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Reduced chondrocyte proliferation, earlier cell cycle exit and increased apoptosis in neuronal nitric oxide synthase-deficient mice

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SUMMARY

Objective: Nitric oxide (NO) has been implicated in the local regulation of bone metabolism. However, the contribution made by specific nitric oxide synthase (NOS) enzymes to skeletal development is unclear. The objective of this study was to examine the effects of inactivation of neuronal nitric oxide synthase (*nNOS*) on cartilage development in mice.
Design: Mice carrying a null mutation in the *nNOS* gene were used to address our objectives. Histological staining, immunohistochemistry and *in situ* analyses were employed along with real-time reverse transcriptase - polymerase chain reaction (RT-PCR).
Results: nNOS-null mice show transient growth retardation and shorter long bones. nNOS-deficient growth plates show a reduction in replicating cells. Reduced chondrocyte numbers may in part be due to slower cell cycle progression and premature cell cycle exit caused by decreased cyclin D1 and increased p57 expression in mutants. In addition, apoptosis was increased as shown by increased cleaved-caspase 3 staining in hypertrophic chondrocytes in mutants. Real-time PCR demonstrated that expression of early chondrocyte markers such as Sox genes was reduced in mutant mice, while expression of prehypertrophic markers such as RORα was increased. Histological sections also demonstrated thinner cortical bone, fewer trabeculae and reduced mineralization in mutant mice.
Conclusions: These data identify an important role of nNOS in chondrocyte proliferation and endochondral bone growth and demonstrate that nNOS coordinates cell cycle exit and chondrocyte differentiation in cartilage development.

Introduction

NO is a small signaling molecule with important regulatory effects in many tissues. It can function as an intracellular messenger, an autacoid, a paracrine substance, or a neurotransmitter[1]. The versatile functions of NO stem from the chemical properties of the compound. NO is a gaseous uncharged free radical with an unshared electron that can react with many molecules and proteins to regulate biological processes[2]. Because it is uncharged, it can freely diffuse to the surrounding cells, making it ideal as a signaling molecule[3]. In most processes, NO activates soluble guanylyl cyclase (GC), resulting in increased levels of the intracellular secondary messenger cyclic guanosine monophosphate (cGMP)[1,2], but other modes of signaling such as protein nitrosylation also contribute to the biological effects of NO[2,4]. Different cells and tissues have different abilities to produce NO. In general, low levels of NO are produced from constitutive NOSs, (endothelial NOS/eNOS and neuronal NOS/nNOS) and high levels of NO stem from inducible nitric oxide synthase (iNOS). The ultimately effects of NO are determined by its concentration, its source, and the availability of molecules in the microenvironment that it can react with. Over years NO has been known to regulate bone cell metabolism, bone remodeling and chondrocyte physiology in osteoarthritis (OA)[3,5,6], but the contributions made by specific NOS enzymes to bone growth and development are unclear[7,8]. In light of the prevalent connections between chondrocyte differentiation and the pathogenesis of OA[9], and the well-known roles of NO in OA, the elucidation of the roles of individual NOS genes in cartilage development is of great importance.

Formation of the skeleton is achieved through two independent mechanisms: intramembranous and endochondral ossification[10]. Endochondral bone formation is a precisely regulated process and

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responsible for the formation of most bones in the adult skeleton. It involves the steps of chondrogenesis, chondrocyte proliferation, differentiation, and hypertrophy and eventually replacement of cartilage tissue by bone tissue and bone marrow[3,10–13]. During postnatal development, proliferation and hypertrophy occur in a controlled fashion in the cartilage growth plate that determines the final length of the adult bone. Within the growth plate, chondrocytes are organized in distinguished zones (resting, proliferating, prehypertrophic and hypertrophic zones) based on their morphologic shapes and gene expression patterns[11]. If any steps during this process are not properly regulated, skeletal diseases will result, such as different types of chondrodysplasias[14]. In addition, links between skeletal developmental and the pathogenesis of OA are becoming evident. For example, both disturbances of normal endochondral ossification and ectopic initiation of chondrocyte hypertrophy in articular cartilage have been implicated in OA[15,16]. Thus, investigations into the mechanisms controlling normal cartilage development are essential for our understanding of OA pathogenesis.

We recently described cartilage development in *eNOS*- and in *iNOS*-deficient mice that both display reduced bone growth[17,18]. This effect appears to be due to a chondrocyte-autonomous role of eNOS in chondrocyte proliferation. However, nNOS mRNA levels were upregulated in *eNOS*-null chondrocytes, suggesting that nNOS partially compensates for the loss of eNOS. *nNOS* knockout (KO) mice have been found to be viable, have enlarged stomachs due to pyloric muscle hypertrophy, exhibit insulin resistance and resistance to neural damage as the result of stroke induced by middle cerebral artery ligation[19]. nNOS expression has been detected during skeletal development and fracture healing, and previous studies showed reduced bone remodeling with a significant reduction in osteoblast and osteoclast numbers in *nNOS*-deficient mice[19]. However, the exact mechanisms and functions of nNOS in earlier stages of skeletal development have not been described. In this study, we investigated the effects of nNOS deficiency on cartilage development and endochondral bone formation.

Materials and methods

Antibodies and reagents

All reagent materials and general chemicals were obtained from Invitrogen, Sigma or VWR unless otherwise stated. The following antibodies were employed in this study: Kip2/p57 #sc8298 (rabbit antibody against human protein), RORα #sc28612 (rabbit antibody against human protein), activating transcription factor 3 (ATF3) #sc188 (rabbit antibody against human protein), Sox9 #sc-17340 (goat antibody against human protein), c-Fos #sc-52 (rabbit antibody against human protein), Goat-anti-mouse #sc2005, Goat-anti-rabbit #sc2004 (all from Santa Cruz Biotechnology); Cyclin D1 (SP4) #9104-S1 (rabbit antibody against human, mouse and rat proteins; NeoMarkers Inc.); proliferating cell nuclear antigen (PCNA) #2586 (mouse antibody against human, mouse and rat proteins; Cell Signaling Inc.).

Mouse breeding and genotyping

Mice with a deletion of the *nNOS* gene (Stock #2986) and control mice in the C57/BL6 background were obtained from Jackson Laboratory (Bar Harbor, ME), and exposed to a 12-h light-dark cycle and fed tap water and regular chow at libitum[19]. All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee. For PCR genotyping, tail snips were used to prepare DNA for PCR analysis. PCR genotyping was performed by simultaneous amplification of the wild type and null nNOS alleles as described[9,19]. PCR fragments were analyzed by agarose gel electrophoresis.

Histology and immunohistochemistry

After dissection of mice, bones were rinsed in PBS, fixed in 4% paraformaldehyde (PFA) overnight, placed in 10% formalin solution and sent for embedding and sectioning into 4 μm sections at the Molecular Pathology Core Facility at the Robarts Research Institute (London, Ontario, Canada). Following sectioning, bones were stained with hematoxylin and eosin or safranin O/fast green using standard protocols[20,21] or used for immunohistochemistry as described below.

For immunohistochemistry, sections unstained prior to use were dewaxed and incubated in 3% H_2O_2 for 15 min at room temperature, followed by boiling for 20 min in 10 mM sodium citrate (pH 6.0) and blocking with 5% goat serum at room temperature for 30 min, as described[17,21–23]. Sections were incubated with primary antibody overnight at 4°C and secondary antibodies according to the manufacturers' recommendations. After washing, sections were incubated for 1–10 min with DAB (3,3′-diaminobenzidine tetrahydrachloride) substrate solution (Dako North America, Inc.), washed and mounted. All images were taken at room temperature with a Retiga EX camera connected to a Leica DMRA2 microscope using OpenLab 4.0.4 software. For cell counts in sections, all cells and cells positive for staining with primary antibody were counted from three different areas of one section. Sections from at least three mice per genotype, and at least three sections from every mouse, were analyzed, and averages and standard deviation from all counts/genotype are shown.

RNA isolation and real-time RT-PCR

Total RNA was isolated from epiphyseal cartilage of long bone from newborn mice using TRIzol (Invitrogen), according to the manufacturer's recommendations. TaqMan real-time PCR was performed as described[21,24] with primers and probe sets from Applied Biosystems (*Sox5* Mm00488381_m1; *Sox6* Mm00488393_m1; *Sox9* Mm00448840_m1; *P57* Mm00438170_m1; *MMP13* Mm00439495_m1; *Igf1* Mm00439560_m1; *Gapdh* Mm99999915_g1). Data were normalized to *Gapdh* mRNA levels and represent averages and SEM from direct comparison of mutant and control littermates from three different crosses.

Statistical analysis

All experiments were performed with at least three independent litters (each litter representing one independent experiment). Statistical significance of experiments was determined by a one-way analyses of variance (ANOVA) with Bonferroni post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA. Data presented in the graph show mean and 95% confidence intervals.

Results

Inactivation of nNOS gene results in reduced bone length

We used *nNOS*-deficient mice to address the role of this gene in cartilage development *in vivo*. KO mice were viable and fertile, and the general appearance of stature and gait was unremarkable, but male KO mice appeared more aggressive. Body weight and length were not significantly changed in $nNOS^{-/-}$ mice at birth. Growth retardation developed in surviving $nNOS^{-/-}$ mice in the postnatal period at day 21–42, as shown by reduced body weight and length [Fig. 1(A)]. Body weight showed significant reduction at postnatal

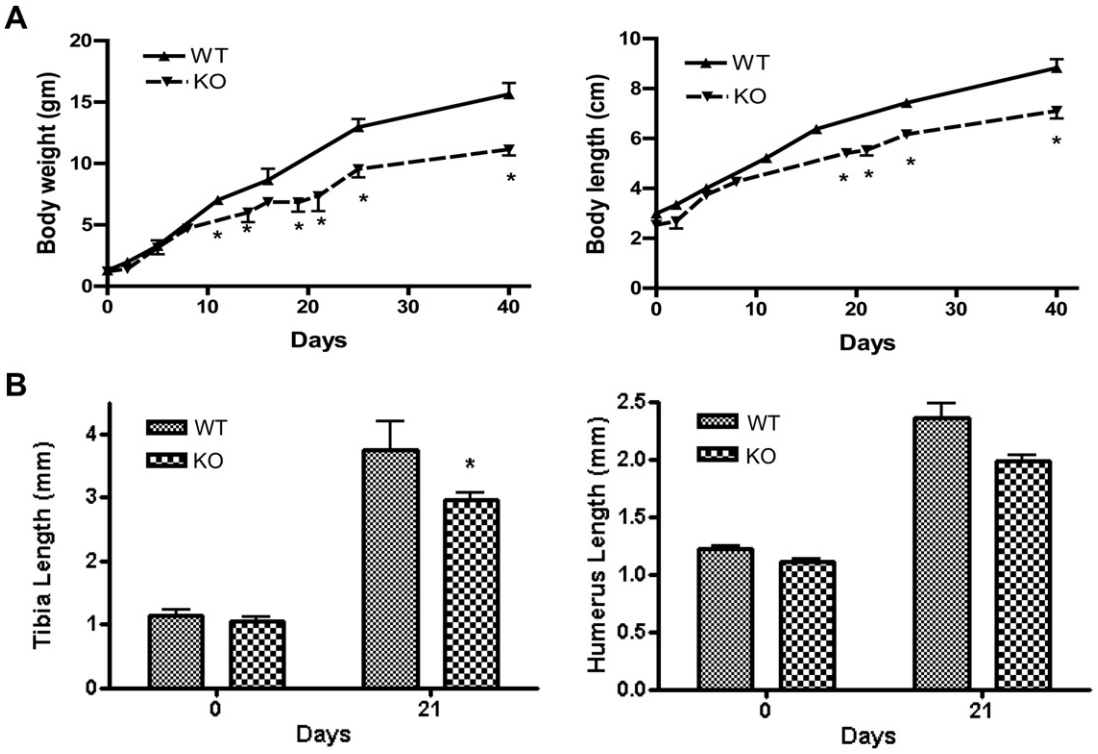

Fig. 1. Loss of *nNOS* results in reduced growth and bone length. Analyses of body weight and length of wild type and *nNOS* homozygote null mice showed that growth retardation developed in *nNOS*-null mice during the first 42 days of their life (A). Measurement of selected individual bones confirmed reduced bone length in tibiae in postnatal day 21 mutant mice (B). At least eight mice per genotype are shown for each data point. ($^*P < 0.05$, mean ± 95% confidence interval) (WT: wild type; KO: knockout).

day 11, before body length was reduced [Fig. 1(A)]. However, analyses at 3-month and 1 year of age demonstrated similar body weight and body length between genotypes (data not shown), suggesting that KO mice catch up during aging. Observation of alcian blue/alizarin red-stained skeletons showed no obvious morphological changes in the axial and appendicular skeleton of mutants (data not shown), but measurement of selected individual bones confirmed reduced bone length in tibiae in mutant mice at postnatal day 21 [Fig. 1(B)].

nNOS deficiency results in reduced chondrocyte proliferation in the growth plate

To elucidate the cellular basis for reduced bone growth, we analyzed growth plate organization at different developmental stages. Growth plates from newborn mutant mice displayed a similar chondrocyte arrangement in resting, proliferative and hypertrophic zones as control mice [Fig. 2(A)]. Measurement of the lengths of resting, proliferating and hypertrophic zones did not show significant differences (data not shown) between genotypes, and cells in the resting and hypertrophic zones of nNOS-null mice did not appear markedly different from controls. However, *nNOS*$^{-/-}$ growth plates appeared slightly disorganized with fewer and shorter columns, especially in the proliferative zone in the center of the growth plates. Some cells also appeared to be smaller in size [Fig. 2(A,B)].

Since our mutant mice showed lower cell numbers in the proliferative zone of the growth plate, we next examined chondrocyte proliferation and apoptosis. Immunohistochemical staining for PCNA demonstrated a reduction in positive cells in mutant mice [Fig. 3(A)] which was confirmed by cell counts [Fig. 3(B)]. Reduced cell numbers could also be due to increased cell apoptosis, which we examined by immunohistochemical staining for cleaved (activated) caspase 3. Staining for cleaved caspase 3 was increased in hypertrophic chondrocytes of mutant mice [Fig. 3(C)].

To elucidate the reason for reduced proliferation in *nNOS*$^{-/-}$ mice, we examined cell cycle protein expression in growth plates. Immunohistochemical staining for cyclin D1, which controls progression through the G1 phase of the cell cycle[11], demonstrated a reduced number of positive cells in mutant mice [Fig. 3(D)]. The transcription factor ATF3 acts as a repressor of cyclin D1 transcription in chondrocytes[25]. Immunohistochemistry demonstrated earlier induction of ATF3 expression in late proliferating chondrocytes and overall stronger ATF3 staining in *nNOS*-deficient growth plates [Fig. 3(E)], suggesting that premature induction of ATF3 expression in mutant mice leads to repression of cyclin D1 transcription and chondrocyte proliferation.

Increased differentiation in nNOS-deficient chondrocytes

Since reduced chondrocyte proliferation is often associated with premature cell cycle exit and maturation[11], we examined the expression of additional markers of prehypertrophic chondrocytes. Expression of the cell-cycle inhibitor p57, which promotes cell cycle exit and is required for normal chondrocyte differentiation, was found strongly increased by immunohistochemistry in mutant mice [Fig. 4(A)]. Immunohistochemistry also showed a similar increase in the number of cells expressing the transcription factors c-Fos [Fig. 4(B)] and RORα [Fig. 4(C)], both of which have been implicated in prehypertrophic/hypertrophic chondrocyte-specific gene expression[26,35].

nNOS deficiency affects cartilage-specific gene expression

In parallel, we analyzed expression of several cartilage marker genes in the mutant mice by real-time reverse transcriptase - polymerase chain reaction (RT-PCR), using RNA directly extracted

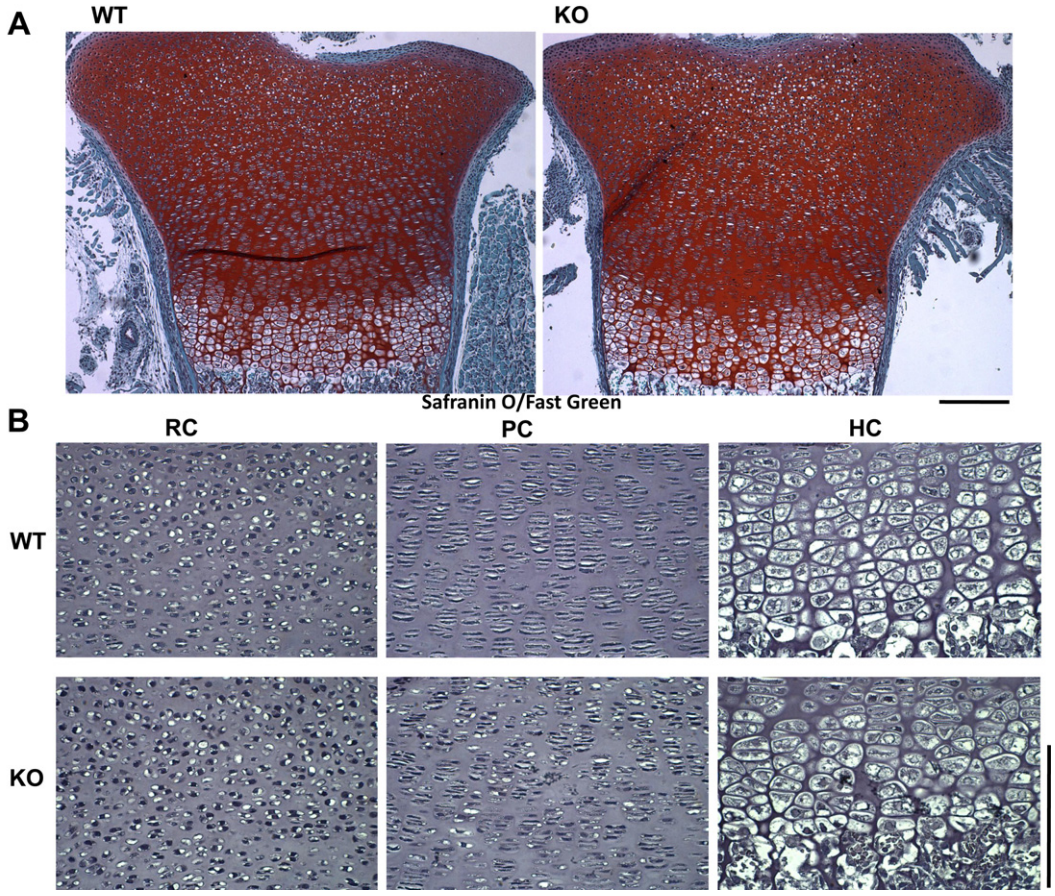

Fig. 2. Lack of *nNOS* causes reduced numbers of proliferating chondrocytes. Safranin O/Fast Green staining of tibia growth plate sections from newborn mice demonstrated similar growth plate architecture in both genotypes (A and B). Detailed analyses of H&E stained sections in the resting, proliferative, and hypertrophic zones showed that the major differences occur in the proliferative zone (B). (RC: resting cells; PC: proliferating cells; HC: Hypertrophic cells). (Scale bar = 200 μm).

from epiphyseal cartilage. Our results revealed decreased expression for *Sox5* and *Sox6* that are important for many stages of chondrocyte differentiation [Fig. 5(A,B)]. Real-time RT-PCR also demonstrated increased expression of prehypertrophic and hypertrophic markers p57 and *Mmp13* [Fig. 5(C,D)]. Transcript levels for *Igf1*, which plays an important role in regulating skeletal development and bone remodeling[21], were also strongly decreased in the mutant mice [Fig. 5(E)]. Interestingly, we also demonstrated upregulated *eNOS* and *iNOS* mRNA levels in the *nNOS* KO cartilage samples [Fig. 5(F,G)].

Loss of nNOS results in reduced trabecular bone and calcium deposition

To analyze whether *nNOS* deficiency had effects on later stages of endochondral ossification, we next examined trabecular bone formation and ossification patterns in mutant mice. Von Kossa staining demonstrated mineralization of trabecular and cortical bone in tibia sections from neonate and postnatal day 21 mice, which was clearly decreased in mutant mice [Fig. 6(A)]. A lack of trabecular structures was also seen by picrosirius red staining for fibrillar collagen [Fig. 6(B)], suggesting that these effects are not simply due to delayed mineralization, but to a delay or reduction in bone formation. Secondary ossification centers were advanced in control mice at postnatal day 12, but less advanced in age-matched KO mice, demonstrating that nNOS deficiency results in delayed secondary ossification [Fig. 6(C)].

Discussion

The signaling pathways that control growth plate chondrocyte proliferation and differentiation during endochondral ossification are incompletely understood. In this study, we provide evidence for an important role of the *nNOS* gene in these processes. Our data show that genetic ablation of *nNOS* results in reduced chondrocyte proliferation and endochondral bone growth *in vivo*. Analyses of these changes in cellular and molecular levels suggest that these effects are likely due to altered expression of several cell-cycle proteins, such as cyclin D 1 and p57, as well as modulation of other genes with known roles in cartilage differentiation, including Sox genes, RORα and c-Fos.

Although there were no obvious morphological changes in the axial and appendicular skeleton, measurement of individual bone confirmed shorter tibiae and femurs in postnatal day 21 mutant mice. Detailed analyses of *nNOS* null tissue sections revealed several abnormalities, most notably reduced chondrocyte proliferation. *nNOS*-deficient growth plates resemble those we observed in mice lacking the *eNOS* or *iNOS* genes[17,18] and also share similarities to those in mice deficient for C-type natriuretic peptide (CNP)[27]. Because both NO and CNP stimulate the production of cGMP via soluble or particulate GCs[27], these similarities are not surprising and further document the importance of cGMP signaling in endochondral bone formation. However, it should be noted that a major phenotype of CNP null mice is a reduction in the length of the hypertrophic zone[27] that we did not observe here. One

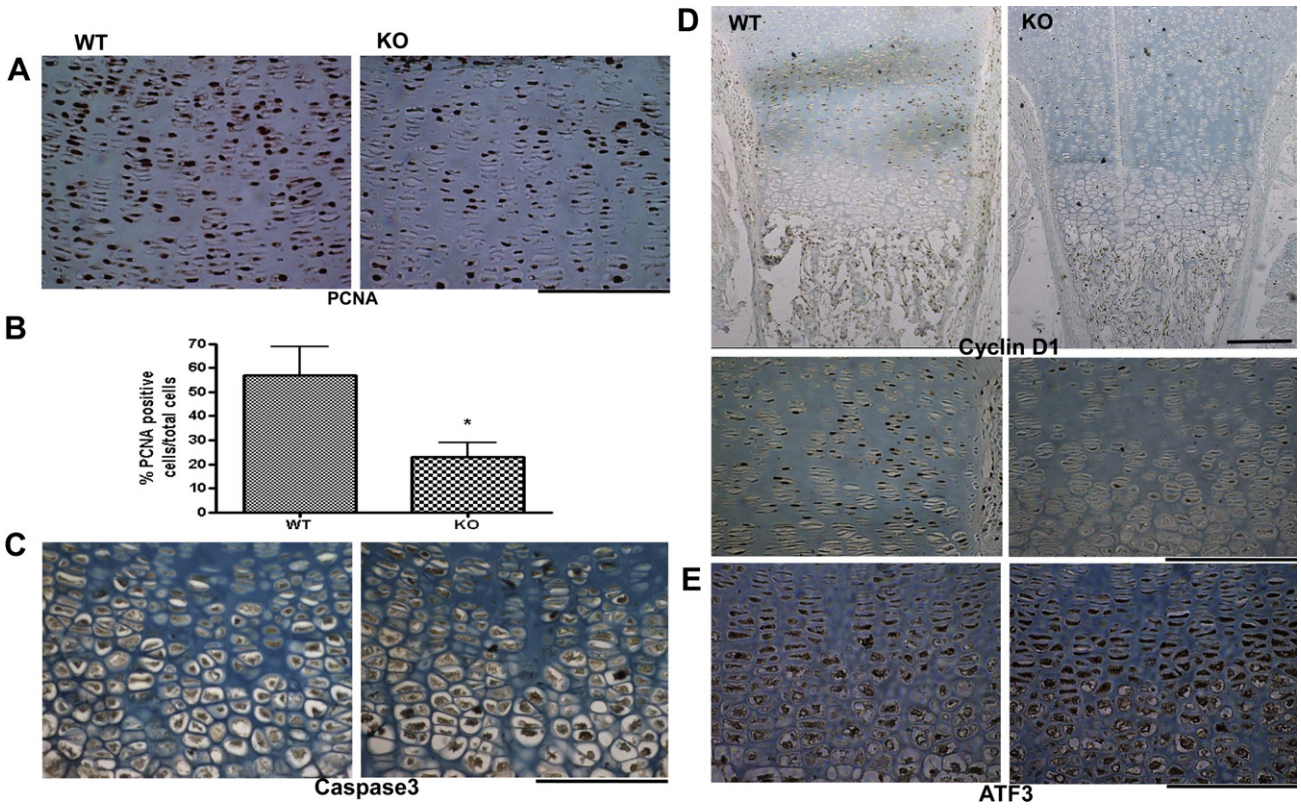

Fig. 3. *nNOS* deficiency reduces chondrocyte proliferation and increases apoptosis. Immunohistochemical staining of newborn tibia sections for proliferating cell nuclear antigen (PCNA; A) showed a reduction in stained cells in mutant mice, which was confirmed by cell counts (B); at least six mice per genotype are shown ($^*P < 0.05$; mean ± 95% confidence interval). Increased staining for cleaved caspase 3 was found in hypertrophic chondrocyte in mutant tibia sections (C). In addition, immunohistochemistry showed that less cells expressed cyclin D1 (D) while expression of ATF3 protein was increased (E) in growth plates from newborn mutant mice (C) (Scale bar = 200 μm). Representative pictures from at least three independent experiments are shown.

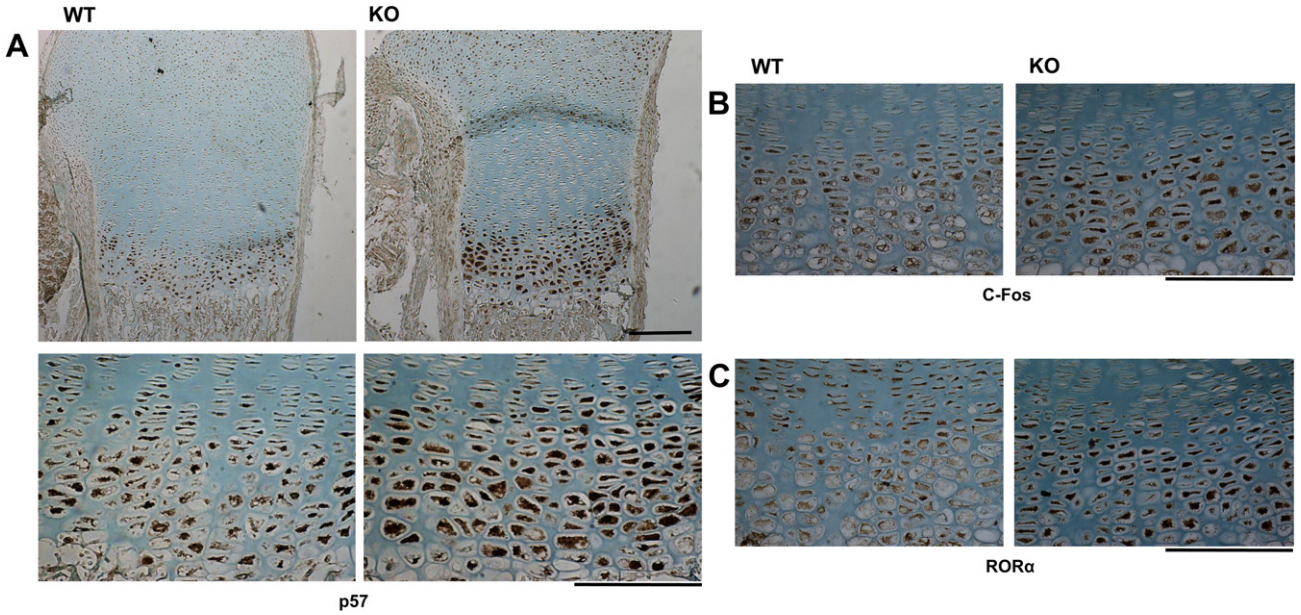

Fig. 4. Increased expression of early hypertrophic markers in *nNOS*-deficient chondrocytes. Expression of the cell-cycle inhibitor p57, which promotes cell cycle exit and is required for normal chondrocyte differentiation, was found increased in mutant mice by immunohistochemical analyses of newborn tibia sections (A). Immunohistochemistry also showed a similar increase in the number of cells expressing the transcription factors c-fos (B) and RORα (C) in sections from newborn mutants (Scale bar = 200 μm). Representative pictures from at least three independent experiments are shown.

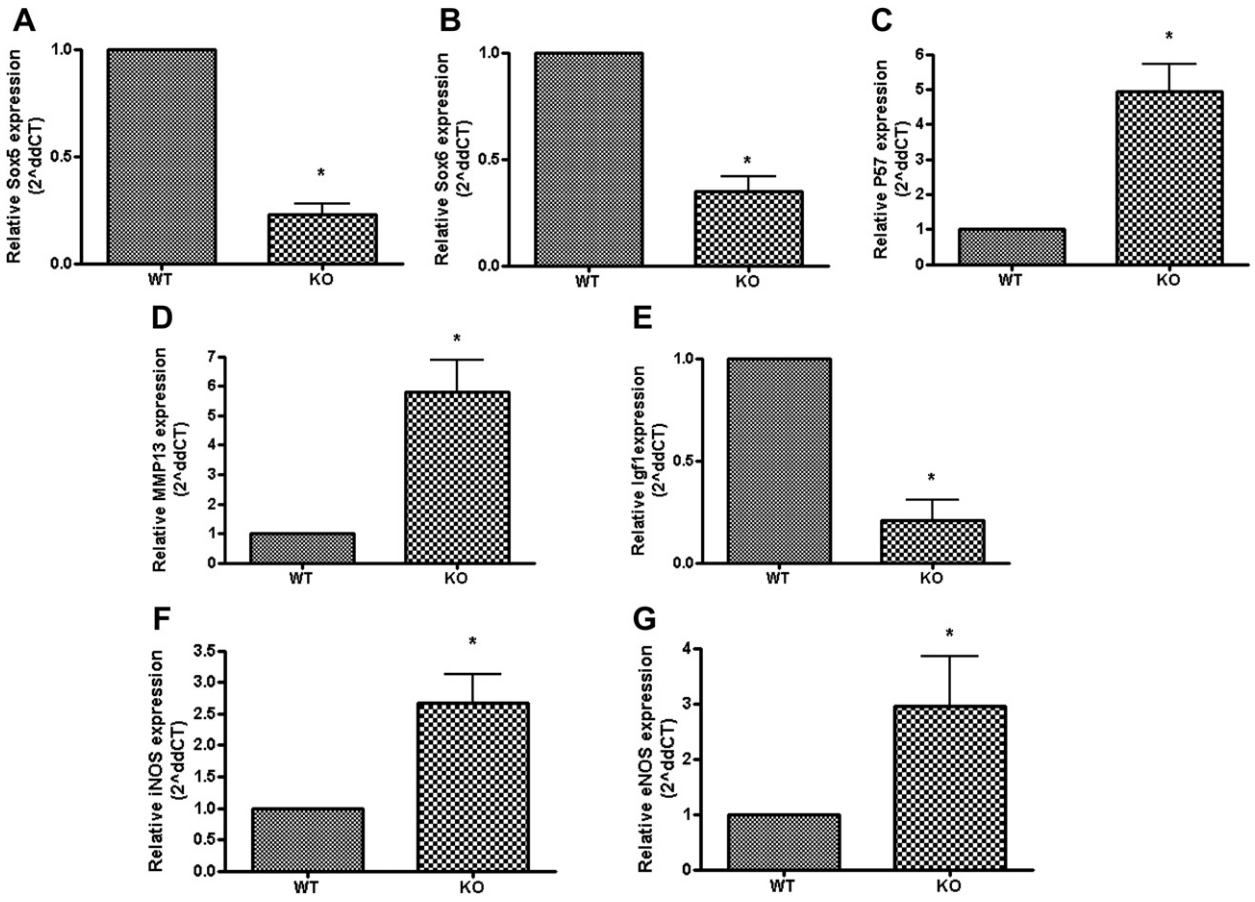

Fig. 5. *nNOS* deficiency affects cartilage-specific gene expression. Real-time RT-PCR revealed decreased expression for *Sox5* and *Sox6* transcripts (A, B) and increased expression of mRNA levels for p57 and *Mmp13* (C, D) in cartilage of KO mice. Transcript levels for *Igf1* were significantly decreased in the mutant mice (E). mRNA levels of *iNOS* and *eNOS* were also found upregulated in *nNOS* KO cartilage (F, G). At least four independent experiments per genotype are shown ($^*P < 0.05$; mean ± 95% confidence interval).

potential explanation for this difference is that during hypertrophy, when all three NOS genes are expressed highly[7], eNOS and/or iNOS (or potentially CNP) can compensate for the loss of nNOS, while they may not be able to do so in the proliferating zone.

The growth plate phenotype of *nNOS* KO mice is similar to that of *eNOS* and *iNOS* null mice[17,18]. Given the similar functions the three NOS proteins and their similar expression in the growth plate[7], the similarities between the phenotypes of the three KO lines were to be expected. In *eNOS* KO mice we only observed the upregulation of the *nNOS* gene, while *eNOS* and *iNOS* genes are both upregulated in *nNOS* KO mice. The compensatory regulation of transcripts of NOS enzymes may explain the relatively minor phenotype we observed in *nNOS* KO mice and provide further evidence for overlapping functions among NOS genes. It is likely that simultaneous inactivation of NOS genes would have more severe outcomes. However, it should be noted that mice lacking all three NOS genes have been described[28]. Although those mice show a very high rate of lethality (85%), the surviving triple NOS KO mice live a surprisingly normal life[29], suggesting that additional compensatory mechanisms may exist.

nNOS inactivation results in reduced chondrocyte proliferation and increased apoptosis. However, increased staining for active caspase 3 was restricted to terminally differentiated hypertrophic chondrocytes, suggesting that apoptosis plays no or only a minor role in the reduced numbers of proliferating chondrocytes. Instead, one of the major reasons for the reduced proliferation appears to be the induction of the transcription factor ATF3, which is able to suppress cyclin D1 transcription[25]. Cyclin D1 expression is induced by many mitogenic stimuli and pathways in chondrocytes[11]. Our studies suggest that in addition to positive regulation through transcription factors such as ATF2 and CREB[30–33], cyclin D1 transcription is also under negative regulation by ATF3[25]. The cyclin-dependent kinase inhibitor p57 is a second cell cycle protein showing altered expression in the absence of eNOS, iNOS or nNOS. Immunohistochemistry showed earlier and broader expression in mutant mice, along with other prehypertrophic markers such as RORα. At the moment, it is unknown whether NO signaling regulates cyclin D1 and p57 expression through a common pathway or through different effectors. It is also possible that premature differentiation to prehypertrophic chondrocytes is due to the reduced expression of *Sox5* and *Sox6* in *nNOS* KO cartilage, but the relationship between these transcription factors and cyclin D1 expression has not been addressed. Finally, reduced expression of *Igf1* could also contribute to the phenotype of *nNOS* KO mice, although the reduced growth in *Igf1*-deficient mice is largely due to smaller size of hypertrophic chondrocytes, as opposed to the primary effect on cell proliferation in our mice[34].

Loss of *nNOS* results in reduced von Kossa staining of cortical and trabecular bone. Picrosirius red staining suggests that this effect is due to paucity of trabecular structures *per se*, not just a delay in mineralization. At the moment, it is unclear whether this phenotype is secondary to defects in cartilage development or due to intrinsic functions of nNOS in osteoblasts or osteoclasts. Cell type-specific KO models will be required to resolve this question.

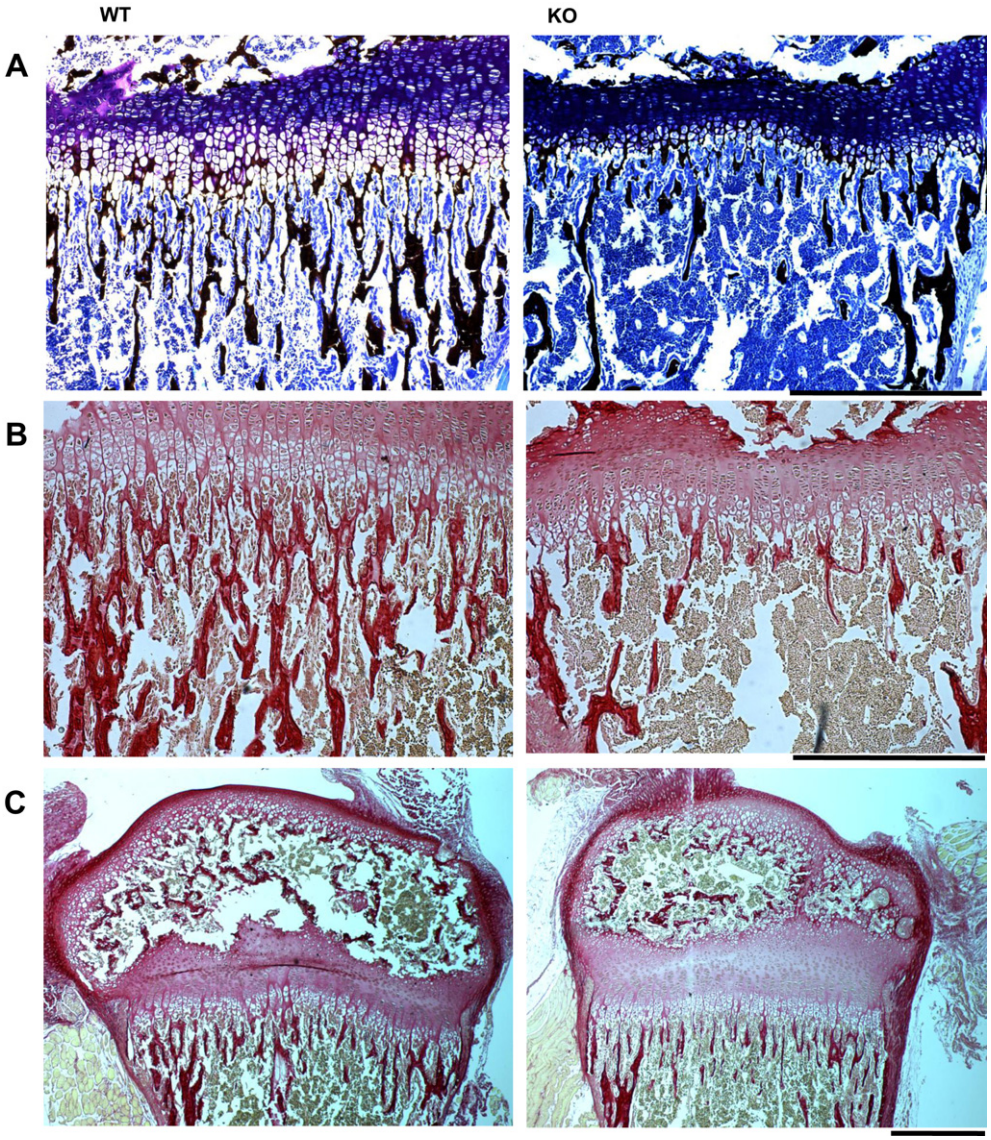

Fig. 6. *nNOS* deficiency leads to reduced trabecular bone and calcium deposition. Mineralization of trabecular and cortical bone shown by Von Kossa staining in tibia sections from postnatal day 21 mice was clearly decreased in mutant mice (A). A similar lack of trabecular structures was also seen by picrosirius red staining for fibrillar collagen (B). Secondary ossification centers were advanced in control mice at postnatal day 12, but delayed in mutant mice (C). (Scale bar: 200 μm for A, B, D; 50 μm for C) Representative pictures from at least three independent experiments are shown.

In summary, our data demonstrate a role for nNOS in chondrocyte proliferation apoptosis and maturation. Loss of nNOS affects numerous aspects of cartilage physiology, including chondrocyte proliferation and gene expression, as well as mineralization of bone. Reduced chondrocyte proliferation may in part be due to premature cell cycle exit shown by decreased cyclin D1 and increased p57 expression in mutants. It appears likely that the overall phenotype of *nNOS*-deficient mice is a combination of alternations in all these aspects. Further investigations into the specific mechanisms involved will result in a better understanding of physiological and pathological skeletal development and associated diseases, such as OA and osteoporosis.

Authors' contributions

QY carried out the experimental work, data collection and interpretation, and drafting the manuscript. QY and FB conceived of the study design and coordinated the studies and data interpretation. Q.F. contributed mutant mice and consultation. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interest.

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