Fibroblast Growth Factor Receptor 2 Promotes Osteogenic Differentiation in Mesenchymal Cells via ERK1/2 and Protein Kinase C Signaling*

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Mesenchymal stem cells (MSCs) are able to differentiate into several lineages including osteoblasts. The signaling mechanisms involved in the osteogenic differentiation of MSCs are however not fully understood. We investigated the role of fibroblast growth factor receptor 2 (FGFR2) in osteoblast commitment and differentiation of murine mesenchymal C3H10T1/2 cells stably transfected with wild type (WT) or activated FGFR2 due to Apert S252W genetic mutation (MT). WT FGFR2 slightly increased, whereas MT FGFR2 strongly increased, FGFR2 tyrosine phosphorylation, indicating activation of the receptor. WT and MT FGFR2 increased C3H10T1/2 cell proliferation but not survival. Both WT and MT FGFR2 increased early and late osteoblast gene expression and matrix mineralization. Forced expression of WT and MT FGFR2 also increased osteoblast gene expression in MC3T3-E1 calvaria osteoblasts. In both cell types, MT FGFR2 was more effective than WT FGFR2. In contrast, WT and MT FGFR2 decreased adipocyte differentiation of C3H10T1/2 cells. WT and MT FGFR2 induced ERK1/2 but not JNK or PI3K/AKT phosphorylation. MT, but not WT, also increased protein kinase C (PKC) activity. Pharmacological inhibition of ERK1/2 prevented cell proliferation induced by WT and MT FGFR2. Using dominant-negative ERK and PKCε vectors, we demonstrated that WT and MT FGFR2 promoted osteoblast gene expression through ERK1/2 and PKCε signaling, respectively. This study identifies FGFR2 as a novel regulatory molecule that promotes osteogenic differentiation in murine MSCs. The promoting effect of WT and MT FGFR2 is mediated by ERK1/2 and PKCε pathways that play essential and distinct roles in FGFR2-induced osteogenic differentiation of mesenchymal cells.

Mesenchymal stem cells (MSCs)† have the potential to differentiate into different lineages, including osteoblasts, chondroblasts, and adipocytes (1–7). The osteoblast differentiation program of MSCs is characterized by cell recruitment, which is followed by timely expressed genes including Runx2, alkaline phosphatase (ALP), type I collagen (ColA1), and osteocalcin (OC), which is associated with extracellular matrix mineralization (8–10). The program of MSC osteogenic differentiation can be induced by soluble molecules such as bone morphogenetic proteins (BMPs) or Wnt proteins that activate several signaling pathways to trigger osteoblast differentiation (11–15). Although various downstream signals are known to promote osteogenic differentiation (16–20), the molecular mechanisms that control the early stages of MSC osteoblast differentiation are not fully elucidated.

Fibroblast growth factors (FGFs) play an important major role in the control of cell proliferation, differentiation, and survival in several tissues including bone (21–24). Notably, FGF2 was found to promote cell growth and osteoblast differentiation in bone marrow-derived mesenchymal cells (25, 26). Consistent with an important role of FGF signaling in the control of osteoprogenitor cells, deletion of FGF2 in mice results in decreased bone marrow stromal cell osteogenic differentiation and altered bone formation (27). The actions of FGFs are highly dependent on high affinity FGF receptors (FGFRs) (28). FGF binding to FGFRs leads to receptor dimerization and phosphorylation of intrinsic tyrosine residues, which leads to activation of several signal transduction pathways including phospholipase Cγ (PLCγ), mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3-kinase (PI3K) (29, 30). In bone, activation of extracellular-related kinase (ERK1/2) MAPK and protein kinase C (PKC) was found to enhance osteoblast gene expression (31, 32). The important role of FGFR signaling in bone formation is highlighted by the finding that gain-of-function mutations in FGFRs results in premature cranial osteogenesis (33, 34). FGFR1 was recently shown to be an important transducer of FGF signals in proliferating osteoblasts (35). In contrast, activated FGFR2 was shown to enhance osteoblast differentiation in Apert syndromic craniosynostosis (36–41). However, the role of FGFR2 signaling in osteogenic differentiation of mesenchymal stem cells is yet to be elucidated.

In the present study, we investigated the specific role of FGFR2 signaling on osteoblast commitment and differentiation extracellular signal-regulated kinase; PKC, protein kinase C; FGFR, fibroblast growth factor receptor; WT, wild type; MT, mutant; FCS, fetal calf serum; JNK, c-Jun N-terminal kinase; ALP, alkaline phosphatase.
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in murine mesenchymal progenitor cells. Our results indicate that wild-type and activated FGFR2 induce osteogenic differentiation in mesenchymal cells through distinct activation of ERK1/2 and PKCα signaling. These data indicate that FGFR2-induced activation of specific downstream signaling pathways mediates osteogenic differentiation of murine mesenchymal cells.

EXPERIMENTAL PROCEDURES

Cells and Materials—Murine pluripotent mesenchymal C3H10T1/2 cells and MC3T3-E1, a recognized osteoblastic cell line, were obtained from the ATCC. Cells were routinely cultured in Dulbecco’s Modified Eagles Medium (DMEM; Invitrogen, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, and penicillin/streptomycin (10,000 units/ml and 10,000 μg/ml, respectively), at 37 °C in a humidified atmosphere containing 5% CO2 in air. Culture media were changed every 2 days. Anti-FGFR2, anti-phosphotyrosine (p-Tyr), anti-ERK, and anti-p-ERK were from Sigma-Aldrich. Other antibodies (anti-AKT, anti-p-AKT, anti-JNK, anti-p-JNK, anti-PI3K p85, and anti-p-PI3K p85) were from Cell Signaling (Sigma–Aldrich). The β-actin antibody, U1026 (an inhibitor of MAPK kinase 1 and 2 (MEK1/2) that blocks phosphorylation and activation of ERK1/2 (42) and G66976, a specific PKCα inhibitor were from Sigma.

Plasmids and Transfections—For stable transfection, C3H10T1/2 cells were grown in tissue culture plates and transfected at 80% confluence with 2.5 μg of the empty vector (EV), FGFR2IIIC-CMV13 (WT), or FGFR2IIICS252W-CMV13 (MT) (40) using Exgen 500 (Euromedex, Mundolsheim, France). After 48 h, the transfectants were selected in DME medium containing 10% FCS and 1 mg/ml G418 (Geneticin, Sigma) for 4 weeks. To analyze whether FGFR2 activation may affect more mature osteoprogenitor cells, we also performed transient transfection experiments with WT or MT FGFR2 in MC3T3-E1 mouse calvaria cells. In parallel experiments, EV, WT, and MT C3H10T1/2 cells were transiently transfected with a dominant-negative ERKp44-MAPK kinase-deficient mutant vector (DN-ERK) (43), a dominant-negative PKCα vector (DN-PKCα) or empty vectors and Exgen (Euromedex) according to the manufacturer’s instructions.

Cell Proliferation and Apoptosis Assays—For the cell proliferation assay, stably transfected C3H10T1/2 cells were cultured in six-well plates (105 cells per well), and cell number was evaluated by cell counting. For the apoptosis assay, stably transfected C3H10T1/2 cells were cultured in the presence of 1 or 5% FCS, and apoptosis was detected using the Apoppercentage apoptosis assay (Biocolor, Belfast, Northern Ireland). Briefly, 20 μl of dye (1/100) were added to the medium. After 30 min, the cells were washed twice with phosphate-buffered saline. Cells were lyzed in DMSO, and absorbance at 550 nm was determined. In some wells, H2O2 (5 mmol/liter) was added to the medium for 2 h before harvesting the cells to induce apoptosis, thereby serving as a positive control.

Protein Kinase C Activity—PKC activity was determined using a PKC Activity Assay kit (Assay Designs, Ann Arbor, MI) based on a solid phase enzyme-linked immuno-absorbent assay (ELISA), according to the manufacturer’s instructions. The assay utilizes a specific synthetic peptide as a substrate for PKC, and a polyclonal antibody that recognizes the phosphorylated form of the substrate.

Quantitative RT-PCR Analysis—Total RNAs were isolated using Trizol reagent (Invitrogen). 3 μg of total RNA from each sample were reverse-transcribed using MMLV reverse transcriptase and oligoDT primers at 37 °C for 90 min. The relative mRNA levels were evaluated by quantitative PCR (LightCycler, Roche Applied Science, Indianapolis, OH) using a SYBR Green PCR kit (ABGen, Courtabœuf, France) and specific primers (supplemental Table S1). Signals were normalized to glyceraldehyde-3-phosphate dehydrogenase as internal control.

Western Blot Analysis—Cell lysates were prepared as described (38). Briefly, proteins (30 μg) were resolved on 4–12% SDS-PAGE and transferred onto polyvinylidene difluoride nitrocellulose membranes (Millipore Corp., Bedford, MA). Filters were incubated for 2 h in 50 mm Tris/HCl, pH 7.4, 150 mm NaCl, 0.1% (v/v) Tween-20, 0.5% (w/v), bovine serum albumin (TBST/BSA), then overnight at 4 °C on a shaker with specific primary antibodies (1/500–1/1000 in TBST/BSA). Membranes were washed twice with TBST and incubated for 2 h with appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000–1:20,000 in TBST/BSA). After final washes, the signals were visualized with enhanced chemiluminescence Western blotting detection reagent (ECL, Amersham Biosciences, Piscataway, NJ) and autoradiographic film (X-OMAT-AR, Eastman Kodak Company, Rochester, NY). Densitometric analysis using ImageQuant software was performed following digital scanning (Agfa). Representative images of immunoblots are shown. For immunoprecipitation analysis, cell lysates were prepared as for Western blot analysis, and aliquots of total protein (150 μg) were incubated overnight under weak agitation at 4 °C with 2 μg of specific anti-FGFR2 and 20 μl of Dynabeads protein G (Invitrogen). Components of the bound immune complex (both antigen and antibody) were eluted from the Dynabeads and analyzed by SDS-PAGE according to the manufacturer’s recommendations.

Alkaline Phosphatase Staining—ALP staining was performed using Sigma FAST kit according to the manufacturer’s recommendations (Sigma). Briefly, cells were fixed in 75% ethanol, rinsed in phosphate-buffered saline, and incubated with the substrate buffer at 37 °C.

Osteogenic, Chondrogenic, and Adipogenic Assays—Cell culture medium was supplemented with 50 μmol/liter ascorbic acid and 3 mm inorganic phosphate (NaH2PO4) to allow matrix synthesis and mineralization. At the indicated time points, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline. Matrix mineralization was evaluated by alizarin red and von Kossa staining as described (44) and microphotographed using an Olympus microscope (Japan). Glycoprotein synthesis was determined by alcian blue staining (44). For adipogenesis, the accumulation of lipid droplets, a hallmark of functional adipogenesis, was detected by oil red staining (44) and quantified at 14 days of culture, as described (45). Briefly, the stain solution was removed, and cells were rinsed in 500 μl of 60% isopropyl alcohol for 5 s. To extract dye, 700 μl of 60% isopropyl alcohol was added per well, and sealed plates were
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Expression of FGFRs in C3H10T1/2 Cells—We first determined the expression levels of FGFR1–4 in growing C3H10T1/2 cells. Quantitative PCR analysis showed that mRNA expression levels of the four FGFRs differed markedly at 3 days of culture (Fig. 1A). FGFR1 was higher than FGFR2 mRNA levels. In contrast, FGFR3 mRNA expression was weak, and FGFR4 level was almost undetectable (Fig. 1A), suggesting that FGFR1 and FGFR2 are major GGF receptors expressed in these mesenchymal cells. The kinetics analysis of change of the two main FGFRs expressed in growing C3H10T1/2 cells showed that the FGFR1 mRNA level rose and then declined in confluent cells whereas the FGFR2 mRNA level increased in confluent cells (Fig. 1, B and C), suggesting that FGFR2 may be an important transducer of FGF signals in post-proliferating osteoblasts.

Expression and Activation of FGFR2 in Stably Transfected C3H10T1/2 Cells—We then analyzed the effect of FGFR2 signaling in C3H10T1/2 cells. To this goal, the cells were stably transfected with wild-type (WT) FGFR2 or a constitutively activating FGFR2 due to Apert S252W mutation (MT). As expected, quantitative PCR analysis showed that transfection with the two vectors increased FGFR2 mRNA levels in C3H10T1/2 cells (data not shown). Western blot analysis showed a 2–3-fold increase in FGFR2 protein levels in WT- and MT-transfected cells, indicating that FGFR2 protein levels increased similarly in WT and MT FGFR2-transfected cells (Fig. 2A). FGFR activation is characterized by phosphorylation of the cytoplasmic domain that transduces intracellular signals. We analyzed tyrosine phosphorylation of FGFR2 in WT and MT FGFR2-transfected cells. As shown in Fig. 2, B and C, WT FGFR2 induced a slight increase in tyrosine-phosphorylated FGFR2 level under basal conditions (i.e. in the absence of exogenous FGF) compared with EV-transfected cells. In contrast, a 2-fold increase in the tyrosine-phosphorylated FGFR2 level was found in MT FGFR2-transfected cells (Fig. 2, B and C). These data indicate that forced expression of WT FGFR2 slightly activated the receptor in the absence of exogenous ligand, whereas MT FGFR2 induced a marked activation of the receptor.

FGFR2 Increases C3H10T1/2 Cell Proliferation but Not Survival—Activation of FGFR signaling induces multiple and complex effects on cell proliferation and survival (28). To determine the cellular effect of FGFR2 activation in mesenchymal cells, we first investigated the changes in cell proliferation in WT and MT FGFR2 stably transfected C3H10T1/2 cells. Over-expression of WT or MT FGFR2 increased cell proliferation compared with EV-transfected cells at all times of culture before and after confluence (Fig. 2D). Using an assay that detects early events involved in cell apoptosis, we then examined whether WT or MT FGFR2 may affect cell survival in C3H10T1/2 cells. As shown in Fig. 2E, MT or WT FGFR2 did
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Quantitative PCR analysis showing that WT FGFR2 and S252W FGFR2 MT increased the expression of osteoblast markers in C3H10T1/2 cells compared with empty vector (EV) transfected cells (A and B). Biochemical analysis showing that MT FGFR2 enhanced ALP activity more than WT FGFR2 in C3H10T1/2 cells (C). Histochemical staining showing that MT FGFR2 induced a greater stimulatory effect on ALP activity than WT FGFR2 in C3H10T1/2 mesenchymal cells and MC3T3-E1 osteoblastic cells at day 2 (D) compared with WT FGFR2. Transient transfection with WT and MT FGFR2 greatly increased mRNA levels of osteoblast markers in MC3T3-E1 osteoblastic cells, as determined by qPCR analysis (E). MT FGFR2 induced a more rapid and greater stimulatory effect on matrix calcification compared with WT FGFR2 in C3H10T1/2 mesenchymal cells (F). Analysis of gene expression by qPCR at late stages of differentiation showing that MT FGFR2 induced a more rapid increase in SOST mRNA expression compared with WT FGFR2 in C3H10T1/2 mesenchymal cells (G). a indicates a significant difference with EV-transfected cells; b indicates a significant difference with WT FGFR2-transfected cells (p < 0.05).

not affect cell apoptosis when cultured in optimal survival conditions (5% FCS) or in culture condition inducing apoptosis (1% FCS) after 3 days of culture. These data indicate that WT or MT FGFR2 overexpression promotes cell proliferation but not cell survival in C3H10T1/2 cells under these experimental conditions.

FGFR2 Promotes Osteogenic Differentiation in MSCs—We then investigated the effects of WT and MT FGFR2 on osteogenic differentiation of C3H10T1/2 cells. Quantitative PCR analysis showed that both WT and MT FGFR2 induced a 3–4-fold increase in mRNA expression of Runx2, a specific osteoblast transcription factor, at 4 and 6 days of culture (Fig. 3, A and B). In contrast, osterix mRNA level was not affected by WT or MT FGFR2. WT and MT FGFR2 induced a similar 2-fold increase in Coll1A1 expression at 4 and 6 days (Fig. 3, A and B). Alkaline phosphatase (ALP) and osteocalcin (OC) mRNA levels were also increased by WT and MT FGFR2 at 6 days. However, the rise in ALP and OC was greater in MT than in WT FGFR2 (Fig. 3, A and B). Consistently, MT FGFR2 induced a greater increase in ALP activity and staining at day 2 than WT FGFR2 (Fig. 3, C and D). To confirm this finding in more mature cells, calvaria-derived MC3T3-E1 osteoblastic cells were transiently transfected with WT or MT FGFR2. Consistent with our data in C3H10T1/2 cells, MT FGFR2 increased ALP staining and osteoblast genes (Runx2, ALP, Coll1A1) in MC3T3-E1 cells at day 2, and the effect was greater than WT FGFR2, except for osteocalcin expression (Fig. 3E), indicating that MT FGFR2 can promote osteoblast differentiation at different stages of development. We also determined the effects of WT and MT FGFR2 on in vitro matrix calcification in long term culture. As shown in Fig. 3F, MT FGFR2 induced a more rapid (7 versus 14 days) and greater extracellular matrix calcification at 21 days than WT FGFR2 (Fig. 3F). To further determine the effect of WT and MT FGFR2 at a terminal differentiation state, we analyzed the changes in SOST, a specific osteocyte marker (46) and DMP1 that is expressed by osteocytes (47). Fig. 3G shows that SOST mRNA expression was increased by MT FGFR2 at 14 days and by WT FGFR2 at 21 days of culture, whereas DMP1 expression remained unchanged. Overall, these results reveal that both WT and MT FGFR2 increase osteoblast marker expression and osteogenic potential in vitro in C3H10T1/2 cells. However, MT FGFR2 is more effective in promoting early and terminal osteoblast differentiation and in vitro osteogenesis than WT FGFR2 in these cells.

We then investigated whether MT and WT FGFR2 overexpression may modulate MSC differentiation toward the adipogenic lineage. Quantitative PCR analysis showed that MT FGFR2 decreased mRNA expression of the main adipocyte transcription factor PPARγ2 at 4, 6, and 14 days of culture (supplemental Fig. S2A). Furthermore, MT FGFR2 decreased the mRNA levels of C/EBPα (at 6 days) and C/EBPβ (at 4 and 6 days) that are transcription factors controlling adipogenesis, as well as aP2 (at 4–14 days), which is expressed in more mature adipocytes. Both WT and MT FGFR2 decreased mRNA levels of LPL, a late adipocyte differentiation marker, at 4 and 6 days (supplemental Fig. S2A). Consistent with these data, WT or MT FGFR2 decreased the amount of oil red-stained lipid droplets in long term culture (supplemental Fig. S2, B and C), indicating that WT and MT FGFR2 down-regulate adipogenic differentiation in C3H10T1/2 mesenchymal cells. In contrast, WT and MT FGFR2 had no major effect on chondrocyte marker genes in C3H10T1/2 cells. We only observed a slight change (30%) in Sox9 and type 10 collagen expression at 6 days in WT or MT cells whereas aggrecan expression was unchanged (data not shown). Consistent with these modest changes, FGFR2 had no detectable effect on chondrogenesis in long term culture, as revealed by alcian blue staining (data not shown), suggesting that WT or MT FGFR2 did not markedly affect chondrocyte differentiation of C3H10T1/2 cells under these experimental conditions.
FGFR2 Induces ERK1/2 MAPK Signaling in C3H10T1/2 Cells—Activation of FGFRs by FGFs is known to elicit several signaling pathways (28). We therefore sought to identify the signaling pathways that are activated by WT and MT FGFR2 in C3H10T1/2 mesenchymal cells. As shown in Fig. 4A, Western blot analysis showed that WT FGFR2 increased ERK1/2 phosphorylation in the absence of exogenous ligand, presumably in response to endogenous FGF expressed by the cells. Similarly, ERK1/2 phosphorylation was activated by MT FGFR2 in these cells (Fig. 4A). In contrast, neither WT FGFR2, nor MT FGFR2 induced detectable effect on p-JNK, p-Pi3K, or p-AKT levels (Fig. 4, B–D), indicating that WT and MT FGFR2 selectively activated ERK1/2 signaling in C3H10T1/2 cells. To determine whether ERK1/2 signaling is involved in the cellular phenotype induced by WT and MT FGFR2, we selectively inhibited the ERK1/2 pathway. As shown in Fig. 4E, ERK1/2 phosphorylation induced by WT or MT FGFR2 was blocked by the MEK inhibitor U0126 (10 μM). Using this efficient inhibitor, we determined the role of ERK1/2 in the induction of cell proliferation induced by WT and MT FGFR2. As shown in Fig. 4F, pharmacological inhibition of ERK1/2 completely inhibited cell proliferation induced by WT and MT FGFR2. These results suggest that ERK1/2 activation mediates the increased C3H10T1/2 cell proliferation induced by WT or MT FGFR2. Activation of ERK1/2 has been recently proposed to be involved in the skeletal phenotype induced by the activating S252W FGFR2 mutation in a mouse model of Apert syndrome (48). To determine the role of ERK1/2 on osteoblast differentiation induced by FGFR2 in C3H10T1/2 cells, the cells were transfected with DN-ERK (43), and the expression of osteoblast differentiation markers was determined. As shown in Fig. 5A, WT and MT FGFR2 cells transfected with DN-ERK showed reduced p-ERK1/2 levels compared with empty vector-transfected cells, reflecting the efficiency of the DN-ERK vector. Remarkably, all osteoblast markers were decreased in WT FGFR2 transfected with the DN-ERK compared with control cells transfected with the empty vector (Fig. 5B). In contrast, the expression of osteoblast markers remained unchanged or were increased (Runx2) in MT FGFR2 cells transfected with the DN-ERK vector (Fig. 5C). These results strongly suggest that ERK1/2 signaling plays a critical role in osteoblast differentiation induced by WT, but not MT FGFR2, in C3H10T1/2 cells.

PKC Signaling Is Involved in MT FGFR2-mediated Osteoblast Differentiation of C3H10T1/2 Cells—The above results suggest that another signaling pathway is involved in osteoblastic differentiation induced by MT FGFR2 in C3H10T1/2 cells. We and others (37, 38) previously found that FGF signaling activates PLCγ-PKC signaling in osteoblasts. To determine the potential role of PKC in osteoblast differentiation induced by FGFR2 activation in mesenchymal cells, we analyzed PKC activity in C3H10T1/2 cells stably expressing WT or MT
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FGFR2. As shown in Fig. 5D, PKC activity was increased in MT FGFR2 cells, but not in WT FGFR2 cells compared with control cells, suggesting a distinct effect of WT and MT FGFR2 on PKC activation in these cells. To determine the role of specific PKC isoenzymes in the induction of osteoblast gene expression induced by FGFR2, C3H10T1/2 cells stably expressing WT or MT FGFR2 were transfected with DN-PKCα and osteoblast gene expression was determined. As shown in Fig. 5E, DN-PKCα reduced ALP mRNA levels induced by WT FGFR2. In contrast, DN-PKCα reduced mRNA expression of all osteoblast marker genes induced by MT FGFR2 (Fig. 5F). These results indicate that the induction of osteoblast markers by MT FGFR2 involves predominantly activation of PKCα in these mesenchymal cells. To confirm these findings, we performed long-term analysis of matrix calcification by WT and MT cells treated with Go6976 at a dose (2.3 nm) that inhibits PKCα activity in osteoblasts (38). Pharmacologic inhibition of PKCα slightly reduced matrix calcification induced by WT and completely blunted matrix calcification induced by MT FGFR2 (Fig. 5G), confirming the predominant role of PKCα in MT FGFR2-induced osteogenic differentiation in these mesenchymal cells. Collectively, these results indicate that FGFR2 induces osteoblast differentiation of C3H10T1/2 mesenchymal progenitor cells through activation of ERK1/2 and PKCα signaling and that these pathways play essential and distinct roles in FGFR2-induced promotion of osteogenic differentiation in murine mesenchymal cells (Fig. 6).

DISCUSSION

Multipotent mesenchymal stem cells are able to differentiate into osteoblasts under appropriate stimulation with BMPs or Wnt proteins. In the present work, we identified another key regulatory signaling molecule, namely FGFR2, which contributes to the osteogenic differentiation of murine mesenchymal progenitor cells. FGFRs are expressed differently in various cells and tissues, which determine the cell response to specific ligands (21–23). Previous studies on the FGFR expression profile indicated that FGFR1 and FGFR2 are the main FGF receptors expressed in osteoblasts whereas FGFR3 is more restricted to chondrocytes (23, 24). However, the expression of specific FGFRs and their role in the osteogenic differentiation of mesenchymal stem cells remain poorly understood. In this study, we found that cultured C3H10T1/2 mesenchymal cells expressed high FGFR1 and FGFR2 levels, whereas FGFR3 was low and FGFR4 almost undetectable, suggesting that FGFR1 and FGFR2 play a prominent role in response to FGFs in these cells. FGFR1 may be the major transducer of FGF signals in proliferating progenitors in which it was most prominently expressed, whereas FGFR2 may be a more important transducer of FGF signals in post-proliferating differentiating cells where it was more abundant. To determine more specifically the role of FGFR2 in cell commitment and osteoblast differentiation, we developed C3H10T1/2 cells stably expressing WT or MT FGFR2 and analyzed the phenotype induced by these vectors. MT FGFR2 induced auto-phosphorylation of the receptor, which is consistent with the reported mechanism by which the S252W genetic mutation activates the receptor (49, 50). Stable overexpression of WT FGFR2 induced a lower tyrosine phosphorylation of the receptor compared with MT FGFR2, which is expected in cells cultured in the absence of exogenous ligand. The activation of the WT FGFR2 is likely to be linked to the endogenous production of FGFs such as FGF2 and FGF9 that are known to functionally activate FGFR2 (21, 23). Although FGF signaling was found to promote cell proliferation in bone marrow derived MSCs (25, 26), the specific function of FGFR2 in this effect was not known. Additionally, the functional effect of FGFR2 activation on osteoblastic cell proliferation remains uncertain since the S252W activating FGFR2 mutation was found to promote cell proliferation in murine (51) but not in human osteoblasts (36). A critical point was thus to determine whether activated FGFR2 may have functional effects on mesenchymal stem cell proliferation. We showed here that both WT and MT FGFR2 promoted C3H10T1/2 mesenchymal cell replication, indicating that activated FGFR2 induces a biological response characterized by increased proliferation in these murine mesenchymal cells. Another functional response to FGF signaling is modulation of cell survival. We previously showed that FGF2 has distinct effects on osteoblast survival depending on the stage of differentiation (52). Additionally, activated FGFR2 mutations were shown to increase osteoblast apoptosis in murine and human osteoblasts (51, 53). However, whether FGFR2 may control mesenchymal cell survival remains unknown. The present data indicate that activated FGFR2 does not affect cell survival in C3H10T1/2 mesenchymal cells under our experimental conditions. These results suggest that the control of cell survival by FGFR2 may differ in progenitor cells and more mature osteoblasts, and indicate that activated FGFR2 in murine mesenchymal cells promotes cell proliferation rather than survival.

An important issue was to determine whether activated FGFR2 may functionally promote osteogenic differentiation in mesenchymal stem cells. We showed that although WT and MT FGFR2 increased osteoblast marker gene expression, MT FGFR2 was more effective than WT FGFR2 to promote osteogenic differentiation in both murine mesenchymal stem cells and osteoblastic cells. Previous studies reported conflicting
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results on the role of FGFR2 in the control of osteoblast differentiation. Genetic manipulation in murine osteoblasts indicated that gain-of-function mutations of FGFR2 can either reduce (51, 54) or increase osteoblast differentiation (55–58). The present data provide novel evidence for a positive role of activated FGFR2 on osteogenic differentiation of murine mesenchymal cells in vivo. Our finding that MT FGFR2 also increased osteoblast gene expression in more mature MC3T3-E1 calvaria osteoblastic cells indicates that FGFR2 can activate osteoblast differentiation at different stages of cell maturation. Based on these findings, we attempted to determine whether WT and MT FGFR2 may promote in vivo osteogenesis. However, C3H10T1/2 cells implanted with beta-tricalcium-phosphate (β-TCP) as osteoconductive material in a standard ectopic assay (59) did not show detectable de novo osteogenesis, independently of FGFR2 expression level or activation (data not shown). The potential impact of activated FGFR2 on osteogenic differentiation in vivo awaits further investigation using osteoprogenitor cells with higher osteogenic potential in vivo. Interestingly, our results suggest that the positive and marked effect of activated FGFR2 on osteoblast differentiation in both C3H10T1/2 and MC3T3-E1 cells was linked to increased Runx2 expression. In contrast, marked activation of osteoelastic cell proliferation by FGF through FGFR1 (35) is expected to reduce Runx2 activity, as mitogenic stimuli and the resulting cell cycle progression into S phase are known to reduce Runx2 levels (60). Because Runx2 is an essential transcription factor involved in mesenchymal cell osteogenic differentiation (8, 10), our finding that FGFR2 increases Runx2 expression provides one mechanism by which activated FGFR2 may promote osteogenic differentiation in mesenchymal cells. This is consistent with our finding that MT FGFR2 increased osteoblastic, but not chondrogenic or adipogenic differentiation in C3H10T1/2 cells, which suggests a lineage specific positive action of FGFR2 on osteoblast differentiation in these cells. Recently, FGFR1 was proposed to play a pivotal role in osteoblast determination in murine stem cells (61). The present results extend this finding to FGFR2 and identify a novel role for this receptor in the osteoblast lineage determination of murine mesenchymal cells.

A key issue was to identify the signaling mechanisms that mediate the positive effect of activated FGFR2 on osteogenic differentiation in mesenchymal stem cells. Recent studies suggest that ERK1/2 and PI3K signaling pathways may play an important role in osteogenic differentiation (16–20). Moreover, a pathogenic role for ERK activation has been established in murine craniosynostosis resulting from activated FGFR2 due to a S252W substitution (48), although the target cells and cellular phenotype remain to be determined. In the present study, we showed that both WT and MT FGFR2 activated ERK1/2 phosphorylation, whereas JNK, PI3K, and AKT pathways were not significantly affected, suggesting a central role of ERK1/2 signaling in the phenotype induced by FGFR2 in these cells. Consistently, we showed that inhibition of ERK1/2 activity abolished the increased cell proliferation induced by WT or MT FGFR2. More importantly, we found that molecular inhibition of ERK1/2 reduced osteoblast marker expression induced by WT FGFR2 but not MT FGFR2, indicating an essential role of ERK1/2 signaling in osteoblast differentiation induced by WT FGFR2 in these cells. These findings indicate that ERK1/2 mediates the positive effect of WT and MT FGFR2 on C3H10T1/2 cell proliferation and contributes to the promoting effect of WT FGFR2 on osteoblast differentiation in murine mesenchymal stem cells.

Additional signaling pathways are likely to play a role in osteoblast gene expression induced by FGFR2. Indeed, we found that MT FGFR2 activated PKC in C3H10T1/2 cells and that inhibition of PKCα reduced osteoblast differentiation markers and in vitro osteogenesis induced by MT FGFR2. These results reveal a prominent role for PKCα in osteoblast differentiation induced by MT FGFR2 in murine mesenchymal cells. A positive role of PKC in the regulation of osteoblast differentiation is supported by the recent finding that PKC activation increases osteoblast gene expression (62, 63). Whether PKCα may up-regulate directly osteoblast gene expression is an interesting question. PKCα was found to be involved in ERK1/2 activation in osteoclasts (64). However, we found that molecular inhibition of PKCα but not ERK1/2 reduced osteoblast gene expression in MT FGFR2 cells, suggesting that ERK1/2 does not act downstream of PKCα in these cells. Functional interactions may also occur between PKCα and the tyrosine kinase Src (65, 66). In osteoblasts, PKCα is known to activate Src signaling (67–69). However, we previously showed that MT FGFR2 reduces Src activity which thereby contributes to promote osteoblast differentiation (39). Thus, PKCα appears to increase osteoblast differentiation in MT FGFR2 cells independently of ERK1/2 and Src signaling pathways. Our results therefore reveal a central role for PKCα in the increased osteoblast differentiation program induced by MT FGFR2 and provide a mechanistic insight how activated FGFR2 may directly lead to acceleration of osteogenic differentiation in mesenchymal cells.

In summary, the present data identify FGFR2 as a novel regulatory molecule that promotes osteogenic differentiation of murine mesenchymal cells and calvarial osteoblastic cells. Furthermore, our results indicate that wild-type and constitutively active FGFR2 promote osteogenic differentiation in mesenchymal cells through distinct pathways involving mainly ERK1/2 and PKCα signaling. These results reveal that activated FGFR2 plays an important role in murine mesenchymal cell osteogenic differentiation and provide insights into the molecular signals involved in the osteogenic differentiation of murine mesenchymal cells induced by FGFR2 activation.

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