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Original Article

GATA4 is essential for bone mineralization via ER α and TGF β /BMP pathways[†]

Running title: GATA4 regulates osteoblast functions

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Abstract

Osteoporosis is a disease characterized by low bone mass, leading to an increased risk of fragility fractures. GATA4 is a zinc-finger transcription factor that is important in several tissues, such as the heart and intestines, and has recently been shown to be a pioneer factor for estrogen receptor alpha ($ER\alpha$) in osteoblast-like cells. Herein, we demonstrate that GATA4 is necessary for estrogen-mediated transcription and estrogen-independent mineralization *in vitro*. *In vivo* deletion of GATA4, driven by Cre-recombinase in osteoblasts, results in perinatal lethality, decreased trabecular bone properties and abnormal bone development. Microarray analysis revealed GATA4 suppression of TGF β signaling, necessary for osteoblast progenitor maintenance and concomitant activation of BMP signaling, necessary for mineralization. Indeed, pSMAD1/5/8 signaling, downstream of BMP signaling, is decreased in the trabecular region of conditional knockout femurs, and pSMAD2/3, downstream of TGF β signaling, is increased in the same region. Together these experiments demonstrate the necessity of GATA4 in osteoblasts. Understanding the role of GATA4 to regulate the tissue specificity of estrogen-mediated osteoblast gene regulation and estrogen-independent bone differentiation may help to develop therapies for post-menopausal osteoporosis.

Key words:

GATA4; Estrogen; TGF beta, BMP, Osteoblast

Introduction

One out of 2 women over the age of 50 will experience an osteoporotic fracture in her lifetime (1). The highest risk factor for osteoporosis is being post-menopausal. Thus, understanding the mechanism of action of estrogens in bone is key to preventing and treating osteoporosis. 17β -estradiol (E2) induces apoptosis in bone resorbing osteoclasts and is anti-apoptotic in osteoblasts, leading to an overall building of bone (2). Towards this end, we showed that E2, *via* estrogen receptor alpha ($ER\alpha$), induces transcription of Fas Ligand (FasL) in osteoblasts resulting in a paracrine signal to induce osteoclast apoptosis (3). E2 also induces transcription of alkaline phosphatase and *Bmp2* in osteoblasts, thereby regulating osteoblast differentiation (4).

The GATA family of transcription factors is a conserved set of proteins that bind to the DNA sequence (A/T)GATA(A/G). *Gata4* is expressed early in embryogenesis and is a key regulator of mesodermal and endodermal development (5). *Gata4* was recently described to be expressed in osteoblasts, and to be regulated by $ER\alpha$ (6). However, a role for GATA4 in the commitment of bone progenitors, differentiation of pre-osteoblasts, or mineralization of bone has not been previously described.

TGF β and BMP family members play important roles in skeletal development (7). TGF β -1, -2 and -3 are important in the maintenance and expansion of osteoblast progenitors, and BMP-2, -4, -5, -6 and -7 induce osteoblast differentiation (8). TGF β signaling leads to the phosphorylation of SMAD2/3, which then stimulates proliferation and early osteoblast differentiation, while inhibiting terminal differentiation (9). BMP signaling leads to the phosphorylation of SMAD1/5/8 and activation of the expression and activity of *Runx2* and other genes (7) necessary for osteoblast differentiation. Although the actions of both TGF β and BMP

signaling in bone have been fairly well characterized, the mechanisms regulating their transcriptional regulation are poorly understood.

GATA4 and SMAD signaling pathways regulate transcription in the heart, gut, and ovaries. In heart development, BMP4 signaling regulates *Gata4* expression (10) and conversely GATA4 regulates *Bmp4* expression (11). Furthermore, GATA4 and SMADs co-activate transcription of heart specific genes such as *Nkx2-5* (12). GATA4 and TGF β signaling crosstalk in the gut, where they synergize to regulate epithelial gene expression (13); similarly, in granulosa cells of the ovary, TGF β upregulates *Gata4* and then GATA4 and SMAD3 cooperate to regulate inhibin-alpha (14).

Global knockout of *Gata4* in the mouse reveals heart and gut defects and lethality between embryonic days 7.5 and 10.5 (15, 16). Due to the early embryonic lethality of these models, the importance of GATA4 for bone development *in vivo* was never studied. In order to investigate osteoblast-specific effects of GATA4, we analyzed the estrogen-dependent and -independent effects of GATA4 *in vitro*, and then selectively ablated GATA4 in osteoblasts *in vivo*. These results show that GATA4 in osteoblasts is necessary for survival and proper bone development. Furthermore, we demonstrate that GATA4 regulates TGF β and BMP pathways in osteoblasts. Together, these studies identify GATA4 as a regulator of osteoblast commitment during early development *via* E2-dependent and -independent pathways.

Materials and Methods

Ethics Statement

All animal work was approved by the Animal Research Committee at UCLA.

Reagents

17β -estradiol (E2) was purchased from Sigma-Aldrich Co. All E2 experiments were performed in media without phenol red and with 5% charcoal dextran-treated fetal bovine serum (CDT-FBS) (Omega Scientific). The following antibodies were used: ER α (Thermo Fisher Scientific, clone Ab-16), GATA4 (Santa Cruz, Clone G4), RUNX2 (R&D Systems), and β -actin (Sigma-Aldrich Co.). SMAD antibodies were obtained from Cell Signaling (pSmad1/5/8 #9511, pSmad2: #3108, Smad2: #3122, Smad3: #9523, Smad5: #9517).

Mice

Gata4 flox/flox mice were purchased from Jackson Labs (*Gata4*^{tm1.1Sad/J}) and backcrossed for 10 generations to the FVB background. *Col1A1* (2.3 kb)-Cre mice (FVB-Tg(*Col1a1-cre*)1Kry/Mmucd) were purchased from the Mutant Mouse Regional Resource Centers (MMRRC).

Primary calvarial osteoblasts

Neonatal CD1 calvaria were obtained two days after birth and incubated for 40 minutes in α MEM-1.0 mg/ml collagenase P-1.25% trypsin at 37°C. These were washed in α MEM, and transferred to α MEM-1.0 mg/ml collagenase P-1.25% trypsin for 1 hour at 37°C(17). Digestion was stopped by addition of α MEM/10% FBS. The cells from the second digest were allowed to attach for 48 hours and then differentiated in mineralization medium with media replacement every three days. Differentiation was confirmed by quantitation of *col1A1*, bone sialoprotein (BSP) and osteocalcin mRNA, alkaline phosphatase positivity, and alizarin red staining for mineralization.

Pericytes

Perivascular cells (pericytes) were obtained from human abdominal subcutaneous fat or lipoaspirate, fetal lung, fetal muscle, or fetal bone marrow as described (18). Since specimens were obtained as anonymous and unidentifiable, the activities of the present research do not involve human subjects and therefore do not require IRB review according to UCLA IRB medical committee standards. For *in vitro* mineralization, cells were differentiated with the HyClone AdvanceSTEM Osteogenic Differentiation Kit (Fisher Scientific).

Lentiviral silencing

For shRNA-mediated knockdown of *Gata4* expression, cells were plated in six-well plates (1 X 10⁵ cells per well) and infected 24 hours later with lentivirus. Cells were treated with virus in 2 mL MEM per well with a final concentration of 8 µg/mL Polybrene. Plates were centrifuged at 1400 X g at 30°C for 45 minutes. The following day the media was replaced with mineralization medium with media replacement every three days. Knockdown of *Gata4* was confirmed by qPCR and immunoblotting. Two different shRNA from The RNAi Consortium (TRC) in pLKO vector were used to knockdown mouse *Gata4* (TRCN0000095215:

CCGGCCCAATCTCGATATGTTTGATCTCGAGATCAAACATATCGAGATTGGGTTTTT

G and TRCN0000095217:

CCGGCATCTCCTGTCACTCAGACATCTCGAGATGTCTGAGTGACAGGAGATGTTTTT

G) and human *GATA4* (TRCN0000020424:

CCGGCCAGAGATTCTGCAACACGAACTCGAGTTCGTGTTGCAGAATCTCTGGTTTTT

and TRCN0000020428:

CCGGCCCGGCTTACATGGCCGACGTCTCGAGACGTCCGGCCATGTAAGCCGGGTTTTT

). pLKO-shGFP was used as a negative control for mouse experiments and pLKO-C (shC, with no mammalian target) was used for human experiments.

RNA and Quantitative PCR (qPCR)

Cells were hormone-deprived by culture for three days in phenol red-free medium (Invitrogen Corporation) supplemented with 5% CDT-FBS. Cells were treated with 10 nM E2 or ethanol as a vehicle control for 3 or 24 hours. Total RNA was converted to cDNA with Superscript III First Strand Synthesis Kit according to the manufacturer's instructions (Invitrogen Corporation). Primers were selected using Primer3 (19) and the sequences are listed in Supplemental Fig. 10. cDNA was subjected to quantitative PCR using SYBR Green Mastermix with ROX. Each RNA sample was collected in triplicate and each PCR reaction was amplified in triplicate.

Microarray

Total RNA was analyzed using the MouseRef-8 v2.0 Expression BeadChips from Illumina. Data was analyzed using GenomeStudio (Illumina). Gene ontology was performed by The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (20). Upon acceptance, microarray data will be submitted to NCBI Geo Database.

Immunohistochemistry

Formalin-fixed paraffin-embedded samples were processed through standard deparafinization protocols. The tissue was then incubated in blocking buffer (5% normal goat serum, 2.5% BSA in PBS at pH 7.5) for 30 min. Primary antibodies were incubated overnight at 4°C in a

humidified chamber followed by either the DAKO Envision+ visualization system and counterstaining with Hematoxylin or by immunofluorescence with DAPI counterstaining.

Protein and Immunoblotting

Cells were hormone-deprived by culture for three days in phenol red-free medium (Invitrogen Corporation) supplemented with 5% CDT-FBS. Cells were treated with 10 nM E2 or ethanol as a vehicle control for 24 hours and then lysed in EBC buffer (50 mM Tris, pH 8, 120 mM NaCl, 0.5% Nonidet P-40) supplemented with a protease inhibitor mixture (Complete, Roche Applied Science) for 30 min on ice. Proteins were subjected to SDS-PAGE and immunoblotting with antisera to the indicated proteins.

Alkaline Phosphatase Assay

Osteoblasts were differentiated for the indicated amount of time and then fixed with 3.7% formaldehyde, and stained for alkaline phosphatase activity with SigmaFast BCIP/NBT (Sigma).

Mineralization Assay

Osteoblasts were fixed in 50% ethanol for 15 minutes at 4°C. 1% Alizarin Red S (w/v with 0.1% ammonium hydroxide) was added for 30 minutes. The stain was washed with water and photographed. The alizarin red was eluted with 10% cetylpyridinium chloride and the OD was measured at 570 nm.

RNA *in situ*

Gata4 mRNA was detected by RNAscope technology (2.0 High Definition, Advanced Cell Diagnostics) in wildtype FVB embryos according to the manufacturer's recommendations.

Recombination Efficiency

The calvariae of P0 mice were placed on a stainless steel grid in a 12-well tissue culture dish in differentiation media for 7 days. RNA from calvariae was obtained and cDNA was synthesized.

Primers were designed to anneal to WT cDNA in exon 5 (which is excised by Cre recombinase) and exon 6 of *Gata4* (GCGGAAGGAGGGGATTCAAA and TGAATGTCTGGGACATGGAGC).

Skeletal Preparations

Skeletal preparations were performed as in (21). Briefly, embryos were eviscerated and fixed in 95% EtOH overnight at 4°C, followed by Alcian Blue staining (0.01% Alcian Blue 8GX [Sigma-Aldrich] [w/v] in 95% EtOH) overnight at room temperature. Samples were then stained for Alizarin Red (0.05% Alizarin Red S [Sigma-Aldrich] [w/v] in 1% KOH) for 3 to 4 hours and cleared in a series of graded KOH in glycerol.

Von Kossa

Deparaffinized sections were incubated with 1% silver nitrate solution under ultraviolet light for 30 minutes. Unreacted silver was removed with 5% sodium thiosulfate. The sections were counterstained with nuclear fast red.

Bone MicroCT Scanning and Analysis

Femurs, tibia/fibula, L5 vertebrae, and skulls from a total of 9 P0 WT and *Gata4* cKO mice were dissected, cleaned of soft tissue, and stored in 70% ethanol before μ CT scanning. MicroCT scanning was performed with a Skyscan 1172 scanner (Skyscan, Kontich, Belgium) with the X-ray energy equaling 30 KVp and 175 μ A and a voxel isotropic resolution of 6 μ m. Prior to scanning, bones were positioned with gauze in the sample holder. To compare femur measurements of trabecular bone microarchitecture and cortical bone morphology, 65 transaxial slices were reconstructed and measured for each sample covering 370 μ m of the distal metaphysis starting 0.290 mm above the distal epiphysis and moving along the shaft, and 66 slices covering 380 μ m (33 slices each above and below the mid-diaphysis), respectively. For trabecular bone measurements contours were drawn in the medullar cavity at a fixed distance from the endosteum to define tissue volume. For L5 vertebrae, 60 slices, covering \sim 345 μ m, were analyzed. Bone volume fraction (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th.) and trabecular separation (Tb.Sp.) were measured for L5 vertebrae and trabecular bone of the femur utilizing the CTAn software (Skyscan, Kontich, Belgium). For cortical bone measurements contours were drawn immediately adjacent to the periosteum bone perimeter to define the periosteal envelope. Total cross sectional area (T.Ar.), cortical bone area (B.Ar.), cortical area fraction (% B.Ar.), and mean cortical thickness (Ct.Th.) were measured utilizing the CTAn software (Skyscan, Kontich, Belgium).

Statistical Analysis

All experiments represent both biological and experimental triplicates. Error bars represent mean \pm 1 standard deviation (S.D.). ** = p value < 0.01. * = p value < 0.05 using an Student's t test.

Results

GATA4 regulates ER α and ER α target genes in osteoblasts

Maximal binding of GATA4 to osteoblast-specific target gene enhancers precedes ER α binding, and GATA4 is necessary for open chromatin (marked by histone 3 lysine 4 dimethylation) at ER α binding sites. In accordance, knockdown of GATA4 led to a reduction of ER α binding to DNA at these sites. Together, these results suggest that GATA4 is a pioneer factor (pioneer factors are a special class of transcription factor that can associate with compacted chromatin to facilitate the binding of additional transcription factors (22)) for ER α in osteoblast-like cells (6). Therefore, we performed knockdown of *Gata4* in primary murine calvarial osteoblasts to determine the effects of GATA4 on ER α and ER α target gene regulation.

Gata4 can be successfully knocked down with lentivirally expressed short hairpins directed at *Gata4* mRNA. Two different short hairpins (designated #2 and #4) reduced the level of *Gata4* mRNA by more than two-thirds (Fig. S1A) compared to shGFP-infected cells. For consistency, all figures show shGATA4 #2 and supplemental figures show shGATA4 #4. ShGATA4 lentivirus induced apoptosis in 5.8% of the cells (Fig. S2) compared to 0% of the shGFP infected cells. However, after two weeks of differentiation, both the shGATA4 and shGFP infected cells were at confluence (data not shown). In vehicle (ethanol, EtOH)-treated cells, shGATA4 reduced the basal levels of ER α mRNA and protein (Fig. 1A and 1B). Additionally, shGATA4 affected the E2 response of ER α in these cells. ER α protein and mRNA levels are negatively regulated following E2 treatment in E2-treated shGFP calvarial osteoblasts, as observed previously in other cell types (23, 24). Loss of GATA4 in primary calvarial osteoblasts treated with E2 led to an increase in ER α mRNA and protein compared to vehicle treated cells (Fig. 1A and 1B). Together, this data demonstrate a mis-regulation of ER α signaling in GATA4 ablated cells.

FasL and alkaline phosphatase are two important targets of ER α in osteoblasts (3, 25) (Fig. 1C and 1D). When GATA4 is knocked-down, E2 can no longer induce FasL and alkaline phosphatase mRNA (Fig. 1C and 1D), suggesting that GATA4 protein is necessary for expression of E2-mediated gene targets in primary calvarial osteoblasts. E2 also increases *Gata4* mRNA, and after shGATA4, *Gata4* mRNA is not increased (Fig. S1B). Therefore, GATA4 is necessary to regulate E2 targets in primary osteoblasts, regulating both osteoblast differentiation (*via* alkaline phosphatase) and osteoclast apoptosis (*via* FasL).

ER α -independent roles for GATA4 in bone mineralization

Because GATA4 regulates osteoblast differentiation genes such as alkaline phosphatase (Fig. 1D), we hypothesized that GATA4 may be necessary for proper differentiation and mineralization. To test this, primary calvarial osteoblasts were differentiated for 14 days following *Gata4* knockdown and alkaline phosphatase activity and mineralization were quantitated (Fig. 2A-B). Knockdown of *Gata4* reduced the amount of alkaline phosphatase activity as early as 3 days after differentiation and throughout the time course of differentiation (Fig. 2A). Furthermore, knockdown of *Gata4* led to a greater than 2-fold reduction in mineralization, as compared to control shGFP ($p < 0.001$, Fig. 2B). An independent shRNA targeting of *Gata4* (shGATA4 #4) confirmed a significant reduction in mineralization (Fig. S3). The data suggest an essential role for GATA4 in differentiation and/or mineralization in primary osteoblasts. Interestingly, E2 does not increase mineralization in calvarial osteoblast cultures *in vitro* (data not shown and (26)). In addition, E2 treatment does not increase expression of bone differentiation markers such as bone sialoprotein (BSP (*Spp1*)), osteocalcin (OCN (*Bglap*)), Col1A1, or RUNX2 (Fig. 2C-F). However, knockdown of *Gata4* significantly reduced the levels

of BSP, OCN, Col1A1 and RUNX2 mRNA, corresponding with the differentiation and mineralization assays, indicating E2-independent effects for GATA4 in osteoblast differentiation.

GATA4 is necessary in mesenchymal stem cells for osteoblast differentiation

The expression of *Gata4* during osteoblast differentiation was further investigated in order to determine if GATA4 has stage-specific effects on osteoblast formation and/or activity.

Gata4 mRNA is reduced by five-fold after one day of osteoblast differentiation, and by more than 10-fold after 16 days of differentiation (Fig. 3A). However, mature osteoblasts still express significantly more *Gata4* mRNA than mammary gland cells utilized as the control tissue for the absence of *Gata4* expression. This suggests that GATA4 might influence early stage “precursor” decisions upstream of mature terminally differentiated osteoblast cells. We next verified that *Gata4* is expressed more highly in human mesenchymal stem cells than in differentiated osteoblasts to assess the translational potential of the model. Human fat adventitial cells, fetal lung pericytes, fetal muscle pericytes and fetal bone marrow pericytes (the native ancestors of mesenchymal stem cells) were left undifferentiated or differentiated into osteoblasts (18). *Gata4* mRNA was significantly lower in terminally differentiated osteoblasts than in undifferentiated cells (Fig. 3B-C). There is an inverse relationship between osteoblast differentiation markers (*Col1A1* and alkaline phosphatase mRNA) and *Gata4* mRNA expression (Fig. 3C). Therefore, *Gata4* is expressed more highly in mesenchymal stem-like cells than in differentiated, mineralizing osteoblasts.

The observation that *Gata4* expression is higher prior to differentiation raises the possibility that GATA4 plays a critical role at the phase of osteoblast commitment. To test this, *Gata4* was either knocked down “early” (in undifferentiated pericytes), or “late” in

differentiating osteoblasts (1 week after initiating differentiation, Fig. 3D). The corresponding alkaline phosphatase levels, indicating the amount of differentiation of wildtype pericytes, is demonstrated in Fig. 3E. The cells were selected with puromycin to ensure efficient lentiviral infection, and then induced to mineralize. Cells infected with control lentivirus showed efficient mineralization, as visualized by alizarin red staining, whether infected early or late (Fig. 3F-G). In contrast, cells infected with shGATA4 before differentiation exhibited a significant reduction in the amount of mineralization compared to control infected cells. However, osteoblasts infected with shGATA4 “late” in differentiation showed no loss of mineralization, suggesting that GATA4 plays a role in either osteoblast progenitors or early in differentiation. Taken together, the above results suggest that *in vitro* in both human and mouse osteoblasts, GATA4 elicits its strongest effects on “early” progenitor and/or precursor cells.

GATA4 expression *in vivo*

GATA4 has been described in detail in the developing heart, intestines and several other tissues (5). Indeed, analysis of *Gata4* mRNA in E18.5 embryos reveal highest expression in these tissues (Fig. S4, and data not shown). However, based on the *in vitro* data described above, *Gata4* expression was analyzed in the developing skull and hind limb. *Gata4* mRNA is abundant in the ventricular zone of the developing brain in E13.5, E14.5, and E16.5 mice, before ossification of the skull utilizing highly sensitive *in situ* RNA hybridization (RNAscope technology) (Fig. 4). At E18.5 *Gata4* mRNA has decreased significantly in the brain and is present near and/or within the ossifying skull. *Gata4* mRNA is also present in proliferating chondrocytes in E13.5, E14.5, and E16.5 hind limbs, which has not been previously reported. At E18.5 *Gata4* mRNA is expressed at the ossification front of the growth plate and periosteum.

While the precise identity of the *Gata4*-positive cells in these locations is as yet unclear, the data are consistent with a direct role for GATA4 at various times and stages in normal embryonic bone development.

GATA4 is necessary for bone development in embryogenesis *in vivo*

To determine the role of GATA4 *in vivo*, *Gata4* floxed (GATA4^{Fl/Fl}) mice were crossed with mice expressing Cre recombinase under control of a 2.3 kb fragment of the rat *Colla1* gene (27). Previous studies have shown that Cre-mediated deletion of exons 3, 4 and 5 of the *Gata4* gene converts the floxed allele into a recombined allele no longer capable of encoding a functional GATA4 protein (28). *Gata4* recombination efficiency in osteoblasts was determined *in vivo* by qPCR of mRNA, using primers that cannot detect recombined *Gata4* mRNA. *Gata4* mRNA was reduced by over 75% in differentiated calvarial organs of GATA4^{Fl/Fl}Cre⁺ mice (conditional knockout, cKO) (Fig. 5A). The actual recombination of *Gata4* in osteoblasts is probably significantly higher, since the calvarial organs consist of other cell types, such as stromal cells.

Gata4 cKO mice were not present at Mendelian ratios after embryonic day 16.5 (Fig. 5B). Mutants were morphologically indistinguishable from control littermates' appearance at any age, including E18.5 (Fig. 5C) or P1 (Fig. 5D), or by weight (Fig. 5E). The mice did not exhibit any obvious heart defects, as might be predicted from leaky *Colla1*-Cre expression. Whole hearts and H&E stained sections of left and right ventricles from P0 animals revealed normal septal structure, and a well-developed myocardium (Fig. S5 and data not shown). In addition, heart weight-to-body weight ratios were the same in wildtype and cKO littermate animals (Fig. S5).

Thus, while we cannot at present determine the cause of death of cKO mice, we have no evidence that heart defects are responsible.

Skeletal preparations of E18.5 mice revealed no gross abnormalities (Fig. 6A-B). However, closer examination revealed multiple skull defects (Fig. 6C-J and S5), including smaller zygomatic bones (Fig. 6C-D, red arrows), a more mineralized occipital one (Fig. 6D and 6F) and decreased thickness of cranial bone (Fig. 6G-H, red lines). Furthermore, Von Kossa staining and microCT analysis demonstrate a reduced mineralization of the skull in the cKO mice (Fig. 6I-J, Fig. S5F-G).

In addition to cranial defects, we also observed vertebral and appendicular defects. Individual lumbar vertebrae were larger and misshapen (Fig. 6K-L), leading to an overall increase in spine length of cKO mice (Fig. 6M). The vertebral bodies of the cKO mice have a decrease in hypertrophic chondrocytes (Fig 6N-O) and irregular mineralization (Fig. 6P-Q). There is a lack of fusion of the distal tibia and fibula in cKO mice as observed by both skeletal preparations (Fig. 6R-S) and microCT (Fig. 6T-U). There is also a decrease in hypertrophic chondrocytes and mineralization in the femurs of cKO mice (Fig. 6V-Y).

To quantify the mineralization effects in Figure 6, microCT analysis was performed on the femurs and vertebrae of WT and cKO newborn mice, which demonstrated that cKO mice had reduced trabecular bone (Fig. 7A-L). The trabecular bone volume (BV/TV), trabecular number (Tb.N.) and trabecular thickness (Tb.Th.) were significantly reduced in cKO femurs compared to wildtype littermates (Fig. 7C-E). Trabecular spacing was increased in the cKO mice, although it did not reach statistical significance (Tb.Sp., Fig. 7F). Cortical bone parameters (total cross sectional area, cortical bone area, cortical bone area fraction or cortical thickness) were not statistically different between wildtype and cKO littermate animals (Fig. S7). MicroCT analysis

of lumbar vertebrae (L5) confirmed the irregular shape of cKO vertebrae (Fig. 7G-H) and revealed a significant reduction in the trabecular bone volume (BV/TV) and trabecular thickness (Tb.Th.) (Fig. 7I, K). Trabecular number was decreased in the cKO mice, although it did not reach statistical significance (Fig. 7J). Together, the skeletal preparations, histology and microCT analysis indicate defects in intramembranous and endochondral bone formation.

GATA4 regulates TGF β and BMP pathways

To investigate mechanisms underlying the role of GATA4 in osteoblasts, we examined global gene expression changes following *Gata4* knockdown in primary murine calvarial osteoblasts (Fig. S1A). Osteoblasts were differentiated for two weeks and RNA was analyzed by genome-wide expression profiling (MouseRef-8 v2.0 Expression BeadChips from Illumina). Loss of *Gata4* resulted in a decrease in expression of 1610 genes by 2 fold or greater and an increase in expression of 2399 genes by 2 fold or greater. Gene ontology analysis by DAVID software revealed several categories of differentially expressed genes. (20). The most down-regulated genes in shGATA4 cells encode extracellular matrix proteins (p value = 3.2×10^{-16}) and drive bone development (p value = 3.6×10^{-8}), thereby confirming a role for GATA4 in osteoblasts.

Loss of *Gata4* resulted in mis-regulation of a large number of TGF β superfamily pathway genes (Fig. S8-9). Key upregulated genes in this pathway were TGF β 2 and β 3, while both BMP pathway genes were down-regulated. Analysis of mRNA expression in shGATA4 infected calvarial osteoblasts confirmed decreased expression of two BMP ligands (*Bmp4* and *Bmp6*), a receptor (*Bmpr1A*) and a downstream effector SMAD (*Smad5*) (Fig. 8A). Because of the down-regulation of BMP pathway genes, BMP pathway activation (pSMAD 1/5/8 as a

downstream target for BMP mediated signaling (7)) was analyzed with a phosphorylated SMAD (pSMAD) 1/5/8 antibody by immunoblotting. Indeed, shGATA4 cells had a reduced amount of pSMAD1/5/8 illustrating that BMP-mediated signal transduction has been severely attenuated. E2 had no effect on the regulation of pSMAD1/5/8 (Fig. 8B). Conversely, *TGFβ2* and -3 were significantly upregulated in shGATA4 cells (Fig. 8C). Consistent with the upregulation of the ligands, pSMAD2/3 were increased in shGATA4 cells (Fig. 8D), demonstrating activation of the TGFβ pathway. E2 induced phosphorylation of SMAD2 and SMAD3 in shGFP cells, but not in shGATA4 cells.

To investigate the possibility of an *in vivo* role for GATA4 regulation of the TGFβ and BMP pathways, the femurs of control (Fl/Fl) and cKO mice were analyzed by IHC for pSMAD1/5/8 and pSMAD 2/3. cKO bones exhibited a decrease in the amount of pSMAD1/5/8 in the trabecular bone (Fig. 8E). Conversely, pSMAD2/3 was increased in the trabecular bone from cKO mice, as compared to wildtype bone (Fig. 8F). Our findings thus indicate that GATA4 is critical for the maintenance of osteoblast function, and raise the possibility that this is in part through regulating the balance between BMP and TGFβ pathway activity.

Discussion

We previously identified GATA4 as a pioneer factor for estrogen receptor in osteoblasts (6). Here we demonstrate that GATA4 is important for the regulation of several E2 targets in osteoblasts, but that GATA4 also has E2-independent functions.

Knockout of GATA4 specifically in osteoblasts leads to embryonic and/or perinatal lethality. Because cKO mice were physically indistinguishable from their wild type littermates at all stages of development, the cause of death remains under investigation. The *Col1A1* 2.3 kb

promoter is expressed as early as E14.5, when analyzed by crossing the Col1A1-cre mouse with the ROSA26 reporter mouse (R26R) (29). The Col1A1-cre;R26R mouse demonstrates a high level of bone specific expression of Cre recombinase (29), as does mRNA from many tissues (27). Furthermore, the *Colla1* (2.3kb)-GFP transgene was not detected in the heart or intestines (30), which are two tissues with high *Gata4* expression. However, it is possible that expression could occur at a different age, or at a level below the detection of a Northern blot. The *Colla1* 2.3kb promoter might be active in some growth plate chondrocytes (27), and the extent of this effect needs to be investigated.

Although rare, Col1A1-Cre-mediated deletion has led to embryonic death (31). Crossing the *Gata4* Fl/Fl mouse with a mouse that expresses Cre later in the osteoblast lineage, such as the osteocalcin promoter (32), could reveal a role for GATA4 in adult mice and potential roles in estrogen deficient osteoporosis. Similarly, crossing the *Gata4* Fl/Fl mouse with a mouse that expresses Cre earlier in development could further define the “early” role for GATA4 suggested by the above data.

Total *Gata4* knockout mice exhibit embryonic lethality from E7.5-10.5 (15, 16), which is much earlier than for the osteoblast-specific knockout described here. Interestingly, specific knockouts of GATA4 in the heart with Cre-recombinase controlled by the β -myosin heavy promoter (β -MHC) or α -MHC promoter were generated at predicted Mendelian ratios and mice were viable up to 16 weeks of age (33). *Gata4* deletion from the developing intestinal epithelium (34), pancreas (35), testicular somatic cells (36) or granulosa cells (37) also resulted in mice born at the expected Mendelian ratios, but with tissue-specific effects.

These results demonstrate that GATA4 is essential for bone mineralization both *in vitro* and *in vivo*. MicroCT analysis shows a decrease in trabecular bone measurements in the cKO

mice. Furthermore, the skeletal preparations and microCT show defects, including those in the skull, legs and spine in these mice.

Mouse models for TGF β and BMP signaling in bone have been generated (reviewed in (8)). TGF β 2 overexpression leads to lower bone mineral density, in agreement with the *Gata4* cKO mice (38). TGF β 2-null mice have a lack of occipital bone formation (39), corresponding with the increase in occipital bone formation in *Gata4* cKO mice and the suppression of TGF β 2 by GATA4. A limb mesenchyme double knockout of both BMP2 and BMP4 (driven by the *Prx1-cre*) leads to severe bone malformation. Interestingly, the tibia and fibula in the BMP4 knockout are not fused (40), as is seen in the *Gata4* cKO. Furthermore, an osteoblast-specific knockout of SMAD1 leads to an osteopenic phenotype (41). Together, these mouse models are in agreement with a role for GATA4 in regulating TGF β and BMP signaling. Genetic epistasis experiments will be required to test the physiological relevance of the altered BMP and TGF β pathway activity we have observed in *Gata4* cKO mice.

GATA4 is currently being used for gene therapy in the heart in combination with heart specific transcription factors (42). MSCs show potential for the treatment of osteoporosis, and their optimization could be realized by estrogens, SERMs and/or GATA4 to better induce osteoblast formation. In conclusion, GATA4 in osteoblasts plays a key role in the regulation of TGF β and BMP pathways to control bone differentiation and/or mineralization.

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Figure Legends

Fig 1. GATA4 regulates ER α and E2 target genes. (A) Calvarial osteoblasts were infected with lentivirus expressing either shGFP or shGATA4. Cells were then differentiated for two weeks and RNA was obtained. qPCR was performed for ER α and normalized to actin mRNA. (B) Calvarial osteoblasts were infected with lentivirus expressing either shGFP or shGATA4. Cells were then differentiated for two weeks and then treated with 10 nM E2 for 24 hours. Protein was obtained and immunoblots for ER α , GATA4 and actin were performed. (C) RNA was obtained as in (A) and qPCR was performed with primers to FasL and normalized to actin mRNA. (D) RNA was obtained as in (A) and qPCR was performed with primers to Alkaline Phosphatase and normalized to actin mRNA. *=p value < 0.05; **= p value < .001.

Fig. 2. GATA4 regulates differentiation and mineralization *in vitro*. (A) Primary calvarial osteoblasts were infected with lentivirus expressing an shRNA directed to GFP (shGFP) or to *Gata4* (shGATA4). The following day the cells were placed in mineralization media and allowed to differentiate for 3, 7 or 14 days. Cells were then fixed and assayed for alkaline phosphatase. (B) Primary calvarial osteoblasts were infected with lentivirus expressing an shRNA directed to GFP (shGFP) or to *Gata4* (shGATA4). The following day the cells were placed in mineralization media and allowed to differentiate for two weeks. Cells were then fixed and stained using alizarin red. Alizarin red was eluted and the mineral content was measured at an OD of 570. Silencing was performed in three wells and the average OD is displayed. (C-F) Calvarial osteoblasts were infected with lentivirus expressing either shGFP or shGATA4. Cells were then

differentiated for two weeks and RNA was obtained. qPCR was performed for the indicated genes and normalized to actin mRNA. *= p value < .05, **= p value < .001.

Fig. 3. GATA4 regulates bone mineralization early in the differentiation process. (A) Bone marrow stromal cells were differentiated for 0, 1, 2 or 16 days. RNA was obtained and qPCR was performed for *Gata4* and normalized to actin mRNA. Mammary gland (MG) RNA was also obtained for comparison. (B) Fat adventitial cells, fetal lung pericytes and fetal muscle pericytes were left undifferentiated or differentiated to mineralizing osteoblasts. RNA was obtained and qPCR was performed for *Gata4* and normalized to actin mRNA. (C) Human fetal bone marrow CD146+ cells (pericytes) were cultured in osteoblast differentiation media for 3, 7, 10 and 14 days. RNA was obtained and qPCR was performed for *Gata4*, *Col1A1* and Alkaline phosphatase cDNA and normalized to actin mRNA. (D) Design of experiment for parts E-G. (E) Human fetal bone marrow CD146+ cells (pericytes) were cultured in osteoblast differentiation media for 3, 7, 10 and 14 days. At the indicated times cells were fixed and assayed for alkaline phosphatase. (F) Pericytes were infected with lentivirus expressing shC or shGATA4 either “early” at day 0 or “late” at day 7. All cells were placed in mineralization media at day 1 and allowed to differentiate for two weeks. Cells were then fixed and stained using alizarin red. (G) Alizarin red from part (F) was eluted and the mineral content was measured at an OD of 570. Silencing was performed in three wells and the average OD is displayed. *=p value < 0.05

Fig. 4. *In situ* analysis of *Gata4* expression. Sagittal sections of E13.5, E14.5, E16.5 and E18.5 wildtype FVB embryos were probed for *Gata4* expression (brown) and counterstained with hematoxylin (blue). Arrows indicate *Gata4* expression in bone.

Fig. 5. GATA4 cKO mice are not born at expected Mendelian ratios. (A) The recombination efficiency of Cre-mediated excision was measured by qPCR from cDNA from differentiated WT and cKO calvarial organ cultures. (B) Tail DNA was obtained from mice at E14.5, E16.5, E18.5, P0, P1 and P21. Genotyping was performed for the presence of Floxed *Gata4* and for Cre recombinase. The percent of mice that were GATA4 Fl/Fl/Cre+ (% cKO) is graphed. *=p value < 0.05; **= p value < .001; ***= p value < .0001. (C) Photograph of representative WT and cKO mice at E18.5. (D) Photograph of representative WT and cKO mice at P1. (E) Weight, in grams, of newborn WT and cKO mice.

Fig. 6. GATA4 cKO mice have skeletal defects. (A-D) Skeletal preparations of E18.5 WT (A, C) and cKO (B, D) mice. (A,B) Whole body. (C,D) Superior view of skull. Zy=zygomatic bone. Red arrow indicates decreased zygomatic bone size in cKO mice. Yellow arrows highlight a suture defect in the cKO skull. (E,F) MicroCT images of WT (E) and cKO (F) skulls. Arrow points to occipital bone. (G,H) H&E staining of sagittal sections of WT (G) and cKO (H) heads. Red line indicates thickness of skull bone. (I,J) Von Kossa staining of sagittal sections of WT (I) and cKO (J) heads. (K,L) Skeletal preparations of spine from WT (K) and cKO (L). (M) Total spine length of n=7 mice. *=p value < 0.05. (N,O) H&E staining of sagittal sections of WT (N) and cKO (O) vertebrae. Von Kossa staining of sagittal sections of WT (P) and cKO (Q) vertebrae. (R,S) Skeletal preparations of tibia and fibula from WT (R) and cKO (S). Green arrow indicates lack of fusion of tibia and fibula. (T,U) MicroCT images of WT (T) and cKO (U) tibia and fibula at their closest distance. (V, W) H&E staining of sagittal sections of WT (V) and cKO (W) femurs. h.ch.=hypertrophic chondrocytes. Von Kossa staining of sagittal sections of WT (X) and cKO (Y) femurs.

Fig. 7. Comparison of trabecular bone structure in P0 WT and cKO mice assessed by microCT.

(A, B) Representative microCT images of WT and cKO femurs. (C) Bone volume/total volume (BV/TV), (D) trabecular number (Tb.N.), (E) and trabecular thickness (Tb.Th.) were decreased in femurs of cKO mice. (F) There was no statistical increase in trabecular spacing (Tb.Sp.) in cKO mice. (G, H) Representative microCT images of WT and cKO L5 vertebrae. Arrows indicate defects in shape. (I) BV/TV, (J) Tb.N., (K) Tb.Th., and (L) Tb.Sp. of L5 vertebrae in WT and cKO L5 vertebrae. N=9; *=p value < 0.05

Fig. 8. GATA4 regulates the TGF β and BMP pathways in osteoblasts. (A) Calvarial osteoblasts were infected with lentivirus expressing either shGFP or shGATA4. Cells were then differentiated for two weeks and RNA was obtained. qPCR was performed for the indicated genes and normalized to actin mRNA. (B) Calvarial osteoblasts were infected with lentivirus expressing either shGFP or shGATA4. Cells were then differentiated for two weeks and then treated with E2 for 24 hours. Protein was obtained and immunoblots for pSMAD1/5/8, SMAD5, GATA4 and actin were performed. (C) RNA was obtained as in (A) and qPCR was performed for the indicated genes and normalized to actin mRNA. (D) Protein was obtained as in (B) and immunoblots for pSMAD3, pSMAD2, GATA4 and actin were performed. (E) IHC with an antibody to pSMAD1/5/8 was performed on femurs from P0 wildtype (Fl/Fl) and cKO (Fl/Fl; Cre+) mice. H.Ch.= hypertrophic chondrocytes. Tr= trabecular bone. (F) Immunofluorescence with an antibody to pSMAD1/5/8 was performed on femurs from P0 wildtype (Fl/Fl) and cKO (Fl/Fl; Cre+) mice. h.ch.= hypertrophic chondrocytes. Tr= trabecular bone. *=p value < 0.05.

**= p value < .001.

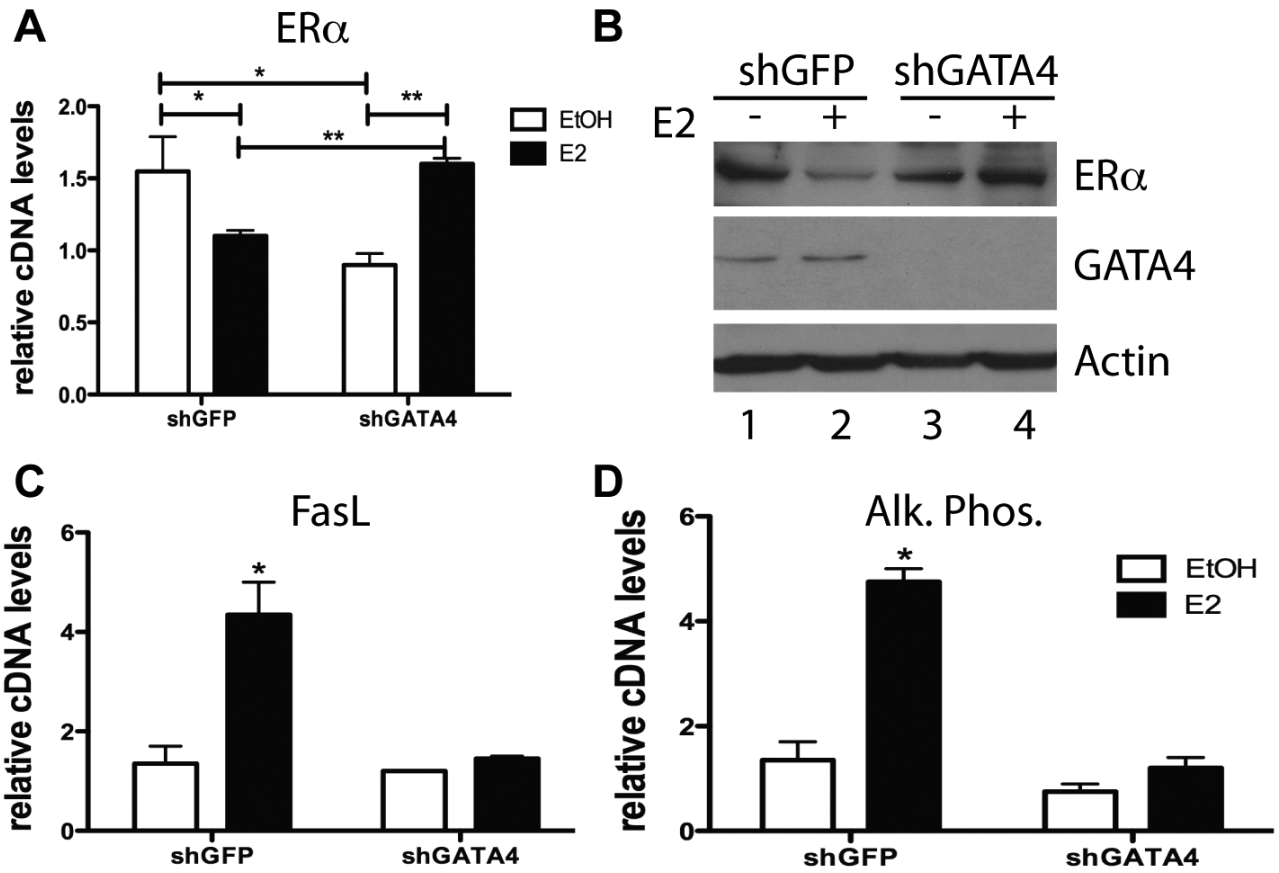


Fig 1

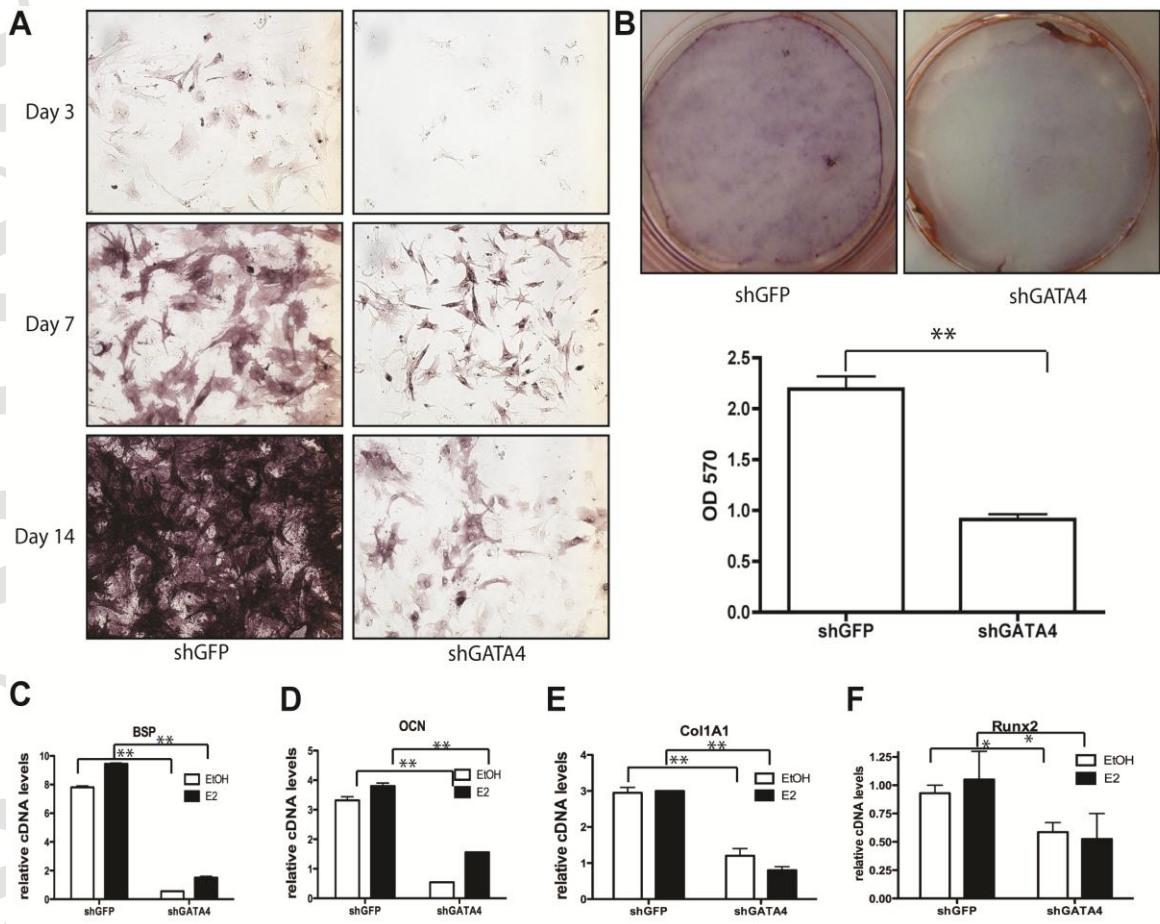


Fig 2

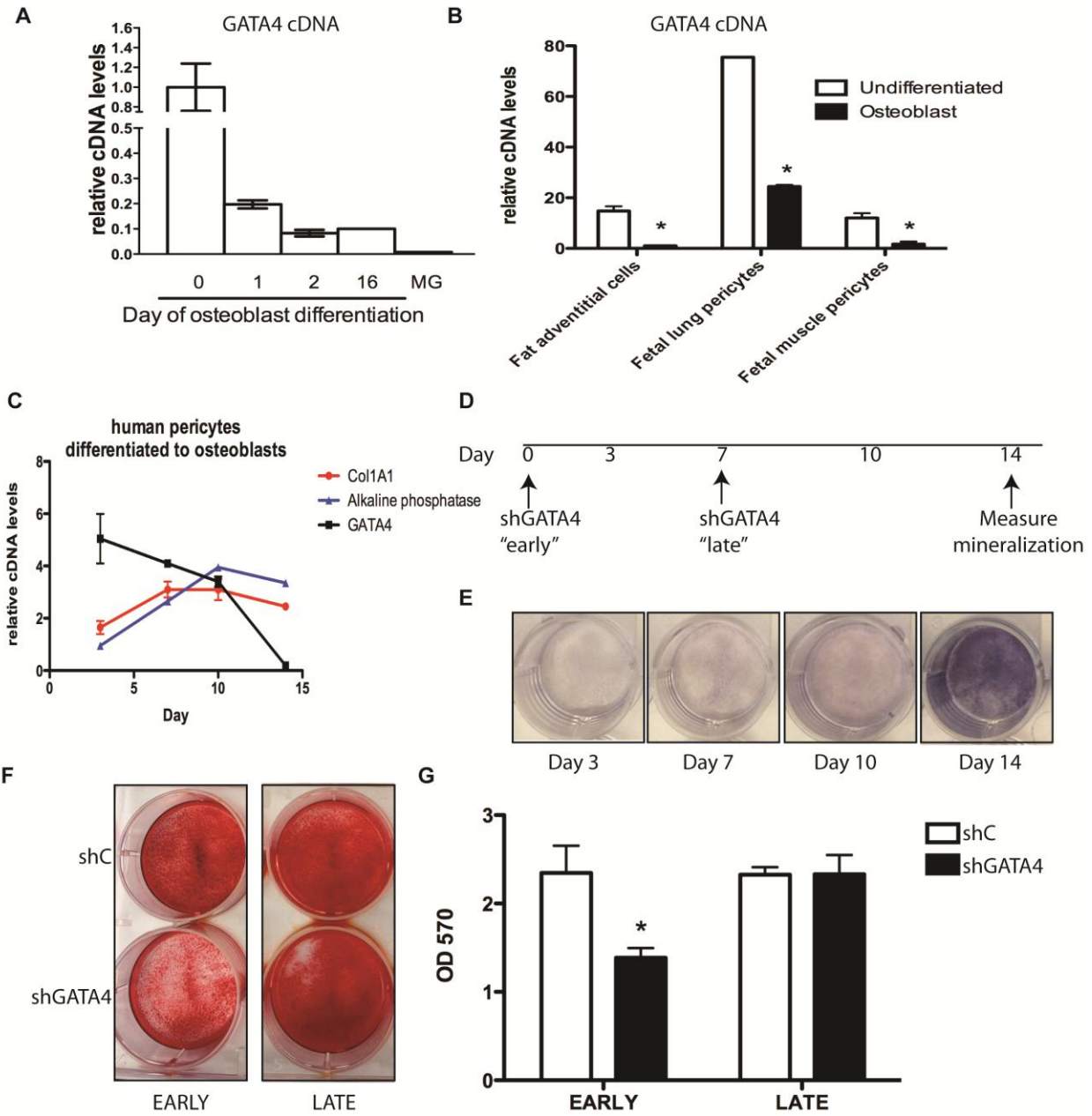


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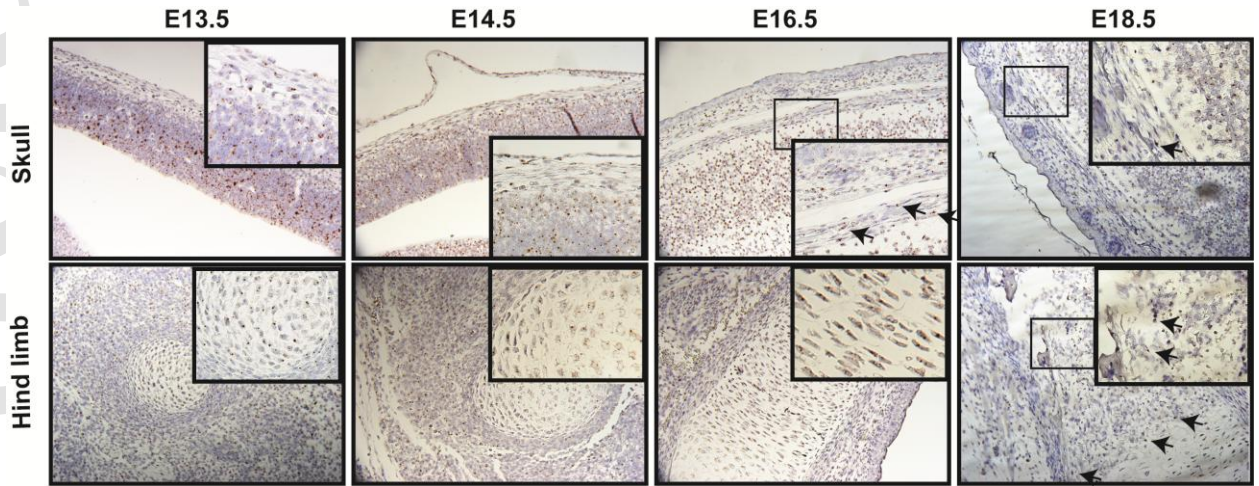


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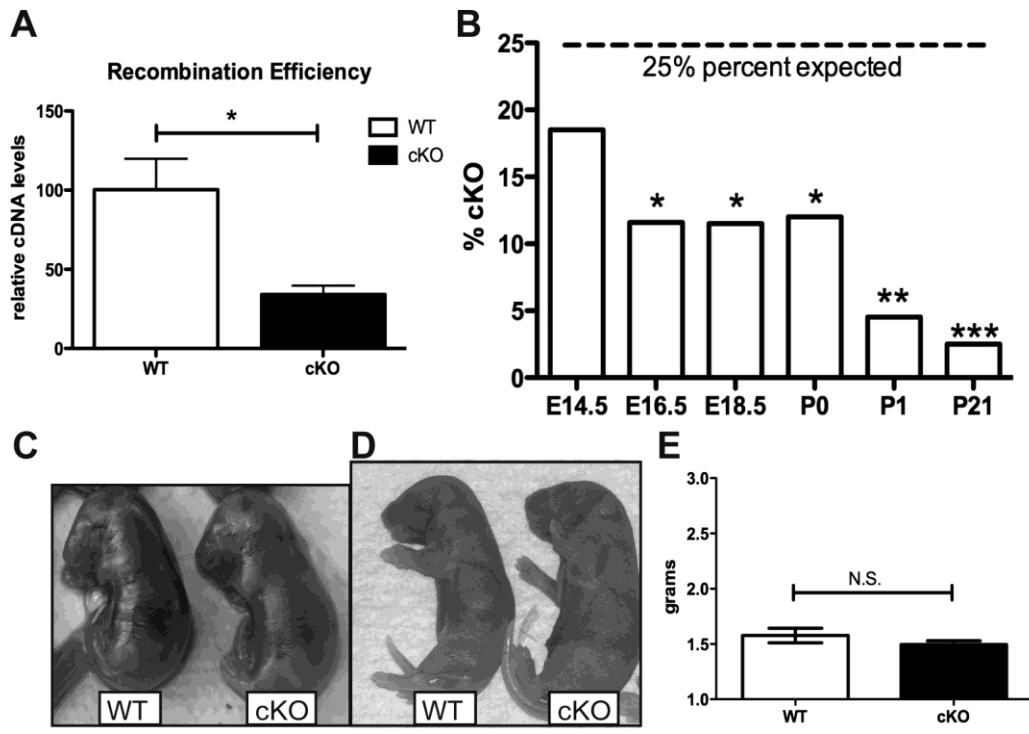


Fig 5

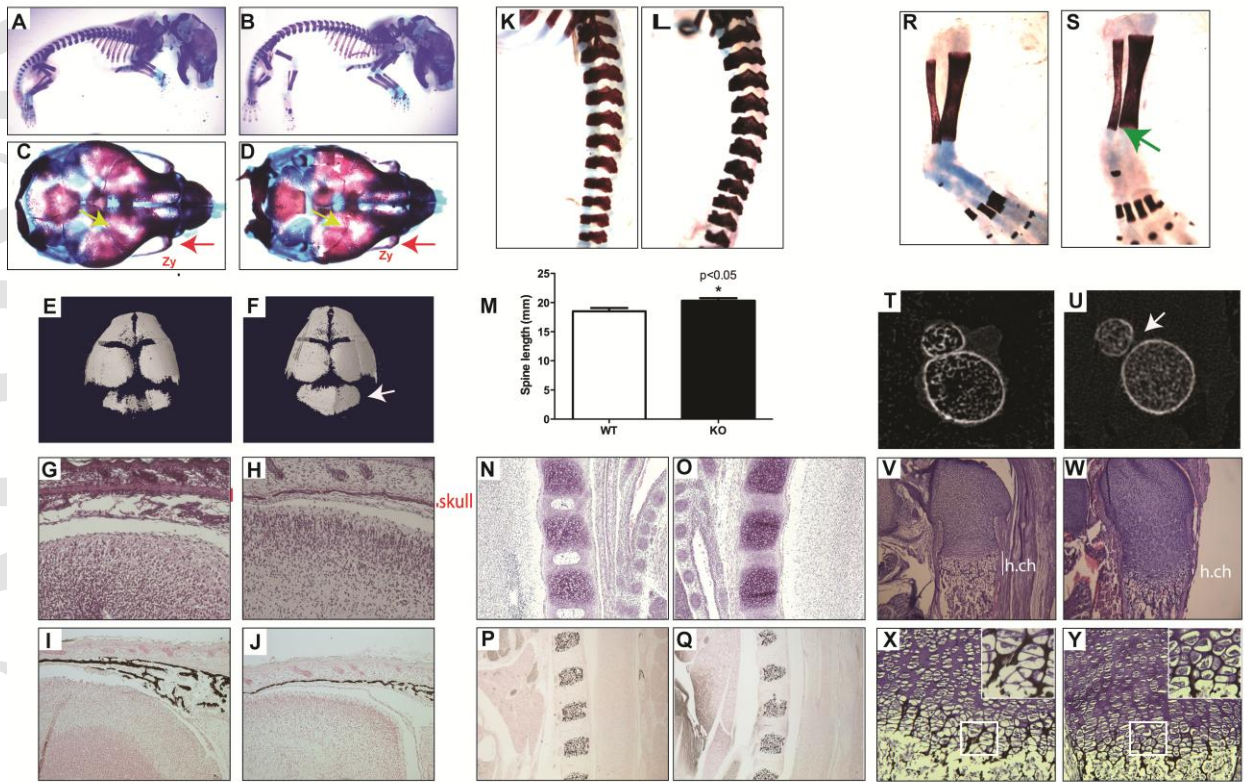


Fig 6

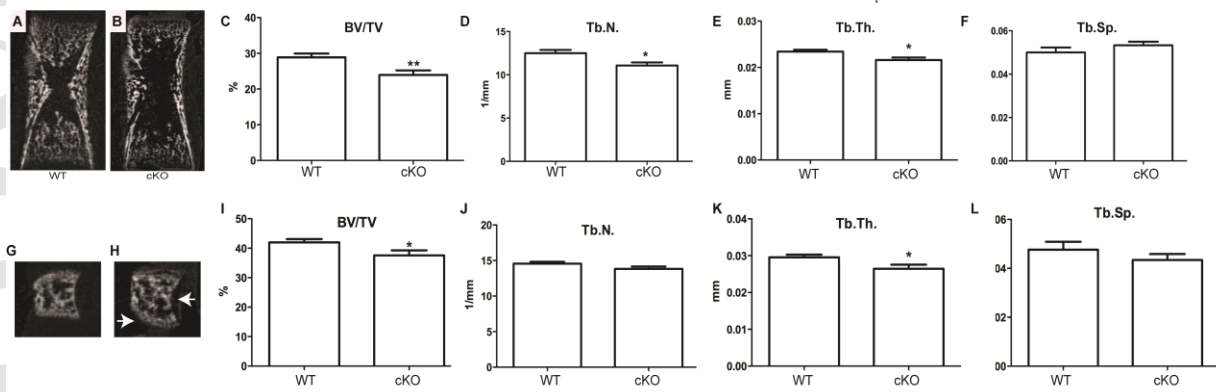


Fig 7

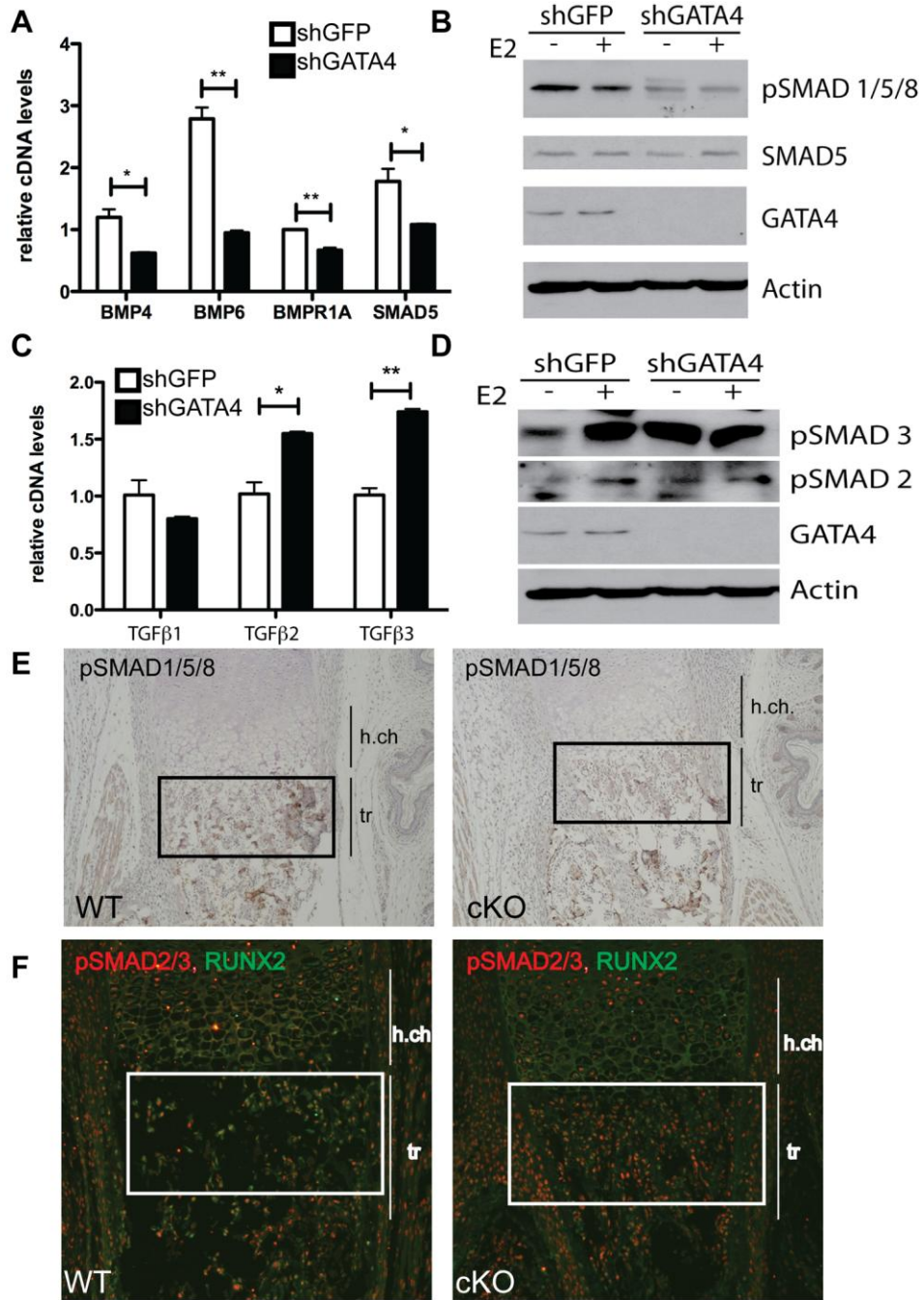


Fig 8