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FGF-8 Stimulates the Expression of NR4A Orphan Nuclear Receptors in Osteoblasts

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ABSTRACT

Nurr1, NGFI-B, and Nor1 form the NR4A subfamily of orphan nuclear receptors. The NR4A receptors are immediate early genes that can be rapidly induced in response to a variety of stimuli in many cell types, for example in osteoblasts. Nurr1 regulates the differentiation of osteoblasts and the expression of several osteoblastic genes. Fibroblast growth factor 8b (FGF-8b) regulates osteoblastic differentiation. We show here that treatment of preosteoblastic MC3T3-E1 cells or mouse bone marrow mesenchymal cells with FGF-8b induces the expression of NR4A receptors rapidly and in a dose-dependent manner. This induction involves MAPK, PI-3K, and PKC pathways. FGF-8b stimulates the proliferation of MC3T3-E1 cells. This effect is enhanced by overexpression of Nurr1 and NGFI-B whereas it is abolished by a dominant negative Nurr1 variant. In conclusion, FGF-8b induces the expression of NR4A orphan nuclear receptors that are involved in mediating the growth promoting effect of FGF-8b in osteoblasts.

INTRODUCTION

Nuclear receptors (NRs)¹ are ligand-inducible transcription factors mediating the effects of small lipophilic ligands such as steroid hormones, retinoids, thyroid hormones, and vitamin D. In addition, there are a large number of NRs lacking identified physiological ligands. These receptors are referred to as orphan NRs (Mangelsdorf et al., 1995; Giguere, 1999; Benoit et al., 2006). NRs regulate important processes in development and homeostasis (Aranda and Pascual, 2001). The NR4A subgroup of orphan NRs consists of NGFI-B (NR4A1), Nurr1 (NR4A2), and Nor1 (NR4A3) (Nuclear Receptors Nomenclature Committee, 1999). Structural analyses of NR4A receptors have demonstrated that these receptors do not possess cavities for ligand binding implying that they are ligand-independent NRs (Wang et al., 2003; Baker et al., 2003; Razzera et al., 2004). The mechanisms modulating the activities of NR4A receptors have remained elusive. The activities of NR4A receptors have been shown to be modulated by cross-talk with other NRs such as retinoid X receptor (RXR) and estrogen-related receptors and by cross-talk with other signaling pathways such as the receptor tyrosine kinase Ret signaling pathway and the nerve growth factor pathway (Perlmann and Jansson, 1995; Katagiri et al., 1997; Aarnisalo et al., 2002; Wang et al., 2003; Lammi et al., 2007). NR4A receptors are expressed for example in the central nervous system, pituitary gland, adrenals, liver, arterial wall smooth muscle cells, macrophages, synovium, and osteoblasts (Zetterström et al., 1996; Maxwell and Muscat, 2006; Mages et al., 1994; Pei et al., 2006). An interesting feature of NR4A receptors is that they are encoded by immediate early genes and thus their expression is rapidly induced in response to various stimuli (Maxwell and Muscat, 2006). For example in osteoblasts, NR4A receptor expression is rapidly increased in response to parathyroid hormone (PTH) (Tetradis et al., 2001a; Tetradis et al., 2001b; Lammi et al., 2004; Pirih et al., 2005). Regulation of the

expression levels of NR4A receptors is most likely an important mechanism modulating their activities.

Fibroblast growth factors (FGFs) regulate a variety of cellular processes during embryonic development and in the adult (Powers et al., 2000; Eswarakumar et al., 2005). During embryonic development, FGFs regulate cell proliferation, differentiation, and migration and thereby control for example the proper development of the central nervous system and skeleton. In the adult, FGFs are important modulators of for example tissue repair, tumor growth, and angiogenesis. FGF-8 was originally identified as androgen-induced growth factor (Tanaka et al., 1998). Eight different FGF-8 isoforms are generated by alternative splicing. Of these, FGF-8b has the greatest ability to activate FGF receptors (FGFRs) in mitogenic assays (MacArthur et al., 1995). FGF-8 has important roles in embryogenesis; it for example regulates limb development, the proliferation and differentiation of osteoblasts, and the development of the midbrain dopaminergic neurons (Crossley et al., 1996; Ye et al., 1998; Moon and Capacchi, 2000; Valta et al., 2006). FGF-8 is also expressed in human breast, ovarian, and prostate cancers (Tanaka et al., 1998; Valve et al., 2000).

FGFs mediate their effects in target cells by binding to specific high-affinity tyrosine kinase receptors designated FGFR1 to 4. Alternative splicing of FGFR1, 2, and 3 generates two functional receptor isoforms – b and c – with different ligand-binding specificities (Powers et al., 2000; Eswarakumar et al., 2005). FGF-8b binds to and activates FGFR2c, FGFR3c, and FGFR4 that are expressed in osteoblasts (MacArthur et al., 1995; Valta et al., 2006). FGFR-mediated signal transduction involves several downstream signaling pathways including mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI-3K), and protein kinase C (PKC) pathways (Eswarakumar et al., 2005).

NR4A receptors have been shown to be expressed in osteoblasts isolated from mouse calvariae and long bones. NR4A receptors are also expressed in several osteoblastic cell lines (Mages et al., 1994; Lammi et al., 2004; Pirih et al., 2005). Nurr1 expression has been reported in the developing limb but the expression pattern of NR4A receptors in developing or mature bone has not yet been analyzed (Zetterström et al., 1996). Nurr1 regulates osteoblastic differentiation and stimulates the expression of osteopontin and osteocalcin genes (Lammi et al., 2004; Pirih et al., 2004; Pirih et al., 2005; Lee et al., 2006). Thus, both FGF-8 and Nurr1 regulate osteoblastic differentiation. FGF-8 regulates the differentiation of the midbrain dopaminergic neurons (Crossley et al., 1996; Ye et al., 1998). Nurr1 plays also a crucial role in the development of these neurons as mice with targeted deletion of the Nurr1 gene fail to develop these cells (Zetterström et al., 1997). FGF-8 and Nurr1 are expressed in mature midbrain dopamine cells. Reduced expression of FGF-8 and Nurr1 has been associated with Parkinson's disease (Tanaka et al., 2001; Le et al., 2003). Differentiation and maintenance of the midbrain dopamine cells seem thus to be processes regulated by both FGF-8 and Nurr1. Mice with targeted deletion of FGF-8 gene present with a severe gastrulation defect (Meyers et al., 1998; Sun et al., 1999), a phenotype that is similar to the one observed in Nurr1 deficient mice (DeYoung et al., 2003). As FGF-8 and NR4A receptors play important roles in the same developmental processes, we studied whether FGF-8 regulated NR4A receptor expression. We show that NR4A receptors are induced as immediate early genes in response to FGF-8b in bone marrow mesenchymal stem cells and in preosteoblasts and that Nurr1 and NGFI-B are involved in mediating the proliferative effect of FGF-8b in preosteoblastic cells.

MATERIALS AND METHODS

Plasmids and chemicals

The expression vectors pCMX-Nurr1, pCMX-NGFI-B, pCMX-Nor1, and pCMX-Nurr1^{DN} were gifts from Dr. Thomas Perlmann (Ludwig Institute for Cancer Research, Stockholm, Sweden). pCMX-Nurr1^{DN} encodes Nurr1 residues 94-356 including part of the amino-terminal domain and the entire DNA-binding domain of Nurr1 fused to the Drosophila repressor protein Engrailed (Castro et al., 2001). The NBRE₃tk-LUC reporter and the pCMX-βgal control plasmid were received from Dr. Ronald Evans (Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA). FGF-8b was purchased from R&D Systems, PD98059 and Calphostin C from Sigma, and LY294002 from Calbiochem. SR11237 was a kind gift from Dr. Thomas Perlmann (Ludwig Institute for Cancer Research, Stockholm, Sweden).

Cell culture

Mouse preosteoblastic MC3T3-E1 and human osteoblastic U2-OS cells were obtained from American Type Culture Collection and maintained in alpha minimum essential medium and Dulbeccos' modified Eagle's medium, respectively. The media were supplemented with 10% fetal calf serum, 25 U/ml penicillin, and 25 µg/ml streptomycin. Primary mouse bone marrow mesenchymal stem cells were isolated from femurs and tibiae of 10 weeks old NMRI mice as described (Qu et al., 1998). The cells were then maintained in alpha minimum essential medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin.

Transfections

U2-OS cells were transfected with the FuGENE transfection reagent (Roche) according to manufacturer's instructions. Briefly, 50 000 cells/well were plated on 12-well plates. After 24 hours, the cells were transfected with 50 ng of the expression vectors, 300 ng of the NBRE₃tk-LUC luciferase reporter plasmid, and 100 ng of pCMX-βgal that was used as an internal control for transfection efficiency. The cells were harvested 24 h later and assayed for luciferase and β-galactosidase activities. The experiments were performed in triplicate dishes and repeated twice.

RNA extraction and RT-PCR

MC3T3-E1 cells (210 000 cells/well) and bone marrow mesenchymal stem cells (500 000 cells/well) were plated on 6-well plates. 24 hours later, the cells received fresh medium supplemented with vehicle, FGF-8b, PD98059, Calphostin C, or LY294002. Total RNA was extracted using the Trizol Reagent (Invitrogen). 2 μg RNA was used for cDNA synthesis with Superscript II (Invitrogen). Real-time RT-PCR was performed with Light Cycler 480 and SYBR Green I dye system according to the manufacturer's protocol (Roche). The program used for PCR amplification was 10 sec at 95°C, 5 sec at 59°C, 10 sec at 72°C, and detection at 84°C. The PCR reactions were performed in triplicates. The expression of NR4A receptors was normalized to the G3PDH values of the respective samples. The results were repeated in three independent experiments. The following primers were used:

Mouse Nurr1 (GenBank [S53744](#))

sense 5'-AAC ACT GAA ATT ACT GCC ACC A-3'

antisense 5'-TTC TAC CTT AAT GGA GGA CTG CT-3'

Mouse NGFI-B (GenBank [NM010444](#))

sense 5'-CTT CAT GGA CGG GTA CAC C-3'

antisense 5'-GCT GGA GGA TAG GGT CTC ATC T-3'

Mouse Nor1 (GenBank [NM015743](#))

sense 5'-GGA GCT ATG CAC CAA GAT CAC-3'

antisense 5'-CTT GTC GAT GAC AGA AGG TGG-3'

G3PDH (Clontech)

sense 5'-ACC ACA GTC CAT GCC ATC AC-3'

antisense 5'-TCC ACC ACC CTG TTG CTG TA-3'.

Cell proliferation assay

MC3T3-E1 cells were plated on 12-well plates (70 000 cells/well) and transfected with 540 ng of the expression vectors or empty vector using the Lipofectamine 2000 transfection reagent (Invitrogen). 24 hours later, the cells received fresh medium supplemented with 10 ng/ml FGF-8b, 1 μ M SR11237, or vehicle. After incubation for 18 hours, 1 μ Ci/ml [3 H]thymidine was added to the cells and the incubation was continued for 2 hours. The cells were then fixed with 5 % TCA, lysed with 0,3 M NaOH, and the incorporated radioactivity was measured using Wallac 1409 Liquid Scintillation Counter (PerkinElmer). The proliferation experiments were performed three times in triplicate dishes.

Apoptosis assay

MC3T3-E1 cells were plated and transfected as described for the proliferation assays. 24 hours after transfection, the cells received fresh medium supplemented with 25 ng/ml FGF-8b or vehicle. Twenty hours later, the cells were trypsinized, counted, and 10 000 cells were analyzed for apoptosis using the Cell Death Detection ELISA^{PLUS} reagent (Roche) according

to manufacturer's instructions. The apoptosis assays were performed three times in triplicate dishes.

Statistical analyses

Statistical analyses were carried out using two-tailed Student's *t* test; $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS

The expression of NR4A receptors is stimulated by FGF-8b in preosteoblastic cells

Preosteoblastic MC3T3-E1 cells express NR4A receptors (Lammi et al., 2004) (Fig. 1). To study the effect of FGF-8b treatment on the expression of NR4A receptors, MC3T3-E1 cells were treated with 10 ng/ml FGF-8b and total RNA was extracted at different time points. The expression of all three members of the NR4A subfamily was rapidly stimulated by FGF-8b treatment (Fig. 1; $p < 0.01$). The mRNA induction was detectable already 30 min after the beginning of the treatment, and the mRNA expression peaked at 1 h. There were slight differences between the receptors in terms of the rate at which the expression declined back to the basal level (Fig. 1.). The expression of Nurr1 and NGFI-B peaked at 1h and then rapidly declined to and below the basal level at 2h and 4 h, respectively (Fig. 1A and 1B). The expression of Nor1 remained elevated until 2h and then declined to the basal level by 4h (Fig. 1C). Similar results were obtained in mouse bone marrow mesenchymal stem cells (data not shown). The expression of all three receptors peaked already at 30 min in these cells. Again, the expression of Nor1 declined more slowly than that of NGFI-B and Nurr1 as it remained elevated at 1h whereas the expression level of Nurr1 and NGFI-B declined already at 1h. These results confirm that the responsiveness of NR4A receptors to FGF-8b is not unique to MC3T3-E1 cells.

Next, we studied whether the induction of the NR4A receptor expression was dependent on the dose of FGF-8b. To that end, MC3T3-E1 cells were treated with increasing doses on FGF-8b for 1 hour and the expression of NR4A receptor mRNAs was determined. FGF-8b induced Nurr1, NGFI-B, and Nor1 expression in a dose-dependent manner (Fig. 2; $p < 0.05$). The expression of NGFI-B and Nurr1 was induced at the FGF-8b concentration 2,5 ng/ml and 5 ng/ml, respectively and the maximum induction was detected at 25 ng/ml (Fig.

2A and 2B). At higher FGF-8b concentrations, the expression levels were slightly lower than at 25 ng/ml. The induction of Nor1 mRNA required slightly higher dose of FGF-8b than the induction of Nurr1 or NGFI-B but treatment with 25 ng/ml FGF-8b yielded the maximum induction also in the expression of Nor1 (Fig. 2C). These results thus show that NR4A receptors are induced as immediate early genes in response to FGF-8b in preosteoblastic cells.

FGF-8b induces NR4A receptors *via* MAPK, PI-3K, and PKC signaling pathways

FGF-8b binds to and activates many of the FGF receptors (FGFRs). FGFRs act through several signaling pathways, including MAPK, PI-3K, and PKC pathways. We next asked which of these pathways is responsible for the induction of the NR4A receptor expression. In order to examine the contribution of these pathways, MC3T3-E1 cells were treated with the MAPK inhibitor PD98059, the PI-3K inhibitor LY294002, and the PKC inhibitor Calphostin C along with FGF-8b. As shown in Figure 3, treatment of the cells with PD98059, LY294002, or Calphostin C significantly reduced the FGF-8b-mediated induction of NR4A receptor expression ($p < 0.05$). This indicates that MAPK, PI-3K, and PKC pathways are involved in mediating the induction of NR4A receptor expression by FGF-8b.

Nurr1 and NGFI-B are involved in FGF-8b-mediated stimulation of the proliferation of preosteoblastic cells

FGF-8b has been reported to stimulate the proliferation of osteoblast precursors (Valta et al., 2006). We examined next the role of NR4A receptors in the proliferation of these cells. To study this, MC3T3-E1 cells were transfected with the expression vectors for Nurr1, NGFI-B, and Nor1, and subsequently, proliferation of the cells was examined by the [3 H]thymidine incorporation assay. Overexpression of Nurr1 and NGFI-B increased the proliferation of

MC3T3-E1 cells by 1,9 fold (Fig. 4B and 4A). In contrast, Nor1 and the dominant negative variant of Nurr1 (Nurr1^{DN}) that abolishes transactivation by all NR4A receptors (Fig. 4A) did not significantly influence proliferation (Fig. 4B and 4D). Treatment of MC3T3-E1 cells with 10 ng/ml FGF-8b stimulated proliferation by 2,2 fold (Fig. 4B; $p < 0.01$). Overexpression of Nurr1 and NGFI-B further increased this effect ($p < 0.01$ and $p < 0.05$, respectively). Nor1, however, did not have this effect. Overexpression of Nurr1^{DN} abolished the proliferative effect of FGF-8b (Fig. 4B; $p < 0.05$).

Nurr1 and NGFI-B form heterodimers with retinoid X receptor (RXR) whereas Nor1 does not (Perlmann and Jansson, 1995; Zetterström et al., 1996). It would have thus been possible that stimulation of proliferation by Nurr1 and NGFI-B involved heterodimerization with RXR. This possibility was studied by transfecting MC3T3-E1 cells with the expression vectors for Nurr1, NGFI-B, and Nor1. Subsequently, the cells were treated with the synthetic RXR agonist SR11237 in order to activate the endogenous RXR in MC3T3-E1 cells. Again, overexpression of Nurr1 and NGFI-B stimulated proliferation whereas Nor1 had no effect. Treatment of the cells with SR11237 did not further enhance proliferation indicating that stimulation of proliferation by Nurr1 and NGFI-B does not involve RXR (Fig. 4E).

NR4A receptors have been reported to regulate apoptosis of different cell types (Cheng et al., 1997; Li et al., 2000; Ke et al., 2004). To study whether they influenced apoptosis of MC3T3-E1 cells and whether FGF-8b was involved in that process, MC3T3-E1 cells were transfected with the expression vectors for Nurr1, NGFI-B, and Nor1. The cells were then treated with 25 ng/ml FGF-8b for 20 hours. Subsequently, the cell cultures were analyzed for apoptosis. Overexpression of Nurr1 and NGFI-B reduced apoptosis of MC3T3-E1 cells by 50% ($p < 0.05$; Fig. 5). In contrast, overexpression of Nor1 did not significantly influence apoptosis (Fig. 5). Treatment of the cells with FGF-8b did not influence apoptosis

in cultures transfected with the empty vector or in cultures transfected with the expression vectors for NR4A receptors. The anti-apoptotic effect of Nurr1 and NGFI-B seemed thus to be independent of FGF-8b signaling. Collectively, these data suggest that Nurr1 and NGFI-B have a growth promoting effect on MC3T3-E1 cells as they reduce apoptosis and as they are involved in mediating the proliferative effect of FGF-8b.

DISCUSSION

NR4A receptors lack identified natural ligands and thus the mechanisms that modulate their transcriptional activities have remained elusive. The activities of NR4A receptors have been reported to be modulated by cross-talk with other NRs and by cross-talk with other signaling pathways (Perlmann and Jansson, 1995; Katagiri et al., 1997; Aarnisalo et al., 2002; Wang et al., 2003; Lammi et al., 2007). As NR4A receptors are encoded by immediate early genes it is plausible that an important mechanism regulating their activities is to regulate their expression levels. Various stimuli induce the expression of NR4A receptors (Maxwell and Muscat, 2006). As both FGF-8 and NR4A receptors play important roles in regulating dopamine cell development and differentiation of osteoblasts, we examined whether FGF-8 regulated the expression of NR4A receptors. We show that FGF-8b induces the expression of all three NR4A receptor family members in preosteoblastic MC3T3-E1 cells and in bone marrow-derived mesenchymal stem cells (Fig. 1 and data not shown). This observation suggests that FGF-8b is a regulator of NR4A receptor activity in preosteoblasts and that FGF-8b and NR4A receptors are involved in the same regulatory pathways.

The stimulation of NR4A receptor expression by FGF-8b involves MAPK, PI-3K, and PKC pathways (Fig. 3). Corticotrophin-releasing hormone was previously shown to induce *Nurr1* expression *via* protein kinase A (PKA), calmodulin kinase II, and MAPK pathways (Kovalovsky et al., 2002). Luteinizing hormone induces NGFI-B expression *via* PKA, PKC, and PI-3K pathways (Song et al., 2001), and PTH induces NR4A receptors through PKA and PKC pathways (Tetradis et al., 2001a; Tetradis et al., 2001b; Pirih et al., 2003). Our observation that several signaling pathways are involved in mediating the induction of NR4A receptor expression by FGF-8b is thus consistent with the previous reports on the induction of NR4A receptors.

FGF-8b regulates the proliferation of bone marrow cells (Valta et al., 2006; Fig. 4). NR4A receptors are involved in the regulation of proliferation and apoptosis of many cell types (Woronicz et al., 1994; Labelle et al., 1995; Castro et al., 2001; Kolluri et al., 2003; Mullican et al., 2007). However, the effect of NR4A receptors on the proliferation of differentiating osteoblasts has not to our knowledge been previously studied. We report here that Nurr1 and NGFI-B stimulate the proliferation of preosteoblastic cells (Fig. 4). Nor1 does not seem to have this effect. Nurr1 and NGFI-B form heterodimers with RXR that are efficiently activated by RXR agonists. Nor1 does not have this property (Perlmann and Jansson, 1995; Zetterström et al., 1996). It would have thus been possible that the stimulation of proliferation required heterodimerization with RXR. However, treatment of the cells with the RXR agonist SR11237 did not further enhance proliferation indicating that Nurr1 and NGFI-B regulated proliferation independent of RXR and thus the inability of Nor1 to regulate proliferation did not result from its inability to heterodimerize with RXR (Fig. 4E). According to our results, FGF-8b stimulates the proliferation of preosteoblastic cells at least partially *via* NR4A receptors as overexpression of Nurr1 and NGFI-B further increased the growth promoting effect of FGF-8b and the dominant negative Nurr1 variant abolished this effect (Fig. 4). In addition to proliferation, Nurr1 and NGFI-B regulated apoptosis of MC3T3-E1 cells as overexpression of Nurr1 and NGFI-B reduced the rate of apoptosis in these cells (Fig. 5). Nurr1 and NGFI-B thus promote the growth of preosteoblasts by two means, by reducing apoptosis and by stimulating cell proliferation.

FGF-8b was recently shown to stimulate osteoblastic differentiation (Valta et al., 2006). Identification of down-stream mediators of FGF-8b signaling is important in order to better understand how FGF-8b regulates osteoblastogenesis. Nurr1 has been shown to regulate osteoblastic differentiation (Lee et al., 2006). The induction of Nurr1 expression by FGF-8b demonstrated in our study links these two pathways. Nurr1 regulates the expression

of several osteoblastic differentiation markers such as alkaline phosphatase (ALP), collagen type I alpha I (COL1A1), osteocalcin (OCN), and osteopontin (OPN) (Lammi et al., 2004; Pirih et al., 2004; Lee et al., 2006). FGF-8b treatment increases ALP activity and enhances bone formation in mouse bone marrow cell cultures (Valta et al., 2006). The effect of FGF-8b on other osteoblastic markers than ALP has not been addressed. However, one could envision that in addition to the proliferation of preosteoblasts, FGF-8b and Nurr1 would also coregulate the differentiation of osteoblasts. In addition to osteoblasts, bone marrow mesenchymal stem cells have the ability to differentiate to other lineages, including adipocytes and chondrocytes (Gregory et al., 2005). Bone marrow mesenchymal stem cells have even been differentiated toward neural cells when incubated with FGF-8 and other growth factors promoting neural differentiation (Long et al., 2005). Bone marrow mesenchymal stem cells express NR4A receptors (Kramer et al., 2006; data not shown). The role of FGF-8 and NR4A receptors in directing the differentiation of bone marrow mesenchymal stem cells to other lineages than osteoblasts remains to be elucidated.

Bone metastases are believed to be caused by factors that are produced by the tumor cells and that stimulate osteoblasts or osteoclasts to form osteosclerotic or osteolytic metastases, respectively (Mundy, 2002). FGF-8 is expressed in breast and prostate tumors (Tanaka et al., 1998). As FGF-8b stimulates the proliferation and differentiation of osteoblasts it may also play a role in the formation of osteosclerotic bone metastases. The potential contribution of NR4A receptors in that process remains to be addressed.

In summary, we have shown in this study that FGF-8b induces the expression of NR4A receptors and that FGF-8b and NR4A receptors are involved in the same regulatory pathways in preosteoblasts.

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FOOTNOTES

¹Abbreviations used: NR, nuclear receptor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; MAPK, mitogen activated protein kinase; PI-3K, phosphatidylinositol-3 kinase; PKA, protein kinase A; PKC, protein kinase C; RXR, retinoid X receptor.

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FIGURE LEGENDS

Figure 1. FGF-8b induces the expression of NR4A receptors in preosteoblasts

MC3T3-E1 cells were treated with 10 ng/ml FGF-8b for the indicated times. Subsequently, the cells were analyzed for Nurr1 (A), NGFI-B (B), and Nor1 (C) expression by real-time RT-PCR. The mean $\pm$ SD of three independent experiments are shown. *Asterisks* indicate statistically significant differences between controls and FGF-8b treated cells; *, $p < 0.05$; **, $p < 0.01$.

Figure 2. FGF-8b induces the expression of NR4A receptors in a dose-dependent manner

MC3T3-E1 cells were treated with increasing concentrations of FGF-8b for 1 h. The expression of Nurr1 (A), NGFI-B (B), and Nor1 (C) was analyzed by real-time RT-PCR. The mean $\pm$ SD values of three independent experiments are shown.

Figure 3. FGF-8b induces the expression of NR4A receptors *via* MAPK, PI-3K, and PKC pathways

MC3T3-E1 cells were treated with 1 μ M PD98059 (PD), 1,25 μ M LY294002 (LY), or 50 nM Calphostin C (CC) for 1 h as indicated. Subsequently, 25 ng/ml FGF-8b was added to the culture medium and the incubation was continued for 1 hour. The expression of Nurr1 (A), NGFI-B (B), and Nor1 (C) was then analyzed by real-time RT-PCR. The mean $\pm$ SD values of three independent experiments are shown. *Asterisks* indicate statistically significant differences between the cells treated with FGF-8b alone and the cells treated with FGF-8b and the inhibitors; *, $p < 0.05$; **, $p < 0.01$.

Figure 4. Nurr1 and NGFI-B are involved in mediating the growth stimulatory effect of FGF-8b

A, U2-OS cells were transfected with empty vector or the expression vectors for Nurr1, NGFI-B, Nor1, and the dominant negative Nurr1 variant (Nurr1^{DN}) as indicated along with the NBRE₃tk-LUC reporter and pCMX-βgal internal control plasmid. The cells were harvested 24 h later and the luciferase activities were measured and normalized to the respective β-galactosidase activities. The mean ± SD values of two independent experiments are shown.

B–D, MC3T3-E1 cells were transfected with empty vector or the expression vectors encoding Nurr1, NGFI-B, Nor1, or Nurr1^{DN} as indicated. The cells were then treated with vehicle or FGF-8b (10 ng/ml) as depicted. After 18 hours of incubation, [³H]thymidine was added to the culture medium and incubation was continued for 2 hours. The cells were then lysed and the radioactivity was measured. The radioactivity is expressed relative to that measured in the cells transfected with empty vector and treated with vehicle (=1). The mean ± SD values of three independent experiments are shown. *Asterisks* indicate statistically significant differences (*, p<0.05; **, p<0.01).

E, MC3T3-E1 cells were transfected with empty vector or the expression vectors for Nurr1, NGFI-B, or Nor1 as indicated. After transfection, the cells were treated with the synthetic RXR ligand 1 μM SR11237 for 18 hours. Proliferation assay was then performed as described in B–D. The radioactivity is expressed relative to that measured in the cells transfected with empty vector and treated with vehicle (=1). The mean ± SD values of three independent experiments are shown. *Asterisks* indicate statistically significant differences (**, p<0.01).

Figure 5. Nurr1 and NGFI-B reduce apoptosis of MC3T3-E1 cells.

MC3T3-E1 cells were transfected with empty vector or the expression vectors encoding Nurr1, NGFI-B, and Nor1 as indicated. The cells were then treated with vehicle or FGF-8b (25 ng/ml) as depicted. After 20 hours of incubation, the cultures were analyzed for apoptosis. Apoptosis in each sample is expressed relative to that measured in the cells transfected with empty vector and treated with vehicle (=1). The mean $\pm$ SD values of three independent experiments are shown. *Asterisks* indicate statistically significant differences (**, $p < 0.01$).

Figure 1 Lammi and Aarnisalo

Figure 4 Lammi and Aarnisalo

Figure 5 Lammi and Aarnisalo