FGF-2 suppresses expression of nephronectin via JNK and PI3K pathways

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Nephronectin (Npnt), an extracellular matrix protein, is a ligand for integrin αβ1 and is involved in the development of various organs, such as the kidneys, bones, liver, and muscles. Previously, we found that Npnt expression was inhibited by various cytokines including transforming growth factor-β (Tgf-β) and oncostatin M (Osm). Fibroblast growth factor (Fgf)-2, otherwise known as basic Fgf, also plays important roles in skeletal development and postnatal osteogenesis. In this study, Npnt expression was found to be suppressed by Fgf-2 in MC3T3-E1 cells, an osteoblast-like cell line, in a dose- and time-dependent manners. Furthermore, Fgf-2-mediated Npnt mRNA suppression was shown to involve the Jun N-terminal kinase (JNK) and phosphoinositide-3 kinase (PI3K) pathways. Together, our results suggest that FGF-2 suppresses Npnt gene expression via JNK and PI3K pathways.

MC3T3-E1 is a cloned mouse osteoblast-like cell line that retains the synthetic functions of bone and has been utilized as an in vitro bone model of development systems. Using MC3T3-E1 cells, Kahai et al. [4] showed that some endogenous miRNAs might repress Npnt expression, resulting in a lower level of osteoblast differentiation. Other studies have also reported that transforming growth factor-β (Tgf-β) and oncostatin M (Osm) downregulate Npnt expression in both dose- and time-dependent manners, while osteoblast differentiation induced by Npnt was found to be inhibited by Tgf-β and Osm in MC3T3-E1 cells [6,7].

Abbreviations
Fgf, fibroblast growth factor; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEMa, alpha-minimum essential medium; Npnt, nephronectin; Osm, oncostatin M; PI3K, phosphoinositide-3 kinase; Tgf-β, transforming growth factor-β.
Results of phylogenetic analysis suggested that 22 different Fgf genes can be arranged into seven subfamilies containing 2–4 members each [8]. In another study, various growth factors, including fibroblast growth factor-2 (Fgf-2), transforming growth factor-β (Tgf-β), insulin-like growth factor-1 (Igf-1), platelet-derived growth factor, and prostaglandin E₃, were shown to act as autocrine and paracrine hormones for regulation of bone cell proliferation [9]. Fgf-2, which is stored in the extracellular matrix and expressed in osteoblasts [10], influences proliferation and differentiation of a variety of cell types in vitro [11,12]. In bone cell culture experiments, Fgf-2 showed increased replication and reduced differentiation markers, such as alkaline phosphatase, in MC3T3-E1 cells is regulated by Fgf-2 via the Jun N-terminal kinase (JNK) and phosphoinositide-3 kinase (PI3K) pathways. Our results revealed that reduced differentiation markers, such as alkaline phosphatase, in MC3T3-E1 cells is regulated by Fgf-2 via the Jun N-terminal kinase (JNK) and phosphoinositide-3 kinase (PI3K) pathways.

Materials and methods

Cell culture

MC3T3-E1 cells were maintained in alpha-minimum essential medium (MEMα) with 2 mM l-glutamine and 10 mg·L⁻¹ phenol red medium (Cat. No. 135-15175; Wako Pure Chemical Industries, Ltd., Osaka, Japan), supplemented with 10% FBS (Cat. No. FB-1365/500; Biosera, Rue de la Calle, France) and 1% penicillin–streptomycin (Cat. No. 15240062; Gibco, Waltham, MA, USA) at 37 °C in a CO₂ incubator (5% CO₂, 95% air). For the experiments, cells were plated at 1.0×10⁵ in 6-well plates (Cat. No. 140675; Thermo Scientific Inc., Waltham, MA, USA).

Reagents

Recombinant murine Fgf-1, Fgf-2, Fgf-8b, Fgf-9, and Fgf-23 were purchased from Pepro tech (Rocky Hill, CT, USA). PD98059 (Cat. No. P215-1 mg), SB203580 (Cat. No. S8307-1 mg), and SP600125 (Cat. No. S5567-10 mg) were from Sigma (St. Louis, MO, USA). LY294002 (Cat. No. 440202-5 mg) was from Calbiochem (Darmstadt, Deutschland), and B6J398 (Cat. No. 872511-34-7-5 mg) was from Selleckchem (Houston, TX, USA).

RT–PCR

Total RNA was extracted using TRizol reagent (Cat. No. 15596018; Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. We synthesized cDNA in a reaction mixture containing RNA using SuperScript III (Cat. No. 18080-044; Life Technologies) and random hexamer (Cat. No. N8080127; Invitrogen, Carlsbad, CA, USA) and then performed incubation at 50 °C for 60 min, followed by inactivation of the reaction by heating at 70 °C for 15 min. PCR was performed with Taq polymerase (Cat. No. M7123; Promega, Madison, WI, USA) using the following specific PCR primers: glyceraldehyde 3-phosphate dehydrogenase (Gapdh), 5′-GAAGGTCGTTGTAACGATTG-3′, and 5′-CATGTAGCGCATGAGGCCAC-3′; Fgfr1, 5′-TGGAGCTTCAATGTAAGGTT-3′ and 5′-ATAAAGGGGTTCTCCTGA-3′; Fgfr2, 5′-AAATACCAAATCTCCAACC-3′ and 5′-GCCGCTTCTCCATCCTC-3′; Fgfr3, 5′-ACTGTACTCAAGACTGCAAGG-3′ and 5′-GTCCCCATGCGATCAT-3′; and Fgfr4, 5′-TACAGTGTCTGCAAACAGTCTGCA-3′ and 5′-ACAAGCAGACAGTTGATC-3′. A 2 μL cDNA sample was used in a 10 μL reaction solution containing Red Taq® PCR mix (Cat. No. R2523-20RXN; Sigma). Primers were amplified using a program starting with 1 min of denaturation at 94 °C, followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 58 °C, and 30 s of extension at 74 °C, with a final extension of 1 min at 74 °C.

Real-time PCR was performed using a StepOne™ Real-time PCR System (Applied Biosystems, Waltham, MA, USA) with SYBR Green Fast PCR Master Mix (Applied Biosystems) with the following specific PCR primers: Gapdh, 5′-AAATGTTGAAAGCTCGTTG-3′ and 5′-TGTAAGGGGTTCTCCTGA-3′, and Npnt, 5′-CACGAATACTCCGGTACACAG-3′ and 5′-CTGCCGTGGAAAGGAGCACAT-3′. The total reaction volume was 10 μL including 2 μL of a cDNA sample. The thermos-cycling parameters employed were holding for 20 s at 95 °C, followed by 40 cycles of denaturation at 95 °C for 1 min, and annealing and extension at 60 °C for 20 s. Amplified products were determined using a standard curve analysis, and the expression level of each gene was normalized against that of Gapdh and expressed as the relative value for each experiment.

Western blotting

Cell lysates were collected using sample buffer solution with reducing reagent (6×) for SDS/PAGE (Cat. No. 09499-14; Nacalai Tesque, Kyoto, Japan), then electrophoresed onto a 10% SDS/PAGE, and blotted onto a poly(vinylidene difluoride) membrane. The membranes were incubated with cancer-specific antibody (Cat. No. AF4298; R&D Systems, Minneapolis, MN, USA) and anti-actin (Cat. No. A5060; MERCK, Darmstadt, Deutschland) as the first antibodies and then further probed with anti-mouse IgG horseradish peroxidase-linked (Cat. No. NA931V; GE Healthcare, Little Chalfont, UK) and anti-goat IgG horseradish peroxidase-linked (Cat. No. NB7352; NOVUS, Littleton, CO, USA) secondary antibodies. Proteins were visualized using
ECL Prime Western Blotting Detection reagent (Cat. No. #RPN2232; GE Healthcare).

Statistical analysis

All results are expressed as the mean ± standard deviation (SD). For results shown in Figs 1A, 2A, 3B and 4A,B, statistical analysis was performed using one-way ANOVA, while those shown in Figs 2B and S1 were analyzed using a two-tailed Student’s t-test. A P value of < 0.05 or < 0.01 was considered to indicate statistical significance.

Results

Fgf-2 strongly suppressed Npnt expression in Fgfs

We attempted to determine whether the expression of Npnt is regulated by members of the Fgf family, including Fgf-1, Fgf-2, Fgf-8b, Fgf-9, and Fgf-23. MC3T3-E1 cells were exposed to each of the investigated Fgfs for 24 h, and then, the level of Npnt mRNA was examined using real-time PCR analysis (Fig. 1A). Fgf-2 showed significant suppression of Npnt mRNA expression, while suppression by Fgf-1 was also noted, although the level was not as great as seen with Fgf-2. With 1 ng·mL⁻¹ of Fgf-2, the expression level of Npnt protein was also suppressed in MC3T3-E1 cells after 24 h of incubation (Fig. 1B). Thus, we exposed MC3T3-E1 cells to various doses of Fgf-2 for 24 h and found that downregulation of Npnt expression occurred in a dose-dependent manner, with the level reaching a plateau at ~ 1 ng·mL⁻¹ (Fig. 2A). Finally, we investigated time-dependent suppression by Fgf-2 at a dose of 1 ng·mL⁻¹ and detected a significant decrease in Npnt mRNA at 6 h after its addition to culture (Fig. 2B). The rate of Npnt expression by cells treated with Fgf-2 as compared to untreated cells was decreased in a time-dependent manner, with the level reaching a plateau at 24 h (data not shown).

Expression of Fgfr genes in MC3T3-E1 cells and their involvement in Npnt expression

The Fgfr (Fgf receptor) gene family is comprised of four members, Fgfr1-4 [8]. We found that each was well expressed in kidney specimens, while Fgfr1-3 were expressed in MC3T3-E1 cells (Fig. 3A). After treating MC3T3-E1 cells with BGJ398, an Fgfr inhibitor, real-time PCR analysis was performed, which showed that 0.1 μM of BGJ398 blocked suppression of Npnt mRNA induced by 1 ng·mL⁻¹ of Fgf-2 and restored its expression (Fig. 3B).

Npnt expression regulated by Fgf-2 via JNK and PI3K pathways

Fgfr-mediated signaling initiates activation of the mitogen-activated protein kinase (MAPK) and PI3K [16]. Downstream of MAPK and PI3K, Fgfr signaling has been shown to regulate several distinct MAPKs, including extracellular signal-regulated kinase (ERK)1/2, p38, and JNK [17–19]. To examine the molecular mechanism of Npnt mRNA downregulation by Fgf-2, we first examined its relationship with the MAPK pathway. MC3T3-E1 cells were treated with Fgf-2 at 1 ng·mL⁻¹, followed by 10 μM of PD98059 (MAPK-ERK kinase inhibitor), SB203580 (p38 MAPK inhibitor), or SP600125 (JNK
inhibitor). Of those, treatment with SP600125 inhibited the suppression of Npnt mRNA expression by Fgf-2 (Fig 4A). To examine the PI3K pathway, MC3T3-E1 cells were treated with Fgf-2, then with 10 μM of LY294002 (PI3K inhibitor), which showed that down-regulation of Npnt mRNA expression was inhibited by treatment with LY294002 (Fig. 4B).

Discussion

This study is the first to show that Fgf-2 strongly inhibits Npnt mRNA expression in a manner related to the JNK and PI3K signaling pathways (Fig. 5). Npnt, which enhances osteoblast differentiation, is expressed in the basement membrane of developing teeth and extracellular matrix of developing jawbones [5]. In addition, Linton et al. [20] showed that embryos lacking a functional Npnt gene frequently display kidney agenesis or hypoplasia, which could be traced to a delay in invasion of metanephric mesenchyme by the ureteric bud at an early stage of kidney development. It has been speculated that kidney disease causes disordered mineral metabolism, resulting in bone disease and ultimately fracture [21]; thus, it is considered that Npnt is closely related to bone metabolism. The relationship of Fgf-2 with skeleton development and bone metabolism has
been reported in several studies. For example, overexpression of human FGF-2 in mice (TgFGF-2) results in dwarfism, with shortening and flattening of long bones and moderate macrocephaly [22], while its deletion has been shown to lead to decreased levels of bone mass, formation, and mineralization in mice [23, 24].

A previous study also demonstrated that Fgfs activate PI3K signaling [8]; thus, we investigated whether Npnt mRNA expression suppressed by Fgf-2 is also regulated via the PI3K pathway. Several different materials, such as Igf-1 [25] and ghrelin [26], have been reported to activate PI3K signaling, while the present results also suggest that these substances may regulate Npnt mRNA expression.

We used the osteoblast-like MC3T3-E1 cell line in the present study and also examined primary osteoblasts obtained from calvaria of 1-day-old mice. Fgf-2 inhibited the expression of Npnt mRNA in primary osteoblasts (Fig. S1). It has also been reported that Npnt enhances osteoblast differentiation, while contrasting findings showed that FGF-2 increases osteoblast differentiation and extracellular matrix mineralization in vitro [27–29]. Similar contradictory results were shown in another study, which found that expression of a transcriptional co-activator with a PDZ-binding motif (Taz) was regulated by Fgf-2 [30]. These results might help to explain the complex mechanisms of Fgfs.

Conclusions

Fibroblast growth factor-2 suppresses Npnt mRNA expression in MC3T3-E1 cells in a dose- and time-dependent manner by activation of the JNK and PI3K pathways.
pathways. Our results suggest novel mechanisms related to Npnt gene expression.

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Author contributions

TK, Ay, MI, YY, KS, NM, AS, TI, DC, HO, and RK involved in study concept. TK, Ay, MI, YY, KS, and RK: collected the data; Ay, MI, YY, KS, and RK: involved in formal analysis; AS, HO, and RK: acquired funding; TK: investigated the study; TK, Ay, and RK: applied methodology for the study; Ay and RK: administrated the project; AS, HO, and RK: collected the resources; Ay and RK: supervised and validated the study; TK and Ay: wrote the original manuscript; TK, Ay, and RK: reviewed and edited the original manuscript.

References

1 Brandenberger R, Schmidt A, Linton J, Wang D, Backus C, Denda S, Mülle U and Reichardt LF (2001) Identification and characterization of a novel extracellular matrix protein nephronectin that is associated with integrin αβ1 in the embryonic kidney. J Cell Biol 154, 447–458.
2 Morimura N, Tezuka Y, Watanabe N, Yasuda M, Miyatani S, Hozumi N and Tezuka Ki K (2001) Molecular cloning of POEM: a novel adhesion molecule that interacts with alpha8beta1 integrin. J Biol Chem 276, 42172–42181.
3 Fujiwara H, Ferreira M, Donati G, Marciano DK, Linton JM, Sato Y, Hartner A, Sekiguchi K, Reichardt LF and Watt FM (2011) The basement membrane of hair follicle stem cells is a muscle cell niche. Cell 144, 577–589.
4 Kahai S, Lee SC, Lee DY, Yang J, Li M, Wang CH, Jiang Z, Zhang Y, Peng C and Yang BB (2009) MicroRNA miR-378 regulates nephronectin expression modulating osteoblast differentiation by targeting GalNT-7. PLoS ONE 4, e7535.
5 Kahai S, Lee SC, Seth A and Yang BB (2010) Nephronectin promotes osteoblast differentiation via the epidermal growth factor-like repeats. FEBS Lett 584, 233–238.
6 Miyazono A, Yamada A, Morimura N, Takami M, Suzuki D, Kobayashi M, Tezuka K, Yamamoto M and Kamijo R (2007) TGF-beta suppresses POEM expression through ERK1/2 and JNK in osteoblasts. FEBS Lett 581, 5321–5326.
7 Kurosawa T, Yamada A, Takami M, Suzuki D, Saito Y, Hiranuma K, Enomoto T, Morimura N, Yamamoto M, Iijima T et al. (2015) Expression of nephronectin is inhibited by oncostatin M via both JAK/STAT and MAPK pathways. FEBS Open Bio 5, 303–307.
8 Ornitz DM and Itoh N (2015) The Fibroblast Growth Factor signaling pathway. Wiley Interdiscip Rev Dev Biol 4, 215–266.
9 Mundy GR, Chen D, Zhao M, Dallas S, Xu C and Harris S (2001) Growth regulatory factors and bone. Rev Endoer Metab Disord 2, 105–115.
10 Globus RK, Plouet J and Gospodarowicz D (1989) Cultured bovine bone cells synthesize basic fibroblasts growth factor and store it in their extracellular matrix. Endocrinology 124, 1539–1547.
11 Hauschka PV, Mavrakos AE, Iafrit MD, Dolemen SE and Klagsbrun M (1986) Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. J Biol Chem 261, 12665–12674.
12 Hurley MM, Kessler M, Gronowicz G and Raisz LG (1992) The interaction of heparin and basic fibroblast growth factor on collagen synthesis in 21-day fetal rat calvariae. Endocrinology 130, 2675–2682.
13 Globus RK, Patterson-Buckendahl P and Gospodarowicz D (1988) Regulation of bovine bone cell proliferation by fibroblast growth factor and transforming growth factor beta. Endocrinology 123, 98–105.
14 Rodan SB, Wesolowski G, Yoon K and Rodan GA (1989) Opposing effects of fibroblast growth factor and pertussis toxin on alkaline phosphatase, osteopontin, osteocalcin, and type I collagen mRNA levels in ROS 17/2.8 cells. J Biol Chem 264, 19934–19941.
15 Mayahara H, Ito T, Nagai H, Miyajima H, Tsukuda R, Taketomi S, Mizoguchi J and Kato K (1997) In vivo stimulation of endosteal bone formation by basic fibroblast growth factor in rats. Growth Factors 9, 73–80.
16 Goetz R and Mohammad M (2013) Exploring mechanisms of FGF signalling through the lens of structural biology. Nat Rev Mol Cell Biol 14, 166–180.
17 House SL, Branch K, Newman G, Doetschman T and Schultz Jel J (2005) Cardioprotection induced by cardiac-specific overexpression of fibroblast growth factor-2 is mediated by the MAPK cascade. Am J Physiol Heart Circ Physiol 289, H2167–H2175.
18 Liao S, Porter D, Scott A, Newman G, Doetschman T and Schultz Jel J (2007) The cardioprotective effect of...
the low molecular weight isoform of fibroblast growth factor-2: the role of JNK signaling. J Mol Cell Cardiol 42, 106–120.

19 Kanazawa S, Fujiwara T, Matsuzaki S, Shingaki K, Taniguchi M, Miyata S, Tohyama M, Sakai Y, Yano K, Hosokawa K et al. (2010) bFGF regulates PI3-kinase-Rac1-JNK pathway and promotes fibroblast migration in wound healing. PLoS ONE 5, e12228.

20 Linton JM, Martin GR and Reichardt LF (2007) The ECM protein nephronectin promotes kidney development via integrin alpha8beta1-mediated stimulation of Gdnf expression. Development 134, 2501–2509.

21 Nickolas TL and Jamal SA (2015) Bone kidney interactions. Rev Endocr Metab Disord 16, 157–163.

22 Coffin JD, Florkiewicz RZ, Neumann J, Mort-Hopkins T, Dorn GW 2nd, Lightfoot P, German R, Howles PN, Kier A, O’Toole BA et al. (1995) Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. Mol Biol Cell 6, 1861–1873.

23 Montero A, Okada Y, Tomita M, Ito M, Tsurukami H, Nakamura T, Doetschman T, Coffin JD and Hurley MM (2000) Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. J Clin Invest 105, 1085–1093.

24 Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoyer JB, Haudenschild CC, Yin M, Coffin JD, Kong L, Kranias EG et al. (1998) Fibroblast growth factor 2 control of vascular tone. Nat Med 4, 201–207.

25 Tan H, Chen R, Li W, Zhao W, Zhang Y, Yang Y, Su J and Zhou X (2017) A systems biology approach to studying the molecular mechanisms of osteoblastic differentiation under cytokine combination treatment. NPJ Regen Med 2, 5.

26 Liang QH, Liu Y, Wu SS, Cui RR, Yuan LQ and Liao EY (2013) Ghrelin inhibits the apoptosis of MC3T3-E1 cells through ERK and AKT signaling pathway. Toxicol Appl Pharmacol 272, 591–597.

27 Noff D, Pitaru S and Savion N (1989) Basic fibroblast growth factor enhances the capacity of bone marrow cells to form bone-like nodules in vitro. FEBS Lett 250, 619–621.

28 Pitaru S, Kotev-Emeth S, Noff D, Kaffuler S and Savion N (1993) Effect of basic fibroblast growth factor on the growth and differentiation of adult stromal bone marrow cells: enhanced development of mineralized bone-like tissue in culture. J Bone Miner Res 8, 919–929.

29 Scutt A and Bertram P (1999) Basic fibroblast growth factor in the presence of dexamethasone stimulates colony formation, expansion, and osteoblastic differentiation by rat bone marrow stromal cells. Calcif Tissue Int 64, 69–77.

30 Eda H, Aoki K, Marumo K, Fujii K and Ohkawa K (2008) FGF-2 signaling induces downregulation of TAZ protein in osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 366, 471–475.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Fgf-2 inhibits expression of Npnt in primary osteoblasts. Real-time PCR analysis was performed using cDNA from primary osteoblasts after treatment with 1ng.ml⁻¹ of Fgf-2 for 24 hours. Values are shown as the mean ± SD of 3 samples as compared to the value without Fgf treatment. *p<0.05, **p<0.01; relative to level in cells without treatment (Student’s t-test).