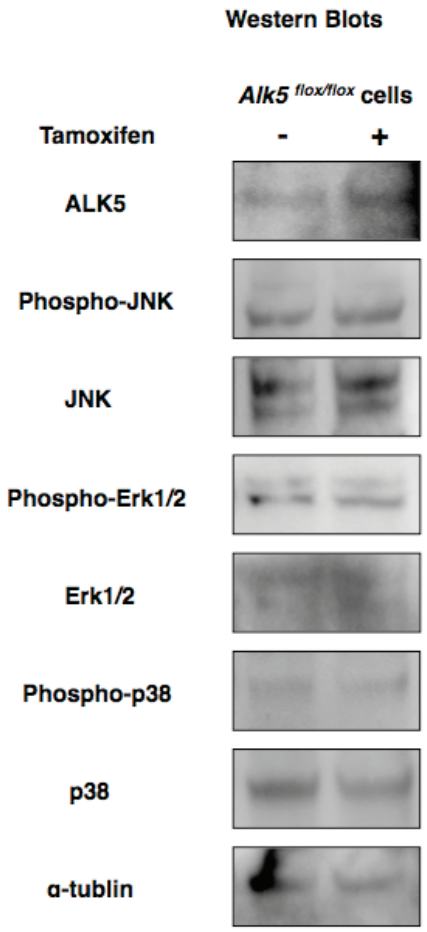


Supplemental Fig. S8. Tamoxifen treatment does not affect proliferation and differentiation of primary CreER-negative *Alk5*$^{flox/flox}$ calvarial cells prepared from *Alk5*$^{flox/flox}$ mice. (A) The number of cells was counted 3 days after treatment with ethanol (- tamoxifen) or tamoxifen (+ tamoxifen). (B) ALP-staining of *Alk5*$^{flox/flox}$ calvarial cells in the absence or presence of tamoxifen at day 3 after osteoinduction. (C) Alizarin Red S staining of *Alk5*$^{flox/flox}$ calvarial cells at day 17 after osteoinduction (left). Quantification of the staining is shown (right).



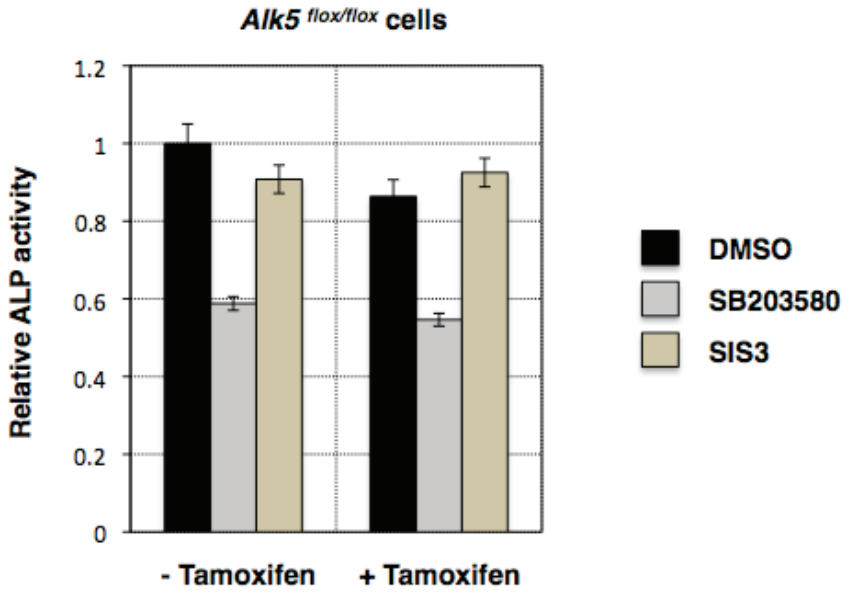
Supplemental Fig. S9. Tamoxifen does not affect adipocytogenesis of CreER-negative *Alk5*$^{flox/flox}$ calvarial cells prepared from *Alk5*$^{flox/flox}$ mice. (A) Representative light and fluorescent microscopic images of Nile Red (green signal) staining of *Alk5*$^{flox/flox}$ calvarial cells in the absence or presence of tamoxifen at day 18 after osteoinduction. There were only a few droplets in light microscopy (left) and Nile Red-positive cells (right) and tamoxifen did not increase the number of droplets and Nile Red-positive cells. (B) Oil Red O-staining of *Alk5*$^{flox/flox}$ calvarial cell cultures in the absence or presence of tamoxifen at day 20 after osteoinduction. Tamoxifen did not increase the number of Oil Red-positive cells. (C) Real-time quantitative RT-PCR for the osteoblast-specific osterix and adipocyte-specific differentiation markers PPARγ and C/EBPα in *Alk5*$^{flox/flox}$ calvarial cells in the absence or presence of tamoxifen. There were no significant effects of tamoxifen on the expression of these genes in *Alk5*$^{flox/flox}$ cells.

Supplemental figure 10.



Supplemental Fig. S10. Tamoxifen does not affect the activation of JNK, Erk1/2, and p38MAPK during osteogenic differentiation of CreER-negative *Alk5*$^{flox/flox}$ calvarial cells. *Alk5*$^{flox/flox}$ calvarial cells were grown for 3 days in osteoinduction medium, in the absence or presence of tamoxifen, and lysates were prepared and subjected to Western blot analysis.

Supplemental figure 11. Matsunobu et al.



Supplemental Fig. S11. Tamoxifen does not affect the inhibitory activity of SB203580 and SIS3 in ALP activity of CreER-negative *Alk5*$^{flox/flox}$ calvarial cells. *Alk5*$^{flox/flox}$ calvarial cells at day 3 after osteoinduction. Tamoxifen was added one day before osteoinduction. SB203580, p38 inhibitor; SIS3 Smad3 inhibitor.

Supplement Table 1.

Oligonucleotide primer sequences

Osterix	**5'-CTGGGGAAAGGAGGCACAAAGAAG-3'** **5'-GGGTTAAGGGGAGCAAAGTCAGAT-3' (497bp)**	
PPARγ	**5'-TTTTCCGAAGAACCATCCGAT-3'** **5'-ACAAATGGTGATTTGTCCGTTG-3' (343bp)**	**Ref.(1)**
C/EBPα	**5'-GATAAAGCCAAACAACGCAACG-3'** **5'-CTAGAGATCCAGCGACCCGAA-3' (257bp)**	**Ref.(1)**

Reference (1): EMBO Rep. 2003 April; 4(4): 374-380.