Cbfa-1 mediates nitric oxide regulation of MMP-13 in osteoblasts

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Summary

During bone development, osteoblast differentiation requires remodeling of the extracellular matrix. Although underlying mechanisms have not been elucidated, evidence points to the participation of the nitric oxide (NO) and cyclic guanosine 3′,5′-monophosphate (cGMP) system. Here, we detected increased matrix metalloproteinase (MMP)-13 mRNA, protein and activity, as well as increased inducible NO synthase (iNOS) and NO production during the differentiation of MC3T3-E1 osteoblasts. Transcriptional activity of the MMP-13 promoter was augmented by NO, 8-bromo-cGMP (8-Br-cGMP), and by a dominant-positive form of protein kinase G (PKG1-α). The stimulatory effect on the MMP-13 promoter was partially inhibited by mutation of the osteoblast-specific element 2 (OSE-2) binding site. Core binding factor-1 (Cbfa-1) expression peaked at 7 days of differentiation, and was phosphorylated by PKG in vitro. Cbfa-1 was localized to cell nuclei, and its translocation was inhibited by the iNOS inhibitor 1400W. Immunohistological examination revealed that MMP-13 and Cbfa-1 expression levels are both reduced in 17-day-old embryos of iNOS-deficient mice. Silencing of Cbfa-1 mRNA blocked MMP-13 expression without interfering with endogenous NO production, confirming its role in NO-induced MMP-13 expression by MC3T3-E1 cells. The results described here suggest a mechanism by which NO regulates osteogenesis.

Key words: Matrix metalloproteinases, MMP-13, Nitric oxide, iNOS, Osteoblasts, cGMP/PKG, Bone development

Introduction

Bone development requires a complex remodeling of the extracellular matrix, and this is in large part mediated by matrix metalloproteinases (MMPs). Osteoblastic cells express several MMPs implicated in bone morphogenesis (Ortega et al., 2003), osteoblast and chondrocyte migration (Blavier and Delaisse, 1995; Onodera et al., 2004), unmineralized matrix degradation (Uchida et al., 2001), and cell invasion (Javed et al., 2005).

Evidence from gene-knockout studies shows that bone formation and resorption are regulated by nitric oxide (NO): mice deficient in endothelial NO synthase (eNOS) or cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent protein kinase (PKG) show bone abnormalities (Aguirre et al., 2001; Chae et al., 1997; Chikuda et al., 2004; Miyazawa et al., 2002; Otsuka et al., 1998; Talts et al., 1998) and inducible NO synthase (iNOS)-null mice show imbalances in bone osteogenenis (van't Hof et al., 2000). However, the mechanisms through which NO influences osteogenesis remain unclear.

The transcription factor core binding factor 1 (Cbfa-1; also called AML-3, Runx-2 and PEBP.2α) is a key mediator of bone differentiation. Cbfa-1 is required for osteoblastic cell differentiation and ossification (Franceschi and Xiao, 2003; Lian et al., 2004), and its expression correlates with the transition of osteoblasts from the proliferative to the differentiated phenotype (Pratap et al., 2003). Cbfa-1 is closely associated with osteogenic processes known to involve MMPs and NO. Cbfa-1 mediates NO-induced expression of genistein in murine bone marrow stromal cells (BMSCs) (Pan et al., 2005) and of osteoprotegerin in ovarectomized rats (Wang et al., 2004b) and the gene encoding MMP-13 is one of many regulated by Cbfa-1 in osteoblasts (Hess et al., 2001; Jimenez et al., 1999; Porte et al., 1999; Selvamurugan et al., 2000; Winchester et al., 2000).

It was already known that NO is involved in bone metabolism, that Cbfa-1 is implicated in mediating MMP expression, and that NO regulates the expression and activity of MMP-13 in endothelium (Lopez-Rivera et al., 2005); this led us to investigate the mechanisms responsible for such actions. Here, we investigated the action of NO during differentiation of the osteoblastic cell line MC3T3-E1. NO induces MMP-13 expression and activity, a process in which Cbfa-1 is required. Our findings further indicate that NO might phosphorylate Cbfa-1 by PKG, thereby suggesting a molecular mechanism through which NO regulates bone metabolism.

Results

MC3T3-E1 differentiation induces iNOS and MMP-13 expression, and NO regulates MMP-13 production

To test the expression of MMP-13 and iNOS in response to cell differentiation, we incubated MC3T3-E1 cells in the presence of 50 μM ascorbic acid and 10 mM β-glycerol phosphate, to

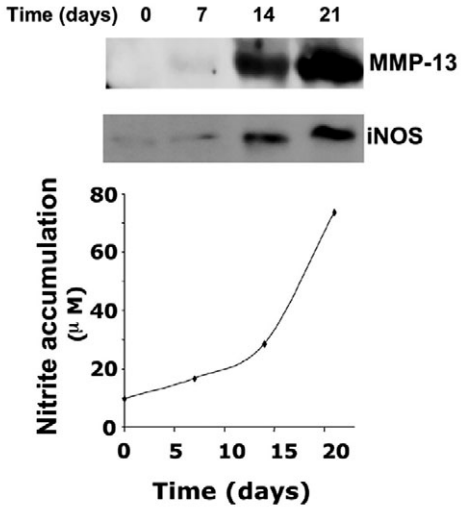

Fig. 1. MMP13 and iNOS protein levels during MC3T3-E1 differentiation. MC3T3-E1 cells were analyzed at the time points indicated in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate. MMP-13 (upper panel) and iNOS (lower panel) were detected by immunoblot (n=3), whereas NO production was detected by Griess reaction (lower graph) (n=3).

induce osteoblast differentiation as reported (Fratzl-Zelman et al., 1998). After 14 and 21 days of incubation, we found a significant increase of MMP-13 protein and iNOS protein as detected by immunoblot, as well as NO production from culture supernatants collected at the time points indicated (Fig. 1).

MMP-13 has been previously reported by others to be induced by ascorbate-mediated differentiation of MC3T3-E1 cells (Mizutani et al., 2001). However, the contribution of NO on such effect is unknown. By incubating differentiated MC3T3-E1 cells with the pharmacological inhibitor of iNOS 1400W (200 μM), we found a decrease in MMP-13 levels (Fig. 2A), gelatinolytic activity (Fig. 2B) and MMP-13-associated activity (Fig. 2C), whereas MT1-MMP (a MMP expressed in MC3T3-E1 cells) did not show variation in response to 1400W (Fig. 2A, lower panel).

iNOS transcriptionally regulates MMP-13 expression in MC3T3-E1 cells

To analyze the effect of NO in the transcriptional regulation of MMP-13, we transiently transfected MC3T3-E1 with pMMP-13 WT, a plasmid containing the MMP-13 promoter region fused to a luciferase reporter gene. Administration of diethylamine NONOate (DEA-NO) (100 μM) induced the activity of MMP-13 promoter significantly above control cells, whereas incubation with 1400W alone (200 μM) brought MMP-13 promoter activity below basal levels (Fig. 3A).

We investigated the relevance of the NO-cGMP-PKG pathway in NO-mediated MMP-13 expression, and found that DEA-NO (100 μM) and the cGMP-soluble analog 8-Br-cGMP (10 μM) stimulated MMP-13 promoter activity. Furthermore, in cells challenged with the pharmacological inhibitor of soluble guanylate cyclase (sGC) [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (20 μM), the stimulatory effect of NO was partially suppressed (Fig. 3B). In addition,

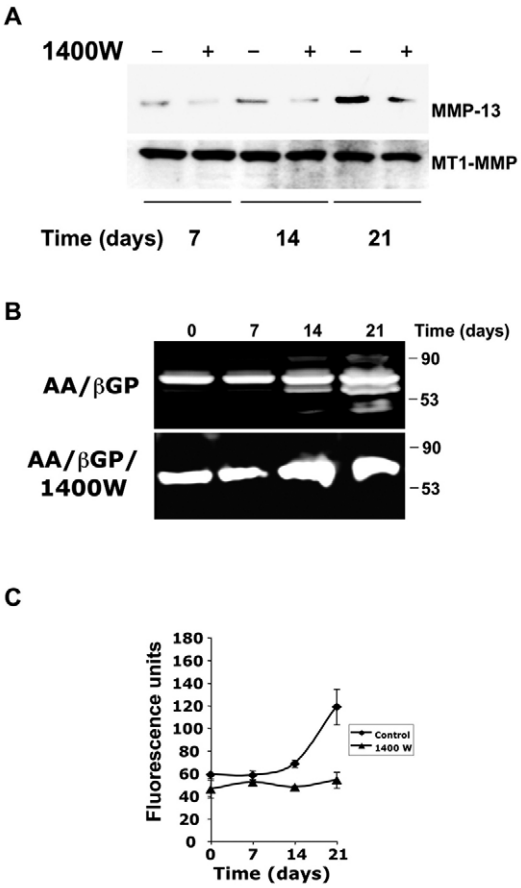

Fig. 2. NO reduces MMP-13 protein and MMP-13 activity during MC3T3-E1 differentiation. MC3T3-E1 cells were induced to differentiate with ascorbic acid (AA) and β-glycerol phosphate (βGP), and analyzed at the time points indicated in the presence or absence of 200 μM 1400W. (A) Immunoblot of MMP-13 and MT1-MMP (n=3). (B) Gelatin zimography (n=3). (C) Detection of MMP-13 activity by using a fluorogenic substrate from culture supernatants (n=3, mean ± s.d.).

overexpression of the PKG1-α dominant-positive isoform (Zaragoza et al., 2002b) also stimulated MMP-13 promoter activity and MMP-13 expression (Fig. 3C).

To test the relevance of Cbfa-1 in MMP-13 expression mediated by the NO-cGMP pathway, MC3T3-E1 cells were transiently transfected with pMMP-13 WT or pMMP-13 OSE2, a plasmid encoding a mutation at the OSE-2 binding site of Cbfa-1. In cells transfected with pMMP-13 OSE2, the stimulatory effects of DEA-NO and 8-Br-cGMP were significantly reduced, as compared with cells transfected with pMMP-13 WT (Fig. 4A and 4B respectively), suggesting that the NO-cGMP pathway regulates the expression of MMP-13 in MC3T3-E1 by the targeted activation of Cbfa-1.

Nuclear localization of Cbfa-1 during MC3T3-E1 differentiation is prevented by inhibition of iNOS, and Cbfa-1 is phosphorylated by PKG in vitro

Cbfa-1 protein expression was markedly increased at day 7 of MC3T3-E1 differentiation (Fig. 5A). Participation of the NO-cGMP pathway seems unlikely since Cbfa-1 levels remained

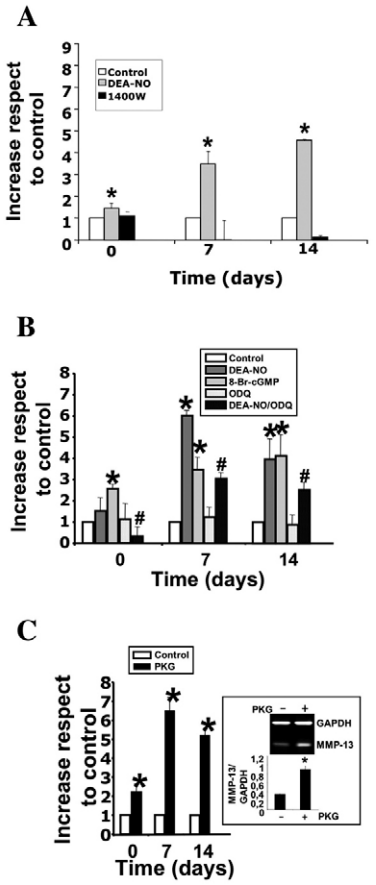

Fig. 3. The NO-cGMP-PKG pathway transcriptionally regulates MMP-13 expression during MC3T3-E1 differentiation. MC3T3-E1 cells were induced to differentiate and analyzed at the time points indicated. (A) MMP-13 promoter activity in MC3T3-E1 transiently transfected with pMMP-13 WT, and incubated with 100 μM DEA-NO, or 200 μM of the iNOS inhibitor 1400W (n=3, mean ± s.d., *P<0.05 versus control). (B) Measurement of MMP-13 promoter activity in MC3T3-E1 cells transiently transfected with pMMP-13 WT, and incubated with 100 μM DEA-NO, 10 μM 8-Br-cGMP, 20 μM ODQ, and 100 μM DEA-NO plus 20 μM ODQ (n=3, mean ± s.d., *P<0.05 versus control, $^{\#}P$<0.05 versus DEA-NO). (C) Measurement of MMP-13 promoter activity in MC3T3-E1 cells transiently transfected with pMMP-13 WT and co-transfected with a dominant-positive construct of PKG1-α (black bars) (n=3, mean ± s.d., *P<0.05 versus control). MMP-13 mRNA was evaluated in these cells by RT-PCR analysis using GAPDH as a control (inset shows a representative experiment and a densitometric analysis of three independent experiments, mean ± s.d., *P<0.05 versus absence of PKG).

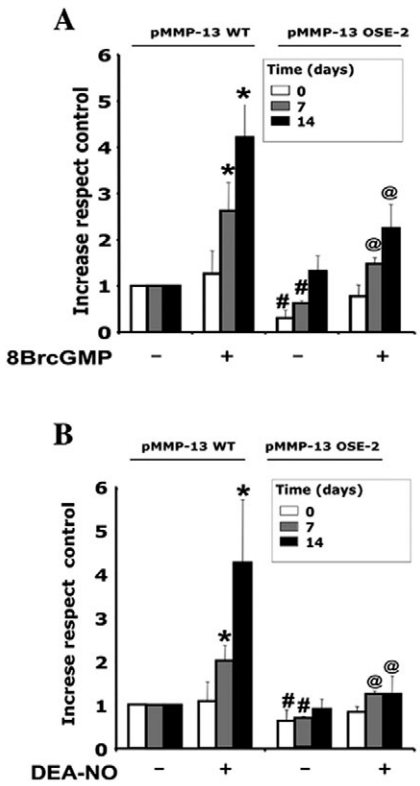

Fig. 4. The NO-cGMP-PKG pathway regulates MMP-13 expression through the Cbfa-1 responsive element OSE-2. Measurement of MMP-13 promoter activity in MC3T3-E1 cells transiently transfected with pMMP-13 WT or pMMP-13 OSE-2. (A) In the absence or presence of 8-Br-cGMP (8BrcGMP). (B) In the absence or presence or DEA-NO (n=3, mean ± s.d., *P<0.05 versus non-treated with 8-Br-cGMP or DEA-NO, $^{\#}P$<0.05 pMMP-13 OSE-2 versus pMMP-13 WT non-stimulated with 8-Br-cGMP or DEA-NO, $^{@}P$<0.05 pMMP-13 OSE-2 versus pMMP-13 WT stimulated with 8-Br-cGMP or DEA-NO).

unchanged in the presence of either NO or 8Br-cGMP (Fig. 5B). Taking into account this result, we evaluated alternative effects of NO, which could explain the effect on Cbfa-1-mediated transcriptional activity of MMP-13. We first monitored the pattern of Cbfa-1 expression by confocal microscopy. Cell differentiation induced Cbfa-1 expression and nuclear localization by day 7 (Fig. 5C, panels i versus panels ii), whereas, strikingly, inhibition of iNOS with 1400W (200 μM) markedly reduced nuclear localization of Cbfa-1 (Fig. 5C, day 7, panels iii versus panels ii), and the result was further confirmed by immunoblot of nuclear and cytosolic extracts from MC3T3-E1 cells at days 7 and 14 of differentiation (Fig. 5D). Taken together, these results suggest nuclear localization of Cbfa-1 as a mechanism elicited by NO during MMP-13 expression in MC3T3-E1 cells.

The fact that the NO-cGMP-PKG signaling pathway regulates Cbfa-1-mediated MMP-13 transcription, and taking into account that iNOS inhibition reduces nuclear levels of Cbfa-1, lead us to investigate if NO might regulate nuclear Cbfa-1 levels by PKG-mediated phosphorylation. During in vitro kinase assays, we found that recombinant purified Cbfa-1 was phosphorylated by recombinant PKG exclusively in the presence of cGMP (Fig. 5E, lane 4 versus lane 5). Autophosphorylation of PKG was also detected as reported by others (Chu et al., 1998; Smith et al., 2000). Thus, the NO-cGMP-PKG pathway might drive Cbfa-1-dependent gene expression in osteoblasts by regulating nuclear translocation of Cbfa-1.

Cbfa-1 expression is reduced in the embryos of iNOS-null mice

To analyze the influence of NO on Cbfa-1 localization in vivo, we detected the expression of Cbfa-1 and MMP-13 over a time

course of embryonic development in wild-type (WT) and iNOS-null mice (Fig. 6, mouse embryos at 17 days post-coitum). Cbfa-1 and MMP-13 levels were mostly detected in bone structures and, to a lesser extent, in surrounding tissues. However, in iNOS-null mice, the overall staining for Cbfa-1 and MMP-13 was significantly reduced (Fig. 6; Cbfa-1, A versus C; MMP-13, B versus D). A more detailed analysis shows a clear reduction in iNOS-null embryos of MMP-13 and Cbfa-1 in the ribs (Fig. 6; MMP-13, J versus L; Cbfa-1, I versus K) and vertebrae (Fig. 6; MMP-13, N versus P; Cbfa-1, M versus O). A similar pattern could also be detected in craniofacial structures. The Meckel's cartilage was negative for MMP-13 and Cbfa-1 in iNOS WT and iNOS-null embryos, as well as chondrogenic regions of vertebrae. However, MMP-13 and Cbfa-1 levels were reduced in the nasal bones, frontal bones and mandibles of iNOS-null embryos (Fig. 6; MMP-13, F versus H; Cbfa-1, E versus G), pointing towards the relevance of iNOS during mouse development. This result was further verified by immunoblot of lysates from 17-day-old WT and iNOS-deficient embryos (Fig. 6Q).

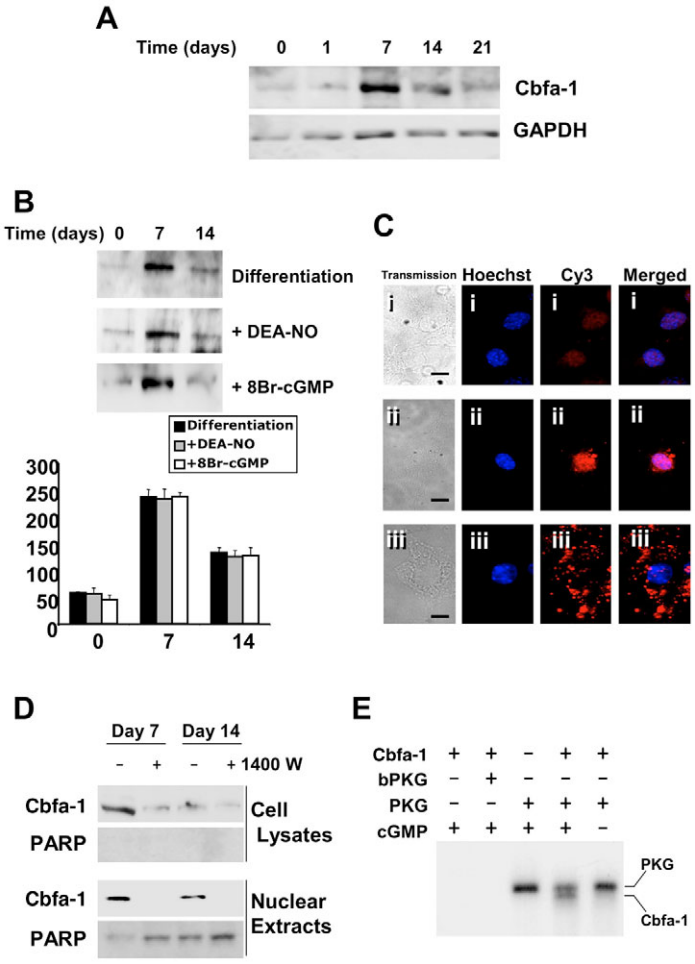

Fig. 5. Effect of the NO-cGMP-PKG pathway on Cbfa-1 regulation in MC3T3-E1 cells. (A) Immunoblot analysis of Cbfa-1 over a time course of MC3T3-E1 differentiation (n=3). (B) Immunoblot analysis of Cbfa-1 over a time course of MC3T3-E1 differentiation in the presence of DEA-NO or 8Br-cGMP (n=3). (C) Immunocytochemistry analysis of Cbfa-1 (red) at day 7 of MC3T3-E1 differentiation. Cells were induced to differentiate (ii), or not (i), or induced to differentiate in the presence of 200 μM 1400W (iii). Nuclei were visualized with Hoechst (blue) (n=3). Bars, 25 μm. (D) Cbfa-1 expression from nuclear extracts and cytosolic extracts of MC3T3-E1 after 7 and 14 days of differentiation. PARP was detected as a control for nuclear integrity (n=3). (E) Recombinant Cbfa-1 (2 μg) was incubated with 100 units of PKG or 100 units of denatured PKG (bPKG) in phosphorylation buffer (see Materials and Methods), containing 1 μCi of [^{32}P]γ-ATP, in the presence or absence of 100 μM cGMP. Samples were electrophoresed and phosphorylation was visualized by autoradiography (n=2).

NO-cGMP-mediated expression of MMP-13 depends on Cbfa-1

To determine the contribution of Cbfa-1 to NO-mediated MMP-13 expression, we silenced the expression of Cbfa-1 by RNA interference. In cells in which Cbfa-1 was silenced, MMP-13 protein abundance was three times lower than in non-silenced cells (Fig. 7, upper left), even when NO levels increased as a result of cell differentiation (Fig. 7, lower graph). By contrast, gene silencing of GAPDH did not affect either MMP-13 or Cbfa-1 expression levels (Fig. 7, upper right). This result suggests that, even when NO levels are kept high, the presence of Cbfa-1 is essential for MMP-13 expression. This is consistent with the concept of Cbfa-1 as a target for NO-cGMP-PKG in the expression of MMP-13 in this osteoblastic cell line.

Discussion

Our results indicate that NO is an important mediator in the expression of MMP-13 during osteoblast differentiation, providing insights into the molecular signaling pathways activated by NO.

The active involvement of NO in bone healing (Corbett et al., 1999; Diwan et al., 2000; Wang et al., 2004a; Zhu et al., 2001), bone development (Aguirre et al., 2001; Armour et al., 2001; Collin-Osdoby et al., 1995; Turner et al., 1997; van't Hof and Ralston, 2001) and bone loss (Cuzzocrea et al., 2003; Wang et al., 2004b) has been reported. Mice lacking eNOS present profound abnormalities in bone formation, and osteoblasts from calvarial explants show retarded proliferation and differentiation. However, the molecular target(s) of NO are not well understood. MMPs, and in particular MMP-13, can be considered good candidates for the actions of NO, owing to their crucial involvement in bone healing (Henle et al., 2005; Yamagiwa et al., 1999; Yang et al., 2004), bone development (Inada et al., 2004; Stickens et al., 2004) and bone loss (Pelletier et al., 2004). Previous work has shown that the transcription factor Cbfa-1, which is fundamental to osteoblast differentiation and regulates the expression of different MMPs, is significantly reduced in osteoblasts from neonatal eNOS-null mice (Afzal et al., 2004). Even when MMP-13 induction in differentiating osteoblasts has been documented (Mizutani et al., 2001), the relationship between NO and MMP-13 expression in bone cells has not been investigated. We found that osteoblast differentiation induced iNOS expression, and that iNOS regulates MMP-13 protein in MC3T3-E1 cells at the transcriptional level through the cGMP pathway, through the activation of the transcription factor Cbfa-1.

The increase of iNOS expression led us to investigate the

effect on MMP-13 in vivo. The analysis of Cbfa-1 and MMP-13 in WT and iNOS-deficient mouse embryos revealed a marked reduction in the expression of both proteins in the absence of NO. Taken together, these results point towards MMP-13 as a target for the role of NO in bone development, and Cbfa-1 as a mediator of its action.

MMP-13 and Cbfa-1 have important roles in bone formation (Stahle-Backdahl et al., 1997). The mechanism leading to increased MMP-13 expression in osteoblasts depends on Cbfa-1 activation (Hess et al., 2001; Jimenez et al., 1999; Porte et al., 1999; Selvamurugan et al., 2000; Winchester et al., 2000). By inhibiting the expression of Cbfa-1 in RNA interference assays, we found that Cbfa-1 regulates NO-mediated MMP-13 expression in MC3T3-E1 cells. Cbfa-1 expression peaked after 7 days of MC3T3-E1 differentiation [as shown by others (Perinpanayagam et al., 2004)], whereas MMP-13 was maximal after 14 days. Cbfa-1 expression is not regulated by NO. However, the presence of Cbfa-1 in the nucleus was still evident at day 14 of differentiation (Fig. 5D), and the process appears to be dependent on NO, thus accounting for the effect on MMP-13 expression. In addition, Cbfa-1 enables cells to evade proliferation (Pratap et al., 2003) and Cbfa-1 expression has been reported to be negatively regulated by Cbfa-1 itself in cultured cells, which could provide a negative-feedback loop that might be used as a repression mechanism (Tou et al., 2003).

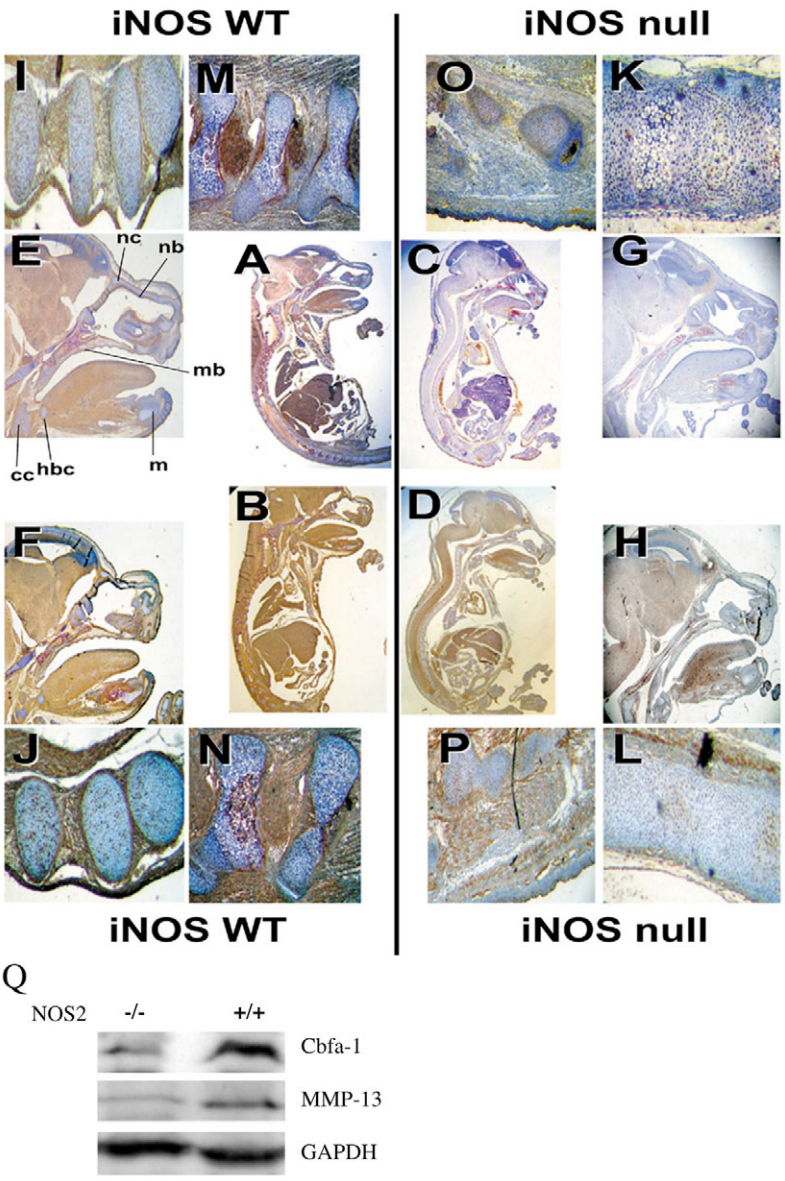

Fig. 6. Cbfa-1 and MMP-13 expression are reduced in iNOS-deficient embryos. Immunohistological detection of Cbfa-1 (A,C,E,G,I,K,M,O) and MMP-13 (B,D,F,H,J,L,N,P) on sections from iNOS wild-type (WT) (left side) and iNOS-deficient (right side) 17-day-old embryos. Experiments were performed in triplicate on sections from three different embryos. Skulls (E,F,GH), ribs (I,J,K,L) and vertebrae (M,N,O,P) are magnified. m, Meckel's cartilage; mb, maxillary bone; nb, nasal bone; hbc, cartilage of the hyoid bone; cc, cricoid cartilage. (Q) Immunoblot detecting the levels of Cbfa-1, MMP-13 and GAPDH from WT (+/+) and iNOS-deficient (NOS2, –/–) mouse embryo lysates (n=3).

Involvement of the NO-cGMP in the activation of Cbfa-1-mediated gene expression has been reported in the case of genistein (Pan et al., 2005). The results shown here, as well as in endothelial cells (Zaragoza et al., 2002a; Zaragoza et al., 2002b), identify cGMP as a mediator of NO-induced expression of MMP-13. In this setting, phosphorylation of Cbfa-1 by PKG might have an important role. Cbfa-1 activity is inhibited by protein kinase inhibitors. In particular, NO increases osteoprotegerin expression through the activation of Cbfa-1 in ovariectomized rats, and the effect is inhibited with herbimycin A (Wang et al., 2004b), a tyrosine kinase inhibitor. Cbfa-1 might become phosphorylated by the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated protein kinase (ERK) pathway, and by protein kinase A (PKA) under certain conditions (Franceschi et al., 2003). However, the kinase(s) responsible for NO-mediated Cbfa-1 activation have not been identified yet. In endothelial cells, MMP-13 expression is regulated by NO through the cGMP-PKG and ERK pathways (Zaragoza et al., 2002b). Taking into account the data shown by others (Franceschi et al., 2003; Pan et al., 2005), and the fact that overexpression of PKG stimulates both the activity of the MMP-13 promoter and MMP-13 expression, we infer that, in osteoblasts, PKG is also able to phosphorylate Cbfa-1. Our in vitro phosphorylation assays confirm that recombinant PKG phosphorylates Cbfa-1 in a cGMP-dependent manner. In silico analysis reveals three putative consensus sequences of Cbfa-1 susceptible to PKG phosphorylation (Thr89, Thr104, Ser340).

Our data provide a link between NO and Cbfa-1, which converge on the expression of MMP-13, and which might have consequences for bone osteogenesis. Future work should contribute to deciphering the pathophysiological relevance of this pathway in animal and human models of disease.

Materials and Methods

Mice and cells

Wild-type C57BL/6 mice and mice disrupted for the gene encoding iNOS were housed in our animal facilities in isolated rooms. Mouse

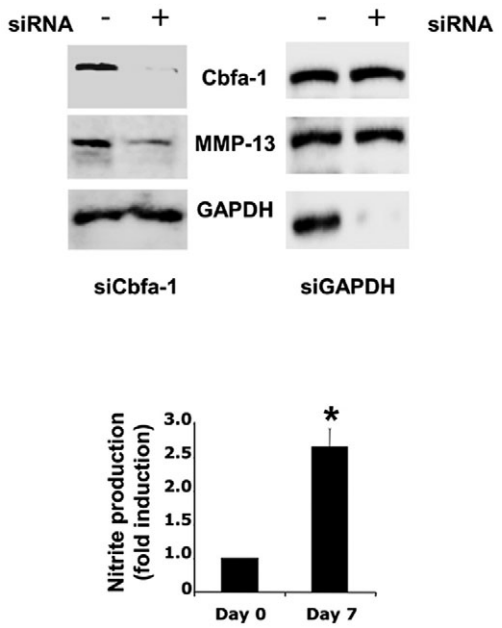

Fig. 7. Cbfa-1 is required for NO-induced MMP-13 expression in differentiating MC3T3-E1 cells. MC3T3-E1 cells were differentiated and, on day 7, Cbfa-1 and GAPDH expression were silenced by RNA interference (siRNA). Cbfa-1, MMP-13 and GAPDH expression were evaluated by immunoblot. The capacity of Cbfa-1-silenced cells to produce NO was evaluated by measuring nitrite accumulation from culture supernatants (lower panel: n=3, mean ± s.d., *P<0.05 versus day 0).

embryos were isolated at regular intervals, embedded in paraffin and sections were obtained for immunohistochemistry.

The osteoblastic cell line MC3T3-E1 was kindly donated by M. Ángel Peñalva (CIB, CSIC, Madrid, Spain). MC3T3-E1 monolayers were grown in 6-well plates, and fed with alpha-minimal essential medium (α-MEM), supplemented with 10% FBS and antibiotics. To differentiate MC3T3-E1, cells were cultured for 21 days in the presence of ascorbic acid (50 μg/ml) and β-glycerol phosphate (10 mM).

Reagents

Cell culture supplies were from Falcon (Beckton-Dickson), serum was from BioWhittaker, antibiotics were from Sigma, RT-PCR reagents were from GIBCO and Taq DNA polymerase was from Applied Biosystems. The Dual Luciferase Reporter System, RNAase inhibitor RNasin, pGL3 plasmids and restriction endonucleases were from Promega. Autoradiography film was from Kodak. Polyvinylidene fluoride (PVDF) protein transfer membranes were from Millipore, and the electrochemiluminescence (ECL)-detecting immunoblot system was from GE. Optimem and Lipofectamine were from GIBCO-BRL. EDTA-free protease inhibitor cocktail tablets were from Roche. Polyclonal antibodies to MMP-13, and active bovine protein kinase G, isoform 1α (form *Spodoptera frugiperda*) were from Calbiochem. Anti-Cbfa-1 monoclonal and anti-iNOS polyclonal antibodies were form SantaCruz Biotechnologies. Anti-MT1-MMP antibody was kindly donated by A. García-Arroyo (CNIC, Madrid, Spain). The Silencer siRNA Construction Kit was from Ambion.

Plasmids

Plasmid pMMP-13 WT contains a functional part of the 5′ promoter of the human MMP-13 (GenBank accession no: NM_002427) located upstream of a luciferase reporter gene. Plasmid pMMP-13 OSE-2 is identical to pMMP-13 WT except for a mutation at the OSE-2 responsive element (Pendas et al., 1997). Dominant-positive PKG, containing the catalytic subunit of cGMP-dependent protein kinase, was expressed in MC3T3-E1 cells as described (Zaragoza et al., 2002b). Recombinant Cbfa-1 was cloned by inserting Cbfa-1 cDNA into the *Sph*I and *Sal*I restriction sites of the pQ30 vector (Qiagen).

Nitrite assay

Nitrite concentration in culture supernatants was determined by a modification of the Griess assay as described (Zaragoza et al., 2002b).

Immunoblot analysis

Cell lysate extraction and protein immunoblots were performed as described (Zaragoza et al., 2002b).

Transient transfection of MC3T3-E1 cells

Transient transfections were conducted with Lipofectamine 2000 reagent as described (Zaragoza et al., 2002b). MMP-13 promoter activity was measured by the expression of the luciferase gene reporter. Experiments were carried out using the Dual Luciferase Reporter system, co-transfecting BAEC with a pGL3 reporter plasmid expressing Renilla under the control of a CMV promoter (pCMV-Renilla).

Gelatin zymography

MC3T3-E1 culture supernatants were collected over a time course of differentiation, and gelatin zymography was conducted using pre-cast Biorad gelatin zymogram gels, according to the manufacturer's instructions.

MMP-13 activity assay

MMP-13 activity was assayed fluorimetrically using a fluorescent substrate from Chemicon as previously described (Zaragoza et al., 2002a).

RNA isolation and RT-PCR

Total RNA was isolated by the guanidinium thiocyanate method as described (Chomczynski and Sacchi, 1987). RNA was detected by real-time quantitative RT-PCR, as previously described (Zaragoza et al., 2002b). The following primers were selected: sense primer, 5′-CCAAATTATGGAGGAGATGC-3′; antisense primer, 5′-CGCCAGAAGAATCTGTCTTTAAA-3′. As a reference gene, we used the following primer set for glyceraldehyde-3 phosphate dehydrogenase (GAPDH): sense primer, 5′-AGTGGGTGATGCTGGTGCTG-3′; antisense primer, 5′-CGCCTGCTTCACCACCTTCTT-3′. The specificity of the amplification was verified by resolution of the PCR products on ethidium bromide agarose gels.

Gene silencing

Cbfa-1 or GAPDH expression were silenced in MC3T3-E1 cells by transient transfection of cells with pre-annealed siRNA oligonucleotides, corresponding to specific regions of the murine Cbfa-1 and GAPDH genes (pre-validated siRNAs; Ambion), as described (Lopez-Rivera et al., 2005).

Confocal microscopy

MMP-13 and Cbfa-1 from MC3T3-E1 were detected by confocal microscopy as previously described (Lopez-Rivera et al., 2005).

In vitro phosphorylation assay

Recombinant Cbfa-1 was purified by nickel chromatograpy as described (Lopez-Rivera et al., 2005). The efficacy of PKG to phosphorylate Cbfa-1 was tested by incubating both proteins in the presence or absence of cGMP in phosphorylation buffer (20 mM Tris HCl, pH 7.5; 10 mM MgCl2; 10 mM DTT; 20 mM ATP, 10 μM NaF), containing 10 μCi [γ-^{32}P]ATP, for 30 minutes at room temperature. Proteins were then electrophoresed on 10% PAGE; the gels were washed with 10% acetic acid, dried and exposed to autoradiography.

Statistical analysis

Unless otherwise specified, data are expressed as means ± s.d., and experiments were performed at least three times in duplicate. Comparisons were made by non-parametric statistics using the Wilcoxon Rank-Sum test whenever comparisons were made with a common control. The level of statistical significance was defined as P<0.05. Error bars represent ±s.d.

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