Inducible nitric oxide synthase–nitric oxide signaling mediates the mitogenic activity of Rac1 during endochondral bone growth

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Summary

Coordinated proliferation and differentiation of growth plate chondrocytes controls endochondral bone growth and final height in humans, and disruption of this process results in diseases of the growing and adult skeleton, such as chondrodysplasias or osteoarthritis. We had shown recently that chondrocyte-specific deletion of the gene *Rac1* in mice leads to severe dwarfism due to reduced chondrocyte proliferation, but the molecular pathways involved remained unclear. Here, we demonstrate that Rac1-deficient chondrocytes have severely reduced levels of inducible nitric oxide synthase (iNOS) protein and nitric oxide (NO) production. NO donors reversed the proliferative effects induced by Rac1 deficiency, whereas inhibition of NO production mimicked the effects of Rac1 loss of function. Examination of the growth plate of iNOS-deficient mice revealed reduced chondrocyte proliferation and expression of cyclin D1, resembling the phenotype of Rac1-deficient growth plates. Finally, we demonstrate that Rac1–NO signaling inhibits the expression of ATF3, a known suppressor of cyclin D1 expression in chondrocytes. In conclusion, our studies identify the iNOS–NO pathway as a novel mediator of mitogenic Rac1 signaling and indicate that it could be a target for growth disorder therapies.

Key words: Rac1, Nitric oxide, iNOS, Proliferation, Growth plate, Chondrocyte

Introduction

Growth of endochondral bones is regulated by the coordinated proliferation and hypertrophic differentiation of growth plate chondrocytes (Kronenberg, 2003; Olsen et al., 2000). Cell volume increase during hypertrophic differentiation provides one driving force for bone growth, whereas ordered proliferation is required for the generation of sufficient numbers of cells to undergo subsequent hypertrophy (Beier, 2005). Thus, inhibition of either proliferation or hypertrophy can result in dwarfism. Disruption of these processes by, for example gene mutations, endocrine imbalances and mechanical or nutritional influences, causes numerous diseases. Many growth factors and hormones have been shown to regulate chondrocyte proliferation, including fibroblast growth factors, parathyroid hormone-related peptide and Indian hedgehog (Ballock, 2003; van der Eerden, 2003). However, the intracellular mechanisms controlling chondrocyte proliferation and hypertrophy are much less understood.

Studies by our group and others have shown that Rho GTPases are important regulators of chondrocyte proliferation and differentiation (Kerr et al., 2008; Kumar and Lassar, 2009; Wang and Beier, 2005; Wang et al., 2004; Woods and Beier, 2006; Woods et al., 2009b; Woods et al., 2005; Woods et al., 2007a; Woods et al., 2007b). Rho family GTPases act as molecular switches that become activated in response to specific extracellular stimuli, including growth factors and integrin ligands, through the activity of guanine nucleotide exchange factors that exchange GTP for GDP (Heasman and Ridley, 2008; Vega and Ridley, 2007; Vega and Ridley, 2008). Activated, GTP-bound Rho proteins then interact with a large variety of downstream effectors, including cytoskeletal regulators and various kinases, to control a number of cellular events such as cell migration, cell cycle progression and gene expression. During the early steps of chondrogenesis, regulation of actin dynamics and cellular morphology appear to mediate many of the effects of Rho GTPases (Beier and Loeser, 2010; Woods and Beier, 2006; Woods et al., 2005; Woods et al., 2007a). However, additional mediators are probably involved in these activities of Rho GTPAses, in particular after cells have differentiated into chondrocytes.

We have previously reported that chondrocyte-specific inactivation of the gene *Rac1*, encoding a Rho family GTPase with a central role in cellular signaling pathways, causes dwarfism and numerous skeletal abnormalities (Wang et al., 2007). A similar phenotype of reduced long bone growth was observed upon limb-specific inactivation of *Rac1* (Suzuki et al., 2009). The defects we observed in cartilage-specific *Rac1* knockout (KO) mice appear to be due, at least to a large extent, to reduced chondrocyte proliferation (Wang et al., 2007), but the downstream mechanisms connecting Rac1 to chondrocyte proliferation are unknown. Rac1 has been shown to control many cellular processes, including actin organization and transcription (Bustelo et al., 2007; Lee and Dominguez, 2010; Pai et al., 2010). In addition, Rac1 activation leads to the generation of reactive oxygen species (ROS) that mediate certain aspects of Rac1

signaling in various tissues (Hordijk, 2006; Sulciner et al., 1996; Sundaresan et al., 1996; Zhang et al., 2011). ROS have also been implicated in the promotion of chondrocyte differentiation (Kim et al., 2010; Morita et al., 2007), prompting us to investigate whether altered ROS levels contribute to the effects of Rac1 deficiency in chondrocytes. There are many interactions between the metabolism of ROS and nitric oxide (NO) (Thomas et al., 2008); however, NO or the enzymes involved in its synthesis have not been identified as major mediators of Rac1 signaling.

Three different nitric oxide synthase (NOS) proteins have been identified, eNOS, iNOS and nNOS (endothelial, inducible and neuronal NOS, respectively) (Bryan et al., 2009). Although pharmacological manipulation of NO levels has been shown to affect chondrocyte differentiation (Teixeira et al., 2005; Teixeira et al., 2001), the roles and regulation of individual NOS genes in cartilage development are largely unknown. iNOS is capable of producing large amounts of NO, often in response to extracellular signals, such as pro-inflammatory cytokines (Teixeira et al., 2008). It has been shown that *Nos2* (iNOS) KO mice show accelerated cartilage degeneration in surgical models of osteoarthritis (Clements et al., 2003), but growth plates of these mice have not been described. Here we identify iNOS as a novel target of Rac1 that mediates the mitogenic activities of this pathway during endochondral bone growth.

Results

Rac1 deficiency results in reduced iNOS expression and NO production

We had previously shown hypocellularity and reduced proliferation in Rac1-deficient growth plates (Wang et al., 2007). We first examined whether we could recapitulate this phenotype in vitro to facilitate biochemical studies on the underlying mechanisms. Primary chondrocytes were isolated from *Rac1*$^{fl/fl}$ mice that carried two alleles of a conditional (floxed) *Rac1* allele. Cells in monolayer culture were infected with adenoviruses expressing green fluorescent protein (GFP; control) or Cre recombinase (Ad-Cre). Infection with Ad-Cre resulted in a marked reduction of Rac1 protein (Fig. 1A); densitometry determined that Rac1 proteins levels were reduced by 70% in Ad-Cre-infected cultures (data not shown). Control cells increased their numbers steadily over the 3-day time course of the experiment, whereas this increase was much slower in cells infected with Ad-Cre (Fig. 1B). This was accompanied by reduced expression of cyclin D1 (Fig. 1C), as we had observed

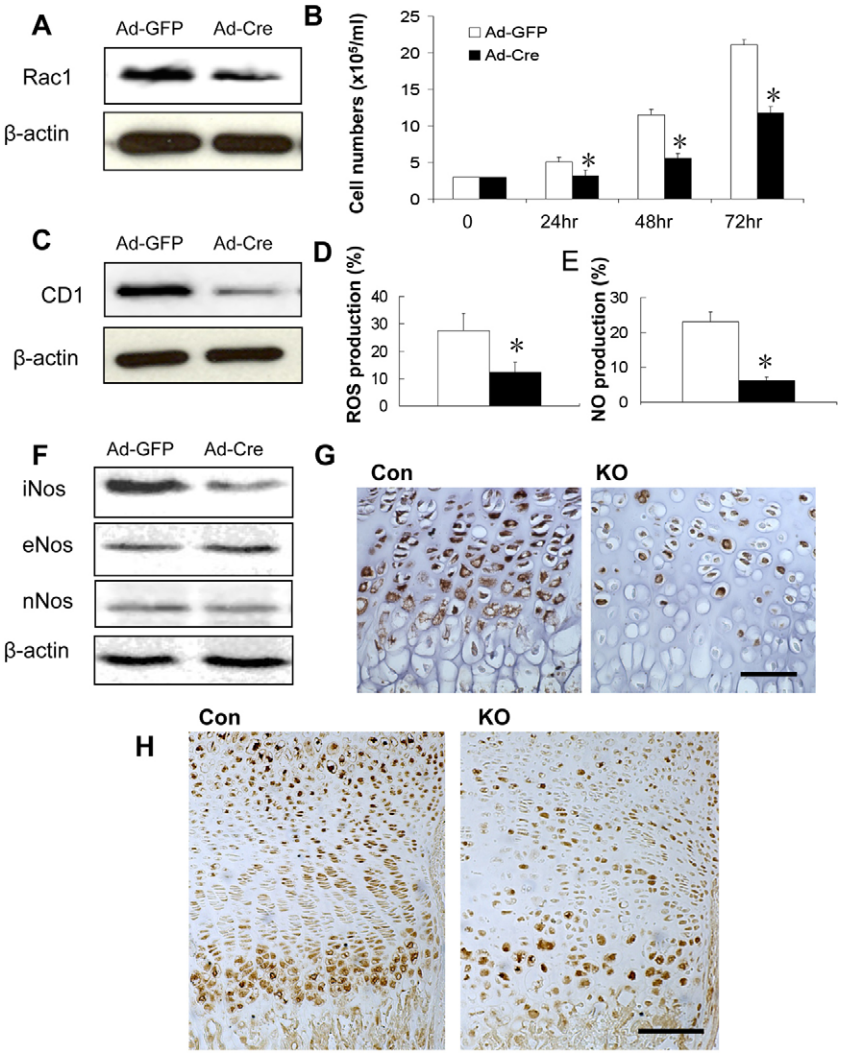

Fig. 1. iNOS expression and NO generation are reduced in Rac1-deficient chondrocytes. Primary chondrocytes were isolated from *Rac1*$^{fl/fl}$ mice and infected with adenovirus expressing GFP (Ad-GFP; control) or Cre recombinase (Ad-Cre). Cells were analyzed by cell counting, indirect fluorescence measurements of ROS and NO levels, and western blotting. (**A**) Western blotting demonstrated that infection with Ad-Cre resulted in approximately 70% reduction of Rac1 protein. (**B**) Cell counts demonstrated that control cells increased their numbers steadily over the 3-day time course of the experiment, whereas cells infected with Ad-Cre multiplied at a much slower rate. (**C**) Western blotting showed that there was also a reduction in the expression of cyclin D1 in mutant cells, compared with control. (**D**,**E**) Rac1 deletion reduced ROS levels (D), but caused a more pronounced reduction in NO production (E) in Cre recombinase-infected cells. (**F**) Western blotting demonstrated markedly reduced expression of iNOS protein in Cre-expressing chondrocytes, whereas eNOS and nNOS expression was similar to those in control cells. (**G**) Immunohistochemistry, showed iNOS staining was notably reduced in growth plates from cartilage-specific *Rac1* KO mice compared with control mice at postnatal day 7 (P7). Scale bar: 50 μm. (**H**) A similar decrease in staining in mutant mice was observed using nitrotyrosine, an indicator of cellular NO levels at P7. Scale bar: 100 μm. Images are representative of three independent trials. In B, D and E, values are means + s.d.; *$P<0.05$.

in vivo. The similarity of these effects to the phenotypes observed in vivo suggested that our in vitro culture system provides an appropriate model to study the mechanisms employed by Rac1 to regulate chondrocyte proliferation.

Because Rac1 is able to stimulate ROS generation, we measured levels of ROS and NO. Rac1 deletion caused an approximately 50% reduction in ROS levels (Fig. 1D), but NO production was inhibited to a much larger degree (Fig. 1E). We therefore examined the molecular cause of reduced NO production and showed that expression of iNOS protein was markedly reduced in Rac1-deficient chondrocytes, whereas the expression of eNOS and nNOS was similar to that in control cells (Fig. 1F). Densitometry showed a significant 51% reduction in iNOS protein levels in Cre-expressing chondrocytes, whereas no significant changes were seen for eNOS and nNOS proteins (data not shown). To analyze the effects of Rac1 deficiency on iNOS expression in vivo, we performed immunohistochemistry on paraffin sections from 7-day old (P7) control and cartilage-specific *Rac1* KO mice. In control mice, iNOS protein was found mostly in late proliferating and prehypertrophic chondrocytes (Fig. 1G). iNOS staining was notably reduced in growth plates from Rac1 mutant mice that also showed disorganization of the proliferative zone, as we described previously (Wang et al., 2007). A similar decrease in staining in mutant mice was observed using an antibody for nitrotyrosine, an indicator of cellular NO levels (Fig. 1H). Collectively, these data suggest that iNOS expression and NO generation are reduced in Rac1-deficient chondrocytes.

NO promotes chondrocyte proliferation in cell and organ culture

We next examined whether addition of exogenous NO could overcome the effects of Rac1 deficiency. We treated cells with the phorbolester, phorbol 12-myristate 13-acetate (PMA; an inducer of ROS that did not change NO levels; data not shown) and observed no effects on numbers of control or Rac1-deficient chondrocytes, suggesting that the mitogenic defects in Rac1-deficient chondrocytes are not due to lower ROS production (Fig. 2A). By contrast, addition of the compound 3-morpholinosydnonimine (SIN-1), which has been show to induce NO synthesis in chondrocytes (Del Carlo and Loeser, 2002; Fay et al., 2006; Mathy-Hartert et al., 2003) and increased both ROS and NO generation in our experiments (supplementary material Fig. S1), restored the number of Rac1-deficient chondrocytes in monolayer culture (Fig. 2A). Similarly, western blotting and subsequent densitometry analysis demonstrated that cyclin D1 expression in Rac1-deficient chondrocytes is restored by SIN-1 treatment, but not by PMA (Fig. 2B).

These data suggested that NO production mediates the mitogenic effects of Rac1 in chondrocytes and that inhibition of NO synthesis by other means would result in similar phenotypes to those resulting from deletion of Rac1, whereas NO donors should promote chondrocyte proliferation. To test this prediction, we incubated chondrocytes with the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) and the NO donors *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 2,2′-(hydroxynitrosohydrazino) bis-ethanamine (NOC18). L-NAME reduced the number of chondrocytes, and both donors increased cell numbers (although only SNAP produced a statistically significant difference in these studies; Fig. 2C,D). In additional experiments, statistically significant increases in the number of chondrocytes was produced by NOC18 and sodium nitroprusside (SNP; supplementary material Fig. S1B). Collectively, these data suggest a model in which Rac1 promotes chondrocyte

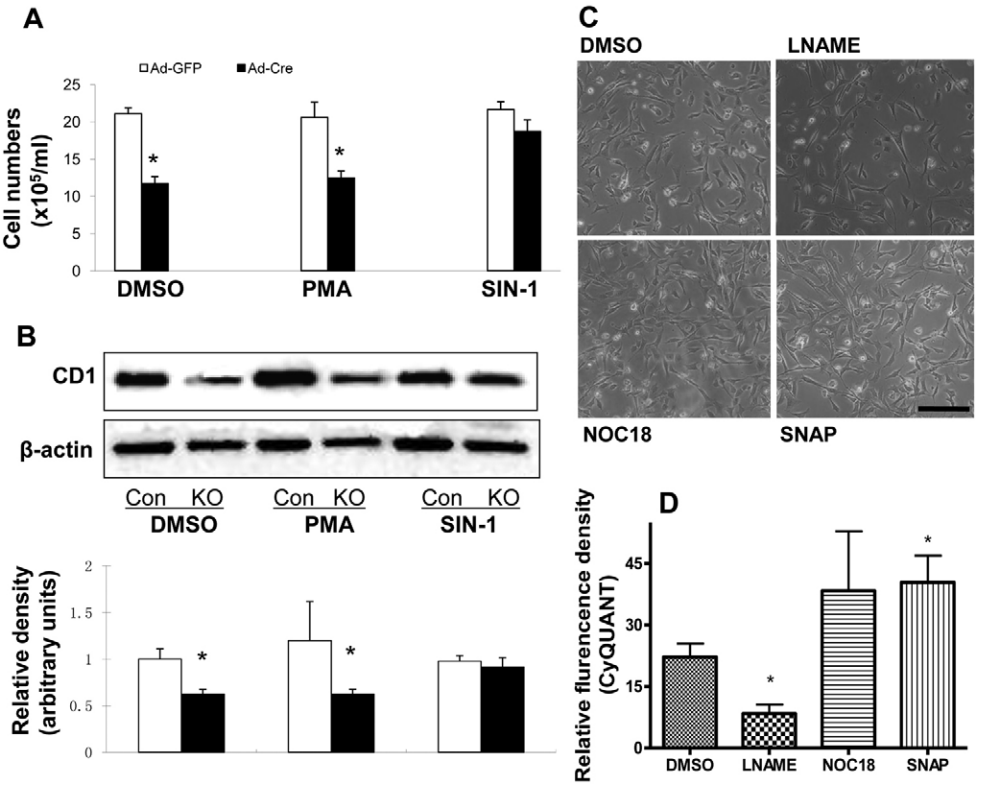

Fig. 2. NO can rescue the effects of Rac1 deficiency. Primary chondrocytes were isolated from *Rac1*$^{fl/fl}$ mice and infected with adenoviruses expressing GFP (control) or Cre recombinase. (**A**) Cell counts demonstrated that addition of SIN-1, but not PMA, in this culture system increased numbers of Rac1-deficient chondrocytes. (**B**) Western blotting showed that cyclin D1 expression in primary chondrocytes was restored by SIN-1, but not PMA. (**C**,**D**) CyQUANT NF cell proliferation assay revealed that incubation of primary chondrocytes with the NOS inhibitor L-NAME reduced cell numbers, whereas the NO donors SNAP and NOC18 caused an increase in chondrocyte cell numbers, although only SNAP caused a statistically significant difference (*P<0.05). Scale bar: 50 μm (C). Images are representative of three independent trials. In D, the means and s.d. are shown.

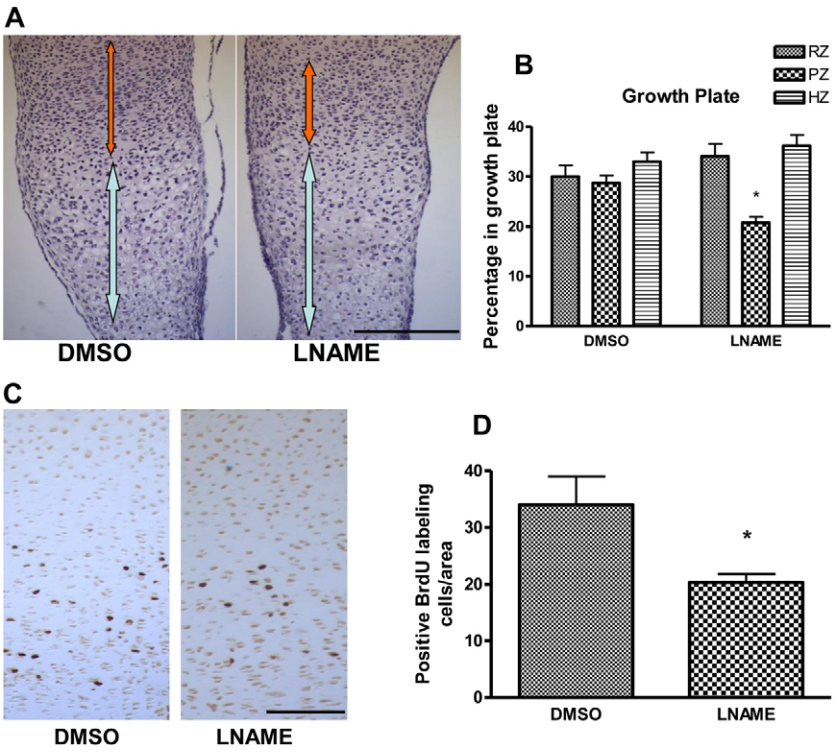

Fig. 3. Inhibition of NO production results in reduced proliferation in tibia organ cultures. Tibiae isolated from E15.5 mice were incubated in organ culture with DMSO or the NOS inhibitor L-NAME (1 mM). After 6 days, tibiae were fixed, embedded and sectioned, and sections were stained with H&E and analyzed using a Leica DMRA2 microscope and OpenLab software. (**A**,**B**) Staining and measurement of zone length demonstrated a shorter proliferative zone in L-NAME-treated tibiae [red arrow, proliferating cell zone and blue arrow, hypertrophic cell zone (A); RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone (B)]. Scale bar: 250 μm. (**C**,**D**) There was also fewer BrdU-incorporating nuclei (dark brown staining). Scale bar: 100 μm. Images are representative of three independent trials. In B and D, values are means + s.d.; *$P<0.05$.

proliferation through iNOS–NO-mediated induction of cyclin D1 expression.

We next examined whether NO regulates chondrocyte proliferation and tissue architecture in intact growth plates, using an organ culture system of embryonic mouse tibiae (Agoston et al., 2007). Tibiae were grown with DMSO (control) and L-NAME for 6 days. Histochemical analyses of Haematoxylin and Eosin-stained paraffin sections from cultured tibiae followed by measurement of the growth plate zones demonstrated that L-NAME treatment resulted in a shortened proliferative zone, whereas the resting and hypertrophic zones of the growth plate were not affected (Fig. 3A,B; supplementary material Fig. S2). This was accompanied by reduced numbers of BrdU-incorporating cells (Fig. 3C,D), providing additional evidence for an important role of NO production in chondrocyte proliferation.

NO donors rescue the effects of Rac1 inhibition on bone growth

To investigate whether NO donors can rescue the effects of Rac1 inhibition in intact growth plates, tibiae were incubated with the pharmacological Rac1 inhibitor NSC23766 (Gao et al., 2004). Our earlier micromass culture studies had demonstrated that this compound has similar effects on early chondrogenesis as genetic inactivation of *Rac1*, providing evidence for the specificity of the inhibitor (Woods et al., 2007b). NSC23766 treatment for 6 days resulted in drastic reduction of tibial growth in our organ culture system (Fig. 4). The NO donor SNP did not affect tibial growth on its own, but completely rescued the effects of Rac inhibition on overall tibial growth (Fig. 4). These data demonstrate that restoration of NO signaling overcomes the effects of Rac1 deficiency in the authentic three-dimensional context of the growth plate.

Reduced chondrocyte proliferation in iNOS-deficient mice

Because our data suggested a role for endogenous Rac1–iNOS signaling in growth plate chondrocyte proliferation, we next analyzed the cartilage phenotype of iNOS-deficient mice (Laubach et al., 1995). Alcian Blue and Alizarin Red staining showed that the skeletons of these mice were morphologically normal, but slightly smaller than those of control littermates (supplementary material Fig. S3). Growth plates from these mice showed normal zonal and columnar architecture, but appeared

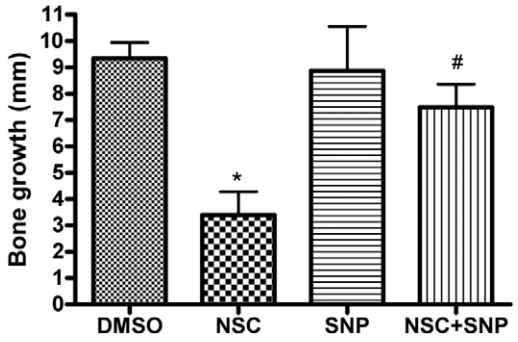

Fig. 4. The NO donor SNP rescues the effects of Rac inhibition in organ culture. E15.5 tibiae were incubated with control medium, the Rac inhibitor NSC23766 (50 μM), the NO donor SNP (10 μM) or both for 6 days. The length of the tibiae was measured at the beginning and end of culture using a dissecting microscope with an eyepiece graticule, and the difference in length between the two time points is shown. Rac inhibition resulted in drastic reduction of tibia growth in our tibia organ culture system. The NO donor SNP (10 μM) did not affect tibia growth on its own, but completely rescued the effects of Rac inhibition on growth. The means and s.d. are shown; *$P<0.05$, compared with DMSO, $^{\#}P<0.05$, compared with NSC treatment alone.

slightly hypocellular in their centers (Fig. 5A; supplementary material Fig. S3), similar to but more subtle than those of mice with cartilage-specific deletion of the *Rac1* gene (Wang et al., 2007). Cell counts confirmed hypocellularity in the proliferative zone in mutant mice (Fig. 5B). Immunohistochemistry and counts of positive cells demonstrated lower numbers of chondrocytes incorporating BrdU (Fig. 5C,D) and staining positively for cyclin D1 protein (Fig. 5E,F) in iNOS-deficient growth plates. Both of these features had also been observed in cartilage-specific *Rac1* KO mice (Wang et al., 2007).

The transcriptional repressor ATF3 is upregulated upon loss of Rac1 or iNOS

We had shown previously that cyclin D1 expression in chondrocytes is positively regulated by the transcription factors ATF2 and CREB and repressed by ATF3 (Beier et al., 2001; Beier et al., 1999; Beier and LuValle, 2002; James et al., 2006). Immunohistochemistry demonstrated limited expression of ATF3 protein in chondrocytes of control mice, with strongest staining at the transition from the proliferating to the prehypertrophic zone and weaker staining in hypertrophic chondrocytes (Fig. 6A). Loss of either Rac1 or iNOS resulted in a marked increase in the number of stained cells, as well as increased staining intensity (Fig. 6A). This suggests increased expression of ATF3 that in turn can repress cyclin D1 transcription and result in earlier cell cycle withdrawal. In agreement with this model, *Atf3* mRNA levels were increased in Rac1-deficient cartilage in vivo (Fig. 6B) and in primary $Rac1^{fl/fl}$ chondrocytes infected with Ad-Cre (Fig. 6C). Western blotting revealed that *Rac1* deletion also increased ATF3 protein levels, whereas SIN-1 treatment slightly reduced ATF3 protein levels in both control and Rac1-deleted chondrocytes, and PMA did not affect ATF3 expression in either case (Fig. 6D). These data suggest that the Rac1–iNOS–NO pathway acts to suppress ATF3 expression in order to maintain cyclin D1 transcription and chondrocyte proliferation.

Discussion

Chondrocyte proliferation is one of the main determinants of endochondral bone growth and thus final height. Although many extracellular signals that control these processes are known, the intracellular signaling pathways connecting cell surface receptors to specific cell cycle genes are less well understood. We had shown that the small GTPase Rac1 is essential for normal chondrocyte proliferation and cyclin D1 expression in mice (Wang et al., 2007), but the molecular mechanisms involved were not known. Here we demonstrate that Rac1 controls chondrocyte proliferation by stimulation of iNOS expression and NO production, which in turn suppresses ATF3 expression and thus allows increased transcription of the cyclin D1 gene (Fig. 7).

Although the regulation of ROS generation by Rac1 is well established, there is much less known about the interactions between Rac proteins and NO signaling. The requirement for Rac1 for normal iNOS expression in our study is consisted with an earlier report showing reduced levels of iNOS (*Nos2*) mRNA upon adenoviral delivery of dominant-negative Rac to rat livers (Harada et al., 2003). However, Rac1 activation or inhibition did not alter iNOS induction by interleukin 1β in smooth muscle cells (Finder et al., 2001), suggesting that the regulation of iNOS expression by Rac1 is stimulus and/or cell-type specific. Moreover, Rac1 deficiency in endothelial cells leads to reduced

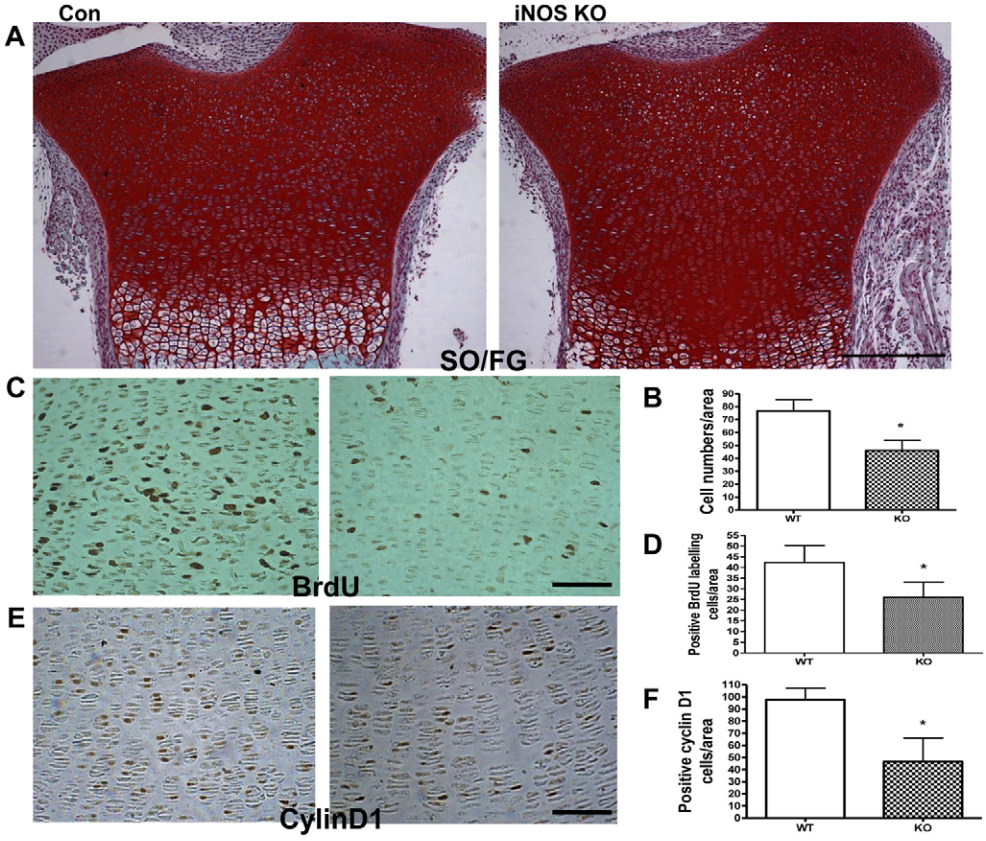

Fig. 5. Reduced chondrocyte proliferation in *Nos2* (iNOS) KO mice. Tibiae were dissected from newborn *Nos2* KO and control mice, processed for histology and analyzed by histochemistry and immunohistochemistry. (**A**) Growth plates from *Nos2* mutant mice were hypocellular, as shown by Safranine O and Fast Green (SO/FG) staining. (**B**) Cell counts confirmed hypocellularity in the proliferative zone of mutant mice. (C–E) Immunohistochemical staining and counts of positive cells demonstrated fewer chondrocytes incorporating BrdU (**C**,**D**) and expressing cyclin D1 (E,F) in iNOS-deficient growth plates. Scale bars: 250 μm (A), 100 μm (**C**,**E**). Images are representative of three independent trials. In B, D and F, values are means + s.d.; $*P<0.05$. Cells were counted from at least three mice per genotype and four sections per mouse.

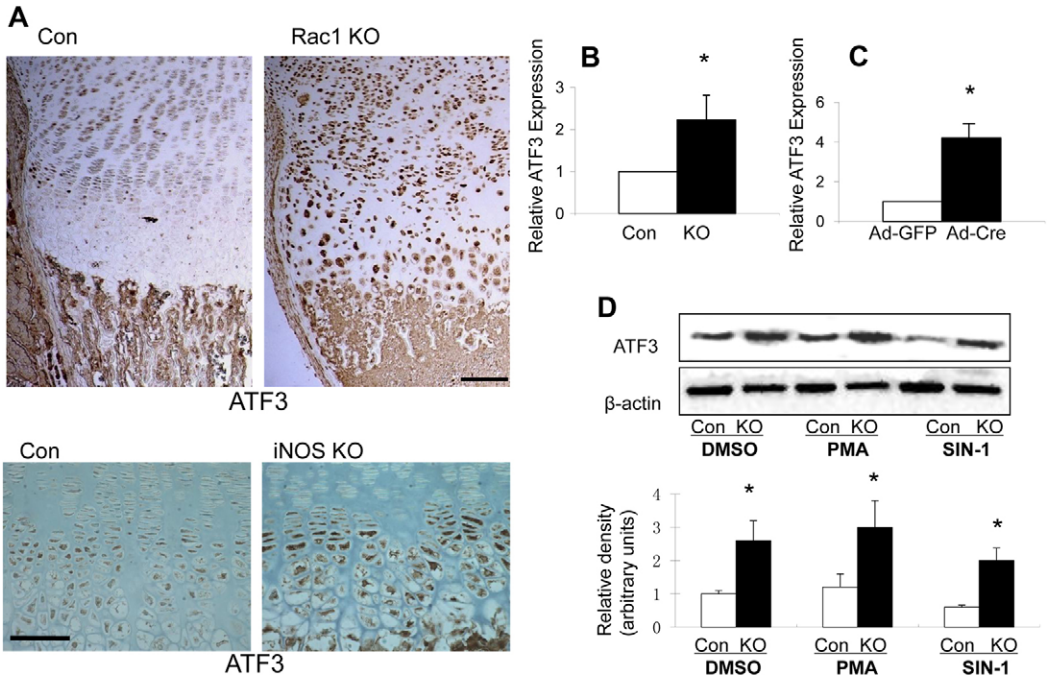

Fig. 6. ATF3 suppresses cyclin D1 expression to inhibit chondrocyte proliferation. (**A**) Immunohistochemistry demonstrated relatively little expression of ATF3 protein in control mice, with markedly increased expression upon loss of Rac1 or iNOS. Scale bar: 50 μm in top panel, 100 μm in bottom panel. Images are representative of three independent trials. (**B**,**C**) Real-time RT-PCR demonstrated that *Atf3* mRNA levels are increased in Rac1-deficient cartilage in vivo (B) and in primary *Rac1*$^{fl/fl}$ chondrocytes infected with Ad-Cre (C). (**D**) Western blotting of primary chondrocytes infected with adenoviruses expressing GFP or Cre demonstrated that loss of Rac1 increased ATF3 protein levels under all conditions, and that SIN-1 treatment reduced expression of ATF3 protein in wild-type and Rac1-deficient chondrocytes. In B, C and D, values are means + s.d.; *$P<0.05$.

expression and activity of eNOS (Sawada et al., 2008), whereas eNOS protein levels were not altered in our system. Together, these data indicate that the connections between Rac1 (and possibly other Rho GTPases) and components of the NO signaling pathway act in a cell-type-specific manner.

Similar to the links between Rho GTPases and iNOS, studies from other tissues suggest that the role of iNOS itself in cellular proliferation is cell-type and/or context specific. For example, iNOS is required for hepatocyte proliferation during liver regeneration (Rai et al., 1998) but suppresses proliferation of smooth muscle cells (Kibbe et al., 2000). Our studies clearly show a requirement for iNOS in chondrocyte proliferation. These in vivo data are further supported by use of the NOS inhibitor L-NAME in vitro, which also resulted in a reduction in the number

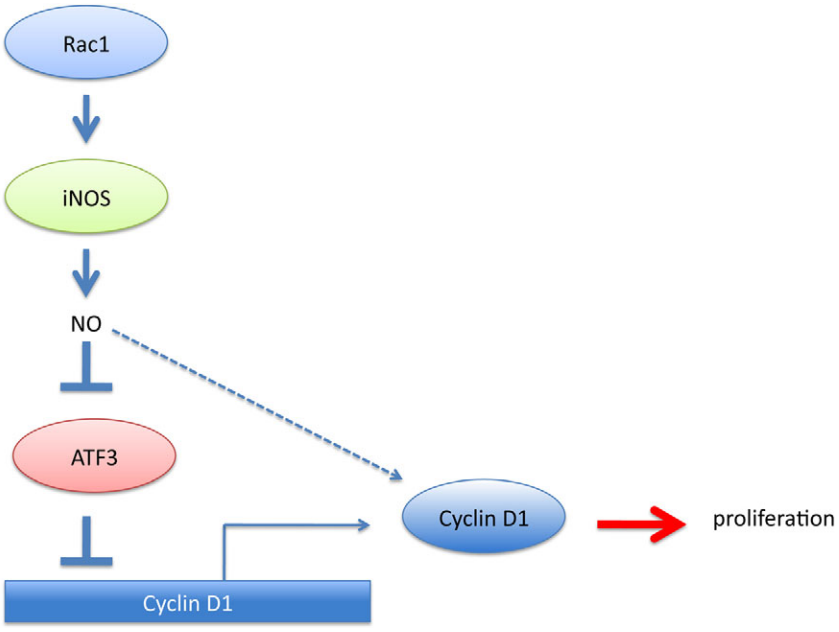

Fig. 7. Model of Rac1–iNOS/NO–ATF3–cyclin D1 signaling in chondrocytes. Our data show that Rac1 is required for normal levels of iNOS expression and NO production in chondrocytes. NO suppresses expression of ATF3, which acts as a transcriptional repressor of cyclin D1. In Rac1- or iNOS-deficient chondrocytes, ATF3 levels are increased, resulting in reduced activity of the cyclin D1 promoter, which in turn slows chondrocyte proliferation and induces premature cell cycle exit. Additional pathways might contribute to NO effects on cyclin D1 expression (dashed line).

of chondrocytes in monolayer culture and reduced proliferation in tibia organ culture.

NO and NOS genes have been studied extensively in the context of bone remodeling and arthritis (Scher et al., 2007; van't Hof and Ralston, 2001), but much less is known about the roles of this pathway in endochondral bone development (Teixeira et al., 2008). Teixeira and colleagues showed that excess NO promotes hypertrophic differentiation and apoptosis of chondrocytes in chicken (Teixeira et al., 2005; Teixeira et al., 2001), whereas our data suggest that basal levels of NO are required for chondrocyte proliferation. Interestingly, we recently demonstrated a similar phenotype as described here (e.g. reduced proliferation and cyclin D1 expression, increased levels of ATF3 and premature cell cycle exit) in eNOS-deficient mice (Yan et al., 2010). Together, these studies suggest a dose-dependent effect of NO in the growth plate where low levels are required for proliferation, but high levels promote terminal differentiation and ultimately apoptosis. This model is in agreement with suggested dose-dependent effects of NO (Thomas et al., 2008). It should be noted in this context that in vitro data from our and other laboratories suggest Rac1 functions during chondrocyte hypertrophy (Kerr et al., 2008; Wang and Beier, 2005); therefore, it is possible that iNOS or other NOS enzymes also contribute to this role of Rac1. However, it appears probable that the Rac1–iNOS interaction demonstrated by us is not exclusive; for example, Rac1 probably acts through additional effectors, including ROS and the actin cytoskeleton, and iNOS activity and expression in chondrocytes is probably controlled by additional upstream regulators.

The similar phenotypes found in this and our earlier study (Yan et al., 2010) also suggest partial overlap of iNOS and eNOS function in chondrocytes. NO generated from either source inhibits the onset of ATF3 expression, allowing continued expression of cyclin D1 and thus delaying cell cycle exit. Because the effects of SIN-1 on ATF3 expression are less pronounced than its rescue of cyclin D1 levels, it is probable that additional transcriptional or post-transcriptional events contribute to the effects of iNOS–NO signaling on cyclin D1 expression. However, eNOS expression was not affected by loss of Rac1, suggesting that the two genes are regulated by different upstream pathways and mediate mitogenic responses to different external stimuli.

In conclusion, this study identified the iNOS/NO pathway as a central mediator of the mitogenic activity of Rac1 in growing endochondral bones. Further research into this will provide us with novel approaches towards the treatment of various skeletal diseases such as chondrodysplasias.

Materials and Methods

Animals and materials

Timed-pregnant CD1 mice were purchased from Charles River Laboratories at embryonic day (E) 15.5. *S*-nitroso-*N*-acetylpenicillamine (SNAP), 2,2′-(hydroxynitrosohydrazino) bis-ethanamine (NOC18) and the Rac1 inhibitor (NSC23766) were obtained from Calbiochem. N(G)-nitro-L-arginine methyl ester (L-NAME), 3-morpholinosydnonimine (SIN-1) and sodium nitroprusside (SNP) were obtained from Sigma and reconstituted in dimethyl sulfoxide (DMSO, vehicle) according to the manufacturer's instructions. Cell culture materials and general chemicals were obtained from Invitrogen, Sigma or VWR unless otherwise stated.

The following antibodies were employed in this study: iNOS ab15323-500, nNOS 1376 and eNOS ab3868-100 (Abcam); Rac1 sc-217, nitrotyrosine sc-32757, ATF3 sc-188, goat anti-mouse sc-2005 and goat anti-rabbit sc-2004 (all from Santa Cruz Biotechnology); cyclin D1 (SP4) 9104-S1 (NeoMarkers Inc.); BrdU 03-3900 (Zymed Laboratories).

Mouse breeding and genotyping

Mice with a deletion of the iNOS gene (*Nos2*^{tm1Lau}; stock no. 002609) (Laubach et al., 1995) and control mice with C57/BL6 background were obtained from Jackson Laboratory (Bar Harbor, ME), and exposed to a 12-hour light–dark cycle and given tap water and regular chow ad libitum. 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 *Nos2* alleles as described previously (Tranguch and Huet-Hudson, 2003). PCR fragments were analyzed by agarose gel electrophoresis.

Primary cell culture and treatments

Primary chondrocytes were isolated essentially as described (James et al., 2006; Woods et al., 2009a). Our previous studies had established that these cells maintain their chondrogenic phenotype for several days, based on their gene expression profile (Bursell et al., 2007; Stanton and Beier, 2007). Long bones from limbs were isolated from E15.5 mouse embryos and placed in α-minimum essential medium (α-MEM; Invitrogen) containing 0.2% bovine serum albumin (BSA), 1 mM β-glycerophosphate, 0.05 mg/ml ascorbic acid and penicillin–streptomycin. Then the medium was removed and the bones placed in 4 ml of 0.25% trypsin–EDTA (Invitrogen) for 15 minutes at 37℃. Trypsin was subsequently replaced with 1 mg/ml collagenase P (Roche) in Dulbecco's modified Eagle's medium (DMEM)–10% fetal bovine serum (Invitrogen), and cells were incubated at 37℃ with rotation at 100 rpm for 90 minutes. Following digestion, the cell suspension was centrifuged for 5 minutes at 1000 rpm, and the collagenase containing supernatant was decanted. Chondrocytes were resuspended in medium containing 2:3 DMEM:F12, 10% fetal bovine serum, 0.5 mM L-glutamine and penicillin–streptomycin (25 units/ml). Cells were seeded in six-well NUNC plates at a density of 5×10^4 cells per ml and incubated overnight. Primary monolayer chondrocytes were treated with 10^{-3} M L-NAME, 10^{-5} M SNAP or NOC-18 or the DMSO control (vehicle) diluted in fresh medium supplemented with 0.25 mM ascorbic acid (Sigma) and 1 mM β-glycerophosphate (Sigma) and incubated for up to 5 days with replacement of the medium and treatments every other day.

Adenoviral infections

Adenovirus expressing Cre recombinase or GFP (control) was purified using the Adeno-XTM virus mini-purification kit (Clontech) and incubated with primary chondrocytes from *Rac1*$^{fl/fl}$ mice at a multiplicity of infection (MOI) of 50. Medium was changed 4 hours after culturing and cells were then cultured as described above.

ROS and NO measurements

Intracellular ROS and NO were measured fluorometrically. The nonfluorescent dye 2′,7′-dichlorofluorescein diacetate (DCF-DA) permeated cells easily and hydrolyzed to fluorescent 2′,7′-dichlorofluorescein (DCF) upon interaction with intracellular ROS. Cultured cells were washed twice with PBS. Cells were then incubated with 20 μM of DCF-DA for 30 minutes and washed with PBS. The DCF fluorescence was analyzed using a fluorometer (SAFIRE microplate reader, TECAN, Australia) at an excitation of 485 nm and an emission of 530 nm. Nitric oxide production in chondrocytes was analyzed using DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate), a fluorescence probe specific for NO, that was diluted in culture medium and added to cells (10 μM). Cells were incubated with DAF-FM for 30 minutes at 37℃ and then washed with PBS. Measurements were made using the SAFIRE microplate reader (TECAN, Australia; excitation, 495 nm; emission, 515 nm).

Cell proliferation assays

Cell proliferation assays were performed using CyQUANT NF Cell Proliferation Assay Kit (Invitrogen) according to manufacturer's protocol, as described previously (Yan et al., 2010). Chondrocytes were isolated as described above and seeded in 96-well NUNC plates at a density of 1000 cells/100 μl/well. After treatment, the medium was discarded and replaced with CyQUANT NF dye reagent diluted in Hanks' balanced salt solution (HBSS) and incubated for 30 minutes in 37℃ incubator. Measurement of the fluorescence intensity of each sample used SAFIRE microplate reader (TECAN, Australia) with excitation at 480 nm and emission detection at 520 nm with a minimum of six individual wells per treatment in duplicate and three independent cell isolations. Images of primary chondrocyte were taken after treatments of 1 day in culture using a Nikon SMZ1500 microscope with Photometric CoolSNAP color digital camera (Nikon Canada) and PTI Image Master 5 program.

Tibia organ culture

Tibiae were isolated from E15.5 embryos from CD1 timed-pregnant mice (Charles River Canada) using the Stemi DV4 Stereomicroscope (Zeiss) as described (Agoston et al., 2007; Ulici et al., 2008). Dissection day was considered to be day 0 and tibiae were allowed to recover from dissection overnight in serum-free α-MEM containing 0.2% BSA, 0.5 mM L-glutamine, 40 units penicillin/ml and

40 μg streptomycin/ml as described (Agoston et al., 2007). The following morning, bones in 24-well Falcon plates were measured using an eyepiece in the Stemi DV4 Stereomicroscope and treated with 10^{-3} M L-NAME or 5×10^{-5} M Rac1 inhibitor (NSC23766) with or without 10^{-5} M SNP, and DMSO as the vehicle. Medium was changed every 48 hours beginning on day 1, and bones were measured on days 1 and 6. Results are expressed as change in length relative to day 1. Experiments were repeated at least three times, with four to six bones per treatment for each trial.

Histology, immunohistochemistry and BrdU labeling

After experiment completion, tibia samples from organ culture or newborn mice were rinsed with PBS and fixed in 4% paraformaldehyde overnight. Bones were then placed in 10% formalin solution and sent for embedding and sectioning at the Molecular Pathology Core Facility at the Robarts Research Institute (London, Ontario, Canada). Following sectioning, bones were stained with Hematoxylin and Eosin (H&E) or Safranin O and Fast Green using standard protocols (Gillespie et al., 2011; Solomon et al., 2009; Ulici et al., 2009; Wang et al., 2007; Yan et al., 2010). For immunohistochemistry, sections were incubated with primary anti-cyclin D1 antibody (1:50 dilution), anti-ATF3 antibody (1:200 dilution), anti-nitrotyrosine antibody (1:200 dilution) or anti-iNOS antibody (1:1000 dilution) overnight at 4°C. For BrdU labeling, newborn mice were injected intraperitoneally 1 hour before being killed with BrdU (Roche) at a dose of 0.01 ml/g. BrdU was detected in paraffin sections using anti-BrdU antibody (1:100 dilution). Bound antibody was visualized using the UltraVision LPValue detection system (Lab Vision) with AEC chromogen substrate (Lab Vision).

RNA isolation and real-time RT-PCR

Total RNA was isolated from epiphyseal cartilage of long bone from newborn mice or primary cells using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations. Taqman real-time PCR was performed as described previously (Woods and Beier, 2006; Woods et al., 2005) with primers and probe sets from Applied Biosystems (*Atf3* Mm00476032_m1; *Gapdh* Mm99999915_g1). Data were normalized to *Gapdh* mRNA levels and given as means ± s.e.m. from direct comparison of mutant and control littermates from three different crosses.

Alcian Blue and Alizarin Red staining

Alcian Blue and Alizarin Red staining of newborn *Nos2* KO and control mice was done as described (Solomon et al., 2009; Ulici et al., 2009; Wang et al., 2007). Briefly, mice were skinned, eviscerated and dehydrated in 95% ethanol and acetone, then stained with 0.015% Alcian Blue, 0.05% Alizarin Red and 5% acetic acid in 70% ethanol for several days. After clearing in 1% KOH and passing through a decreasing KOH series, skeletons were stored in a 1:1 mixture of glycerol and ethanol.

Statistical analysis

All studies were done in at least three independent cell isolations or mice. Statistical significance was determined by Student's *t*-test or by one-way ANOVA with Bonferroni post test, using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA; www.graphpad.com). In figures where values were shown as percentages, data were first log-transformed before applying ANOVA.

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