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The FGFR/MEK/ERK/brachyury pathway is critical for chordoma cell growth and survival

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Recent evidence suggests that the expression of brachyury is necessary for chordoma growth. However, the mechanism associated with brachyury-regulated cell growth is poorly understood. Fibroblast growth factor (FGF), a regulator of brachyury expression in normal tissue, may also play an important role in chordoma pathophysiology. Using a panel of chordoma cell lines, we explored the role of FGF signaling and brachyury in cell growth and survival. Western blots showed that all chordoma cell lines expressed fibroblast growth factor receptor 2 (FGFR2), FGFR3, mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK), whereas no cell lines expressed FGFR1 and FGFR4. Results of enzyme-linked immunosorbent assay indicated that chordoma cells produced FGF2. Neutralization of FGF2 inhibited MEK/ERK phosphorylation, decreased brachyury expression and induced apoptosis while reducing cell growth. Activation of the FGFR/MEK/ERK/brachyury pathway by FGF2-initiated phosphorylation of FGFR substrate 2 (FRS2)-α (Tyr196) prevented apoptosis while promoting cell growth and epithelial-mesenchymal transition (EMT). Immunofluorescence staining showed that FGF2 promoted the translocation of phosphorylated ERK to the nucleus and increased brachyury expression. The selective inhibition of FGFR, MEK and ERK phosphorylation by PD173074, PD0325901 and PD184352, respectively, decreased brachyury expression, induced apoptosis, and inhibited cell growth and EMT. Moreover, knockdown of brachyury by small hairpin RNA reduced FGF2 secretion, inhibited FGFR/MEK/ERK phosphorylation and blocked the effects of FGF2 on cell growth, apoptosis and EMT. Those findings highlight that FGFR/MEK/ERK/brachyury pathway coordinately regulates chordoma cell growth and survival and may represent a novel chemotherapeutic target for chordoma.

Introduction

Chordoma, a rare cancer believed to originate from malignant transformation of notochordal remnants, accounts for 2–4% of all bone malignancies (1). A population-based study using the Surveillance, Epidemiology, and End Results database demonstrated an overall median survival for chordoma of ~6 years (1). Although histologically considered to be a low-grade neoplasm, chordoma commonly recurs after surgical resection. Distant metastasis occurs in 20–40% of patients with chordoma of the spine and <10% of patients with skull-base tumors (2–4). Median survival after metastasis is ~1 year. Chordoma is resistant to chemotherapy and radiotherapy (5).

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; FGF, fibroblast growth factor; FGF2, basic FGF; FGFR, fibroblast growth factor receptor; FRS2, FGFR substrate 2; MEK, mitogen-activated protein kinase kinase; PBS, phosphate-buffered saline; shRNA, small hairpin RNA.

The occurrence of familial chordoma has recently been linked to duplication of the T (brachyury) gene (6). Brachyury, an important transcription factor within the T-box family of genes, is expressed in normal, undifferentiated embryonic notochord in the axial skeleton and plays an evolutionarily conserved function in mesoderm development (7,8). Brachyury is also expressed in chordoma and other neoplasms (9–15). Functional studies have showed that *in vitro* suppression of brachyury abrogates cell proliferation in two different chordoma cell lines (16,17), whereas overexpression of brachyury in a lung cancer cell line results in enhanced proliferation, motility and invasiveness (18). Those results imply that brachyury may act as an important oncogene in the pathogenesis of chordoma.

Recent data suggest that brachyury is a critical regulator of a large network of oncogenic transcription factors in chordoma (19). It follows that the high expression of brachyury in chordoma cells may play a critical role in tumorigenesis. However, it is unclear what drives brachyury overexpression in chordoma. To date, there is no evidence of mutations or duplications of the brachyury gene in chordoma patients outside of the rare instances of familial susceptibility. It is possible that upstream regulation of brachyury may play a critical role in chordoma pathogenesis. In notochord development, fibroblast growth factor (FGF) and fibroblast growth factor receptor (FGFR) signaling are required for brachyury expression (20,21). Activation of FGFR initiates multiple signal transduction pathways, one of which is mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling. Activated ERK regulates growth factor-responsive targets in the cytosol and also translocates to the nucleus where it phosphorylates a number of transcription factors regulating gene expression. This key signaling pathway controls normal cell proliferation, differentiation and apoptosis (22). The activation of FGFR/MEK/ERK is necessary for mesodermal induction by brachyury (23,24). Furthermore, blocking FGFR- or ERK-mediated signaling significantly inhibits mesoderm induction and angiogenesis (25), which is relevant given the fact that chordoma is thought to derive from mesodermal cells. Based on these data, a complete understanding of the FGFR/MEK/ERK as it relates to brachyury regulation may elucidate the mechanisms of chordoma initiation and progression and may represent a novel therapeutic target.

In this study, we investigate the role of the FGFR/MEK/ERK/brachyury pathway in chordoma cell growth and survival. Furthermore, we investigate the impact of brachyury downregulation using a small hairpin RNA (shRNA)-mediated approach. We found that chordoma cells produce FGF2, and neutralization of FGF2 inhibits MEK/ERK phosphorylation, decreases brachyury expression and induces apoptosis while reducing cell growth. Exogenous human recombinant FGF2 promotes cell growth, inhibits apoptosis and upregulates factors associated with epithelial-mesenchymal transition (EMT) through activation of FGFR/MEK/ERK phosphorylation. In addition, brachyury knockdown by shRNA inhibits FGF2 secretion and blocks the FGF2-induced effects. These results shed new light on the role of FGF2 activation in chordoma growth through the FGFR/MEK/ERK/brachyury pathway and may help to develop novel strategies for the treatment of chordoma.

Materials and methods

Materials

Anti-MEK 1/2 (47E6), anti-phosphorylated MEK 1/2 (S217/221), anti-ERK 1/2, anti-phosphorylated ERK 1/2 (T202/Y204) and anti-FGFR substrate 2 (FRS2)-α (Tyr196) were purchased from Cell Signaling Technology (Danvers, MA). Anti-FGFR2 was purchased from GeneTex (San Antonio, TX). Anti-FGFR1, 3 and 4, anti-brachyury, anti-FGF2, anti-horseradish peroxidase-conjugated secondary antibody against rabbit, PD0325901 (a MEK inhibitor), and PD184352 (an ERK inhibitor) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Normal rabbit IgG was purchased from R & D Systems (Minneapolis, MN). Human recombinant FGF2 was purchased from Life Technologies

(Grand Island, NY). PD173074 (a FGFR inhibitor) was from Cayman Chemical Company (Ann Arbor, MI). CellTiter 96® AQueous One solution Assay and Caspase-Glo 3/7 Assay were from Promega (Madison, WI). Human FGF2 ELISA Kit was from Sigma–Aldrich Co. LLC (St Louis, MO). DNA Cell Death Detection ELISA PLUS Kit was from Roche Diagnostics (Indianapolis, IN).

Cell cultures, transfection and treatments

Human foreskin fibroblast cell line BJ (CRL-2522) was purchased from the American Type Culture Collection (Manassas, VA) and cultured in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS). Chordoma cell line JHC7 was obtained from Johns Hopkins University and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% FBS at 37°C in 5% CO_2. Chordoma cell lines UCH1 and UCH2 cell lines were obtained from Duke University/Chordoma Foundation and grown in 10% FBS of Iscove's modified Dulbecco's media/DMEM (4:1) medium. Prior to stimulation with FGF2, chordoma cells were serum-starved overnight. FGF2 was added at the final concentration of up to 100 ng/ml or with FGFR inhibitor (PD173074, 1 µM). MEK (PD0325901) and ERK inhibitors (PD184352) were added to the cultures at indicated concentrations. The plasmid expressing brachyury-specific shRNA (Johns Hopkins University High Throughput Biology Center) or a control scramble shRNA (Addgene) were generated by inserting the reported sequences into the pLKO.1 vector, and chordoma cells were transiently transfected using X-tremeGENE HP DNA transfection reagent (Roche) for 48 h.

FGF2 enzyme-linked immunosorbent assay

Human foreskin fibroblast BJ cells and chordoma cells were seeded in 12-well plates in triplicate for 24 h. Medium was changed with serum-free cell culture medium for 3 days. The medium was collected and the cell monolayers were washed with 1× phosphate-buffered saline (PBS) and lysed in 300 µl of cell lysis buffer. Cell protein was measured by the Bradford assay. FGF2 in culture medium was quantified with the human FGF2 ELISA Kit (Sigma–Aldrich). Secreted FGF2 measured by enzyme-linked immunosorbent assay (ELISA) was normalized to cellular protein and presented as picograms of FGF2 per microgram of cell protein.

Cell growth assay

Chordoma cells were plated in 96-well plates in triplicate at a density of 2×10^3 cells per well in 100 µl of medium. After treatment with FGF2, normal IgG, anti-FGF2, PD173074, PD0325901, PD184352 and/or transfected with brachyury shRNA or scramble shRNA, cell growth was measured by MTS using a CellTiter 96® AQueous One solution (Promega) based on the manufacturer's protocol. Data represent the mean absorbance of three wells, and these are presented relative to control.

Detection of apoptosis

Chordoma cells were cultured in 96-well plates in triplicate or quadruplicate at 2×10^3 cells per well in 100 µl of medium for 24 h. Cells were treated with FGF2, normal IgG, anti-FGF2, PD173074, PD0325901, PD184352 and/or transfected with brachyury shRNA or scramble shRNA for 48 h. Apoptosis was measured by caspase 3 activity and cytoplasmic DNA fragmentation using Caspase-Glo 3/7 assay and a DNA Cell Death Detection ELISA PLUS Kit following the manufacturer's protocol, and these are presented relative to control.

Western blot assay

Chordoma cells were lysed in ice-cold lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1 mg/ml phenylmethanesulfonyl fluoride) with protease inhibitor cocktail (cOmplete, Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor cocktail (PhosStop, Roche Applied Science). Protein extracts were electrophoresed by 12.5% sodium dodecyl sulfate–polyacrylamide

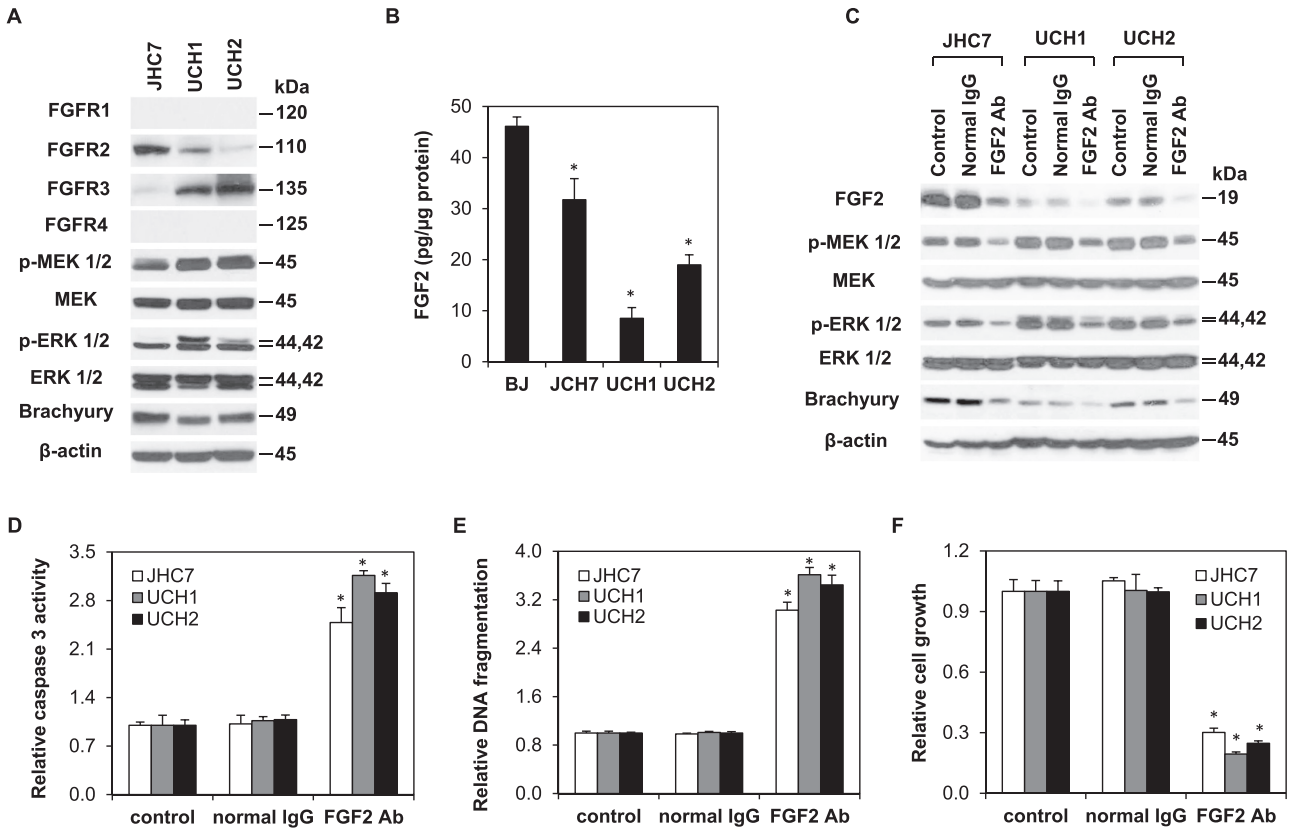

Fig. 1. Expressions of FGF2 and FGFR/MEK/ERK/brachyury in chordoma cells. (**A**) Protein extracts from JHC7, UCH1 and UCH2 cells were used for western blot analysis of FGFR1, 2, 3 and 4, MEK phosphorylation (p-MEK 1/2), MEK 1/2, ERK phosphorylation (p-ERK 1/2), ERK (ERK 1/2) and brachyury. (**B**) Human foreskin fibroblast BJ cells (as a control) and chordoma cells (JHC7, UCH1 and UCH2) were cultured for 3 days in serum-free medium. FGF2 secreted into the medium was measured by ELISA and normalized to protein measured in cell lysates. (**C**) JHC7, UCH1 and UCH2 cells was incubated in serum-free medium (control) and treated with neutralizing antibody to FGF2 (FGF2 antibody, 20 µg/ml) or normal IgG (20 µg/ml) for 48 h. Protein extracts from cells were used for western blot analysis of FGF2, MEK phosphorylation (p-MEK 1/2), MEK 1/2, ERK phosphorylation (p-ERK 1/2), ERK (ERK 1/2) and brachyury. (**D**, **E** and **F**) JHC7, UCH1 and UCH2 cells were incubated in triplicate serum-free medium (control) and treated with neutralizing antibody to FGF2 (FGF2 antibody, 20 µg/ml) or normal IgG (20 µg/ml) as negative control for 48 h (D & E) or 5 days (F). Caspase 3 activity (D), DNA fragmentation (E) and cell growth (F) were measured by the Caspase-GloR 3/7 assay, a DNA Cell Death Detection ELISA PLUS Kit and MTS assay. Values represent means ± SD ($n = 3$). *$P < 0.05$, compared with control group.

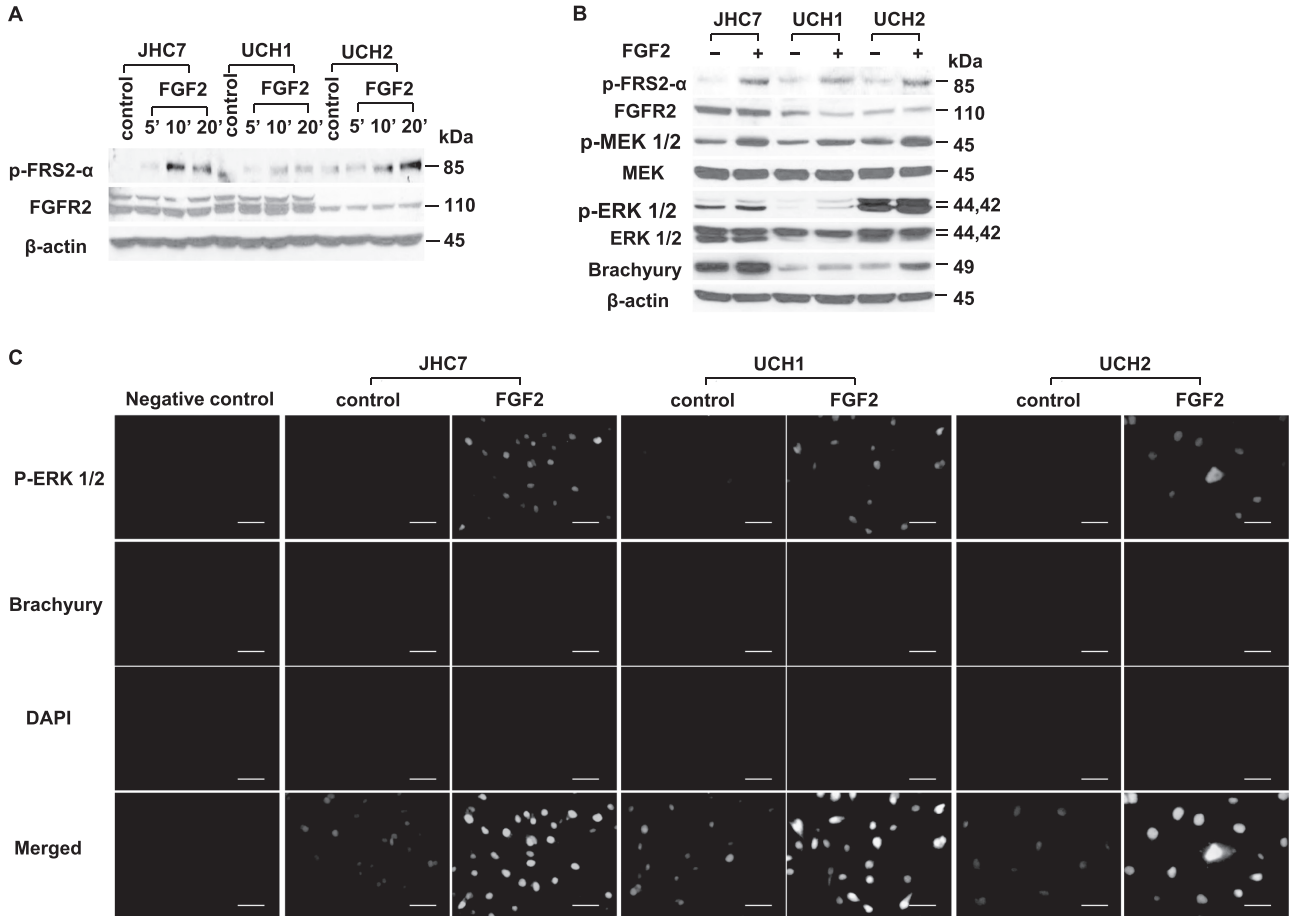

Fig. 2. FGF signaling and response to FGF2 in chordoma cells. (**A**) JHC7, UCH1 and UCH2 cells were seeded in six-well plates for 24 h, then were starved overnight and treated with FGF2 (100 ng/ml) for 5, 10 and 20 min. Cell protein extracts were used for western blot analysis of FGFR2 and its phosphorylation (p-FRS2-α). Representative bands from three independent experiments are shown. (**B**) JHC7, UCH1 and UCH2 cells were seeded in six-well plates for 24 h, then were starved overnight and treated with FGF2 (10 ng/ml) for 24 h. Cell protein extracts were used for western blot analysis of FGFR2, p-FRS2-α, MEK 1/2, ERK 1/2, p-MEK 1/2 and p-ERK 1/2. Representative bands from three independent experiments are shown. (**C**) JHC7, UCH1 and UCH2 cells were starved overnight and treated with FGF2 (10 ng/ml) for 24 h. Cells were fixed and double immunofluorescence stained for p-ERK 1/2 with anti-p-ERK 1/2 (green) and for brachyury with anti-brachyury (red). Meanwhile, a negative control from JHC7 cells incubated second antibodies alone was included. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Yellow color indicates the co-localization of p-ERK 1/2 and brachyury. Scale bars = 50 µm. Representative images from three independent experiments are shown.

gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 5% non-fat dry milk, the membrane was washed three times with Tris-buffered saline/Tween-20 and incubated with the primary antibody diluted in 3% bovine serum albumin or 5% milk at 4°C overnight. The blot was washed in Tris-buffered saline/Tween-20 and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody diluted 1:2000 for goat anti-rabbit (Cell Signaling) or 1:3000 for donkey anti-goat (Promega). The signal was detected using the ECL Prime Western Blotting Detection reagent (GE Healthcare Bio-Sciences Corp., Pittsburgh, PA). Band densities on photographic films were analyzed using Image J 1.47v (National Institutes of Health, Bethesda, MD). Data are presented as means ± standard error of the mean of three independent experiments.

Double immunofluorescence staining

JHC7, UCH1 and UCH2 cells were plated on coverslips in wells of a 24-well plate containing DMEM/F12 and Iscove's modified Dulbecco's media/DMEM medium with 10% FBS. Cells were starved overnight and treated with FGF2 (10 ng/ml) for 24 h. Cells were then fixed with 10% formalin for 15 min. Coverslips were rinsed with PBS and permeabilized with 0.2% Triton X-100 for 20 min, then washed three times with PBS and incubated with anti-phosphorylated ERK 1/2 (1:200; Cell Signaling) and anti-brachyury antibodies (1:100; Santa Cruz Biotechnology) for 1 h. After washing with PBS, cells were incubated with Alexa Fluor 488 goat anti-rabbit and anti-goat immunoglobulin G (Invitrogen, Carlsbad, CA) for 1 h along with 4′-6-diamidino-2-phenylindole (30 nM) for 15 min in the dark and washed three times with PBS. Cell images were captured under a fluorescent microscope (Olympus IX70) with a digital camera and processed using Image-Pro Plus 5.1 software. Data are representative of three independent experiments.

Quantitative real-time PCR

Total RNA from chordoma cells plated in triplicate was isolated using TRIZOL (Life Technologies Chemical, Ann Arbor, MI) according to manufacturer's protocol, and RNA concentrations were measured at A260. Reverse transcription was performed with Omniscript RT Kit (Qiagen, Valencia, CA), Oligo (deoxythymidine) 12–18 Primer (Invitrogen) and ribonuclease inhibitor (Promega). Amplification reactions were performed in triplicate in Applied Biosystems 7500 Real-Time PCR System using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The primers used for real-time PCR were as follows: brachyury forward: 5′-AGACTGGAGAGTTGGG-3′ and reverse: 5′- CAGGTGGTCCA CTCGGTACT-3′; E-cadherin: forward: 5′-CC CACCACGTACAAGGGTC-3′ and reverse: 5′-ATGCCA TCGTTGTTCAC TGGA-3′; Snail forward: 5′-GAGGCGGTGGCAG ACTAG-3′ and reverse: 5′-GACACATCGGTCAGACCAG-3′; Slug forward: 5′-ATATTCGG ACCCACACATTACCT-3′ and reverse: 3′-GCAAATGCTCTGTTGCAGTGA-5′ and glyceraldehyde 3-phosphate dehydrogenase forward: 5′-CATGA GA AGTATGACAACAGCCT-3′ and reverse: 5′-AGTCCTTCCACGATACC AAAGT-3′. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). Each assay included a standard curve of five serial dilutions to quantify gene expressions in samples. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase, and these are presented relative to control.

Statistical analysis

Data are expressed as mean ± SD of at least triplicate measurements or standard error of the mean of three independent experiments. Statistical analysis was performed by SPSS V.10.0 for Windows using one-way analysis of variance with Bonferroni *post-hoc* test. $P < 0.05$ was considered statistically significant.

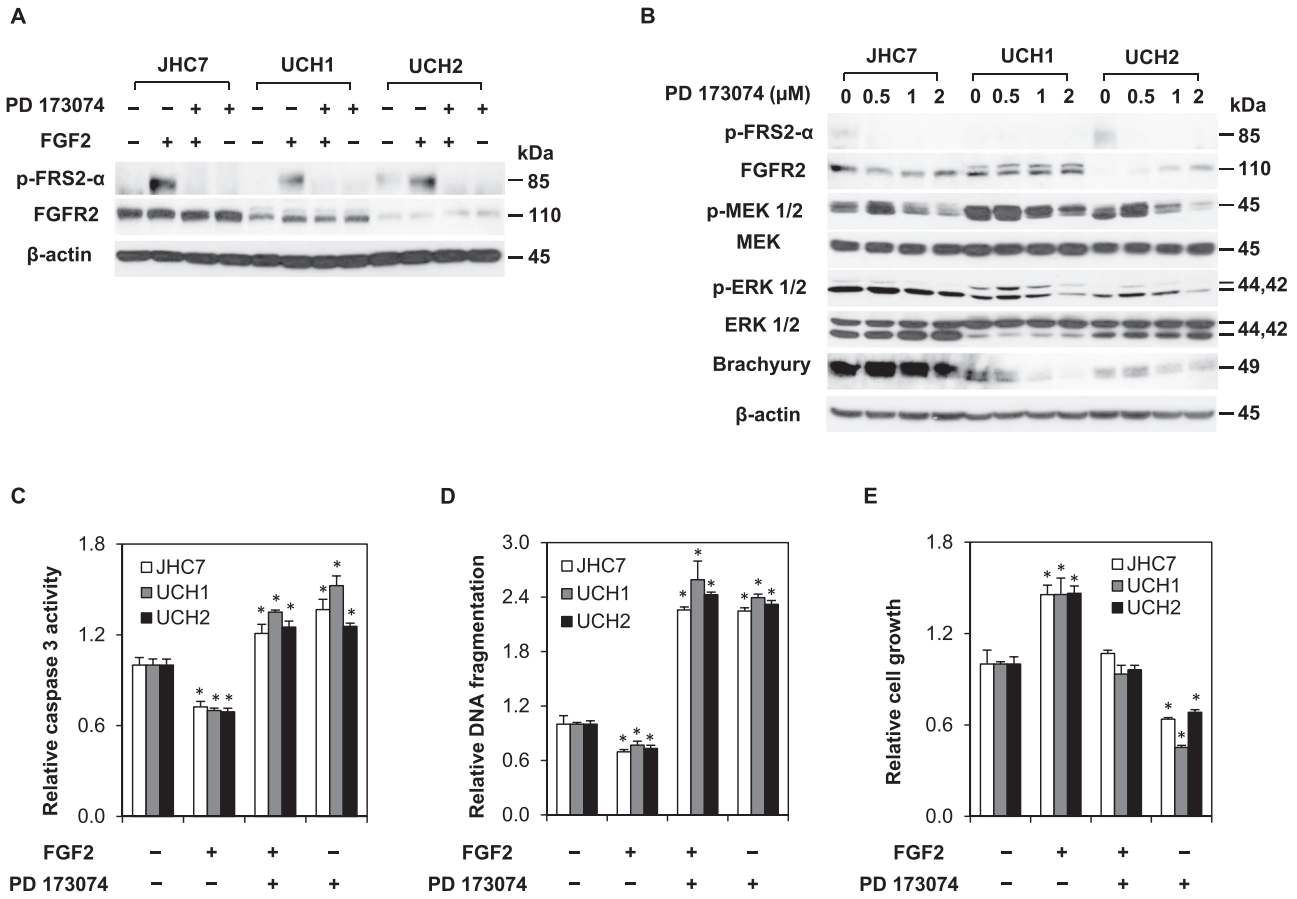

Fig. 3. The inhibition of FGFR reduced MEK/ERK phosphorylation, brachyury expression and the growth of chordoma cells. (**A**) JHC7, UCH1 and UCH2 cells were seeded in six-well plates at density of 2×10^5 cells per well for 24 h, then cells were starved overnight and treated with FGF2 (100 ng/ml) for 10 min following PD173074 treatment (1 μM) for 30 min. Cell protein extracts were used for western blot analysis of FGFR2 and its phosphorylation (p-FRS2). Representative bands from three independent experiments are shown. (**B**) JHC7, UCH1 and UCH2 cells were treated with FGFR inhibitor (PD173074) at dose of 0.5, 1 and 2 μM for 48 h. Cell protein extracts were used for western blot analysis of p-FRS2-α, FGFR2, p-MEK 1/2, MEK 1/2, p-ERK 1/2, ERK 1/2 and brachyury. Representative bands from three independent experiments are shown. (**C**, **D** and **E**) JHC7 and UCH1 cells were seeded in 96-well plate in at least triplicate and treated with FGF2 (100 ng/ml) for 48 h (C & D) or 5 days (E) followed by PD173074 (1 μM) for 30 min. Caspase 3 activity (C), DNA fragmentation (D) and cell growth (E) were measured by the Caspase-GloR 3/7 assay, a DNA Cell Death Detection ELISA PLUS Kit and MTS assay. Values represent means ± SD ($n = 3$ for DNA fragmentation and cell growth, $n = 4$ for caspase 3 activity).*$P < 0.05$, compared with control group.

Results

FGFR/MEK/ERK/brachyury is expressed and FGF2 is produced in chordoma cells

Western blots showed that JHC7, UCH1 and UCH2 cell lines expressed detectable levels of FGFR2, FGFR3, MEK, ERK and brachyury (Figure 1A and Supplementary Figure 1A, available at *Carcinogenesis* Online), whereas no cell lines expressed FGFR1 and FGFR4. Analysis of supernatant medium from the above cell lines by ELISA revealed that FGF2 protein was secreted into the medium by chordoma cells, but the levels of FGF2 secreted by those cells were lower than that secreted by human foreskin fibroblast BJ cells (Figure 1B). Neutralizing FGF2 antibody significantly inhibited MEK/ERK phosphorylation and decreased brachyury expression (Figure 1C and Supplementary Figure 1B, available at *Carcinogenesis* Online). Further, neutralization of FGF2 increased caspase 3 activity (Figure 1D) and DNA fragmentation (Figure 1E) while suppressing cell growth (Figure 1F), suggesting a role of endocrine FGF2 signaling in chordoma progression.

The FGFR/MEK/ERK/brachyury pathway is activated by exogenous FGF2

Western blot assay showed that human recombinant FGF2 activated phosphorylation of FRS2-α Tyr196 (a key FGFR adapter protein required for MEK/ERK pathway activation, Figure 2A and Supplementary Figure 1C, available at *Carcinogenesis* Online), induced MEK and ERK phosphorylation, and upregulated brachyury expression (Figure 2B and Supplementary Figure 1D, available at *Carcinogenesis* Online). Immunofluorescence staining further demonstrated that FGF2 activated ERK and increased p-ERK 1/2 and brachyury expressions in the nucleus (Figure 2C).

The inhibition of FGFR reduces MEK/ERK phosphorylation, decreases brachyury expression, induces apoptosis and suppresses cell growth

We examined FGF signaling in chordoma by exposing chordoma cells to FGF2 and/or PD173074, an FGFR inhibitor. PD173074 blocked FGF2-induced FRS2-α phosphorylation (Figure 3A and Supplementary Figure 1E, available at *Carcinogenesis* Online), reduced the phosphorylation of MEK 1/2 and ERK 1/2 and decreased the level of brachyury at a dose of 1 or 2 μM (Figure 3B and Supplementary Figure 1F, available at *Carcinogenesis* Online). The different inhibitory effects of PD173074 on brachyury expression between JHC7 cell line and UCH1/UCH2 cell line need to be further investigated. We also examined the effects of FGF2 and/or PD173074 on apoptosis and the growth of chordoma cells. Apoptosis was evaluated by caspase 3 activity and DNA fragmentation. We found that FGF2 significantly decreased caspase 3 activity (Figure 3C) and DNA fragmentation (Figure 3D) while promoting cell growth (Figure 3E). PD173074 blocked the effects of FGF2 on

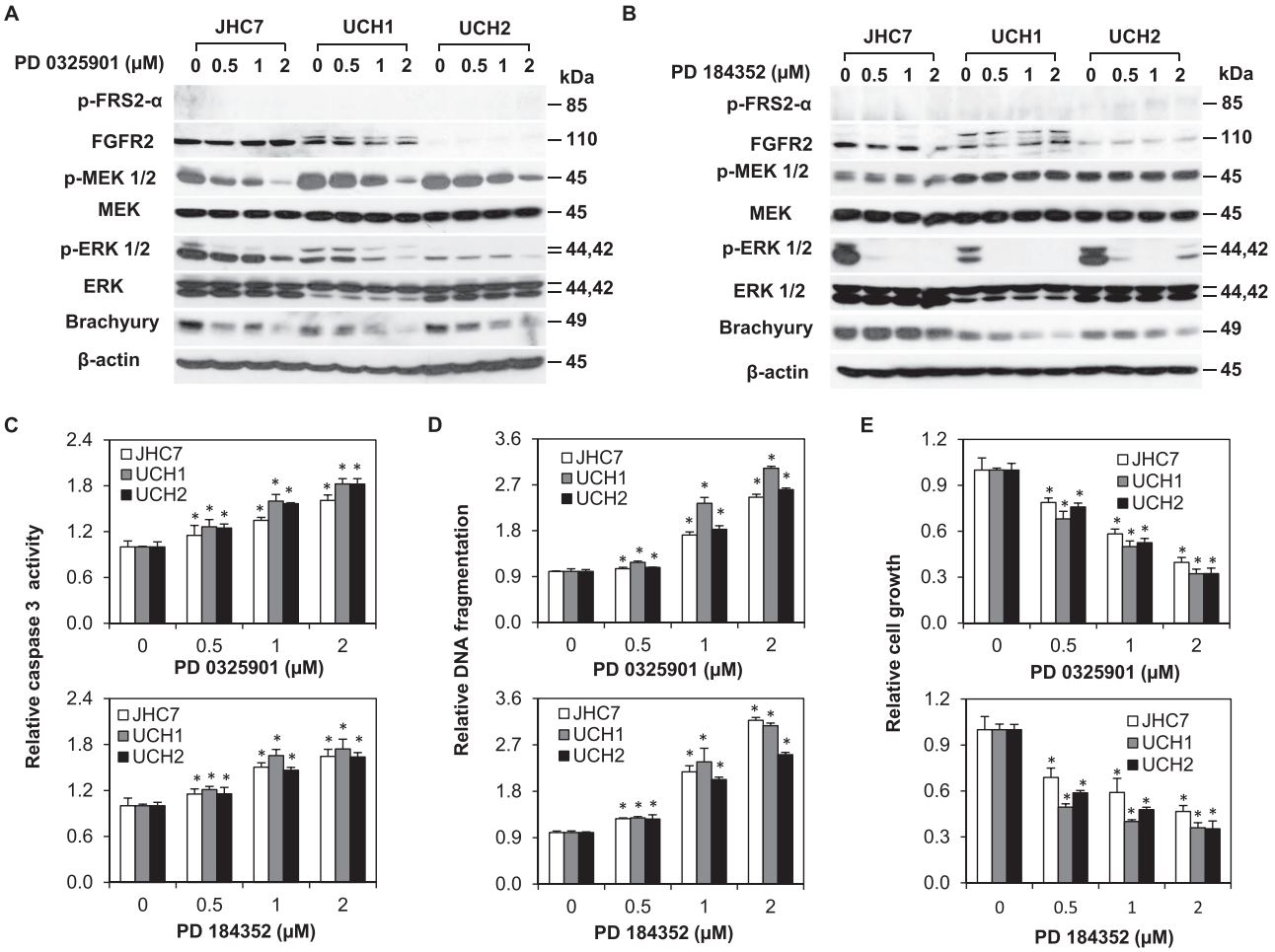

Fig. 4. The inhibition of MEK/ERK phosphorylation suppressed brachyury expression, induced apoptosis and suppressed the growth of chordoma cells. (**A**) UCH1, UCH2 and JHC7 cells were seeded in six-well plates at density of 2×10^5 cells per well and treated with MEK inhibitor (PD0325901) at dose of 0.5, 1 and 2 μM for 48 h. Cell protein extracts were used for western blot analysis of p-FRS2-α, FGFR2, p-MEK 1/2, MEK 1/2, p-ERK 1/2, ERK 1/2 and brachyury. Representative bands from three independent experiments are shown. (**B**) UCH1, UCH2 and JHC7 cells were treated with ERK inhibitor (PD184352) at doses of 0.5, 1 and 2 μM for 48 h. Cell protein extracts were used for western blot analysis of p-FRS2-α, FGFR2, p-MEK 1/2, MEK 1/2, p-ERK 1/2, ERK 1/2 and brachyury. Representative bands from three independent experiments are shown. (**C**, **D** and **E**) JHC7, UCH1 and UCH2 cells were seeded in 96-well plate in triplicate and treated with PD0325901 or PD184352 at doses of 0.5, 1 and 2 μM concentration for 48 h (C and D) or 5 days (E). Caspase 3 activity (C), DNA fragmentation (D) and cell growth (E) were measured by the Caspase-GloR 3/7 assay, a DNA Cell Death Detection ELISA PLUS Kit and MTS assay. Values represent means ± SD ($n = 3$). *$P < 0.05$, compared with control group.

caspase 3 activity (Figure 3C), DNA fragmentation (Figure 3D) and cell growth (Figure 3E). PD173074 alone led to an increase in caspase 3 activity and DNA fragmentation while inhibiting chordoma cell growth.

Inhibition of MEK/ERK phosphorylation decreases brachyury expression, induces apoptosis and suppresses cell growth

We treated JHC7, UCH1 and UCH2 cells with MEK or ERK inhibitors to evaluate the role of these proteins in chordoma pathophysiology. The inhibitor of MEK phosphorylation, PD0325901, had no effect on FGFR2 and its phosphorylation, p-FRS2-α, but decreased ERK phosphorylation as well as brachyury expression in all chordoma cell lines (Figure 4A and Supplementary Figure 2A, available at *Carcinogenesis* Online). Specific inhibition of ERK phosphorylation by PD184352 also led to a reduction in brachyury expression in all chordoma cell lines (Figure 4B and Supplementary Figure 2B, available at *Carcinogenesis* Online). Both PD0325901 and PD184352 led to increased caspase 3 activity (Figure 4C), increased DNA fragmentation (Figure 4D) and reduced cell growth (Figure 4E) in a dose-dependent manner. These data suggest that MEK/ERK/brachyury pathway plays a critical role in chordoma cell growth and apoptosis.

Brachyury knockdown reduces FGF2 secretion and FGF2-induced FGFR/MEK/ERK phosphorylation

Previous studies have demonstrated that brachyury requires endogenous FGFR/MEK/ERK signals for mesodermal maintenance and differentiated mesodermal formation (23,24), whereas FGF-mediated mesodermal formation and maintenance are dependent on brachyury (26). To detect the regulatory effect of brachyury on FGF signaling in chordoma, chordoma cells were transfected with brachyury shRNA or scramble shRNA and subsequently treated with FGF2. As shown in Figure 5, brachyury knockdown reduced FGF2 secretion (Figure 5A), inhibited FGF2-induced phosphorylation of FRS2 (Figure 5B and Supplementary Figure 2C, available at *Carcinogenesis* Online) and reduced MEK/ERK phosphorylation (Figure 5C and Supplementary Figure 2D, available at *Carcinogenesis* Online). Brachyury inhibition also blocked the effects of FGF2 on caspase 3 activity (Figure 5D), DNA fragmentation (Figure 5E) and cell growth (Figure 5F). These data suggest that brachyury is required for FGFR/MEK/ERK-mediated chordoma cell growth and survival.

Brachyury knockdown blocks FGF2-driven EMT

The EMT has emerged as a process of relevance during carcinoma progression and metastasis. Studies have showed that FGF may be

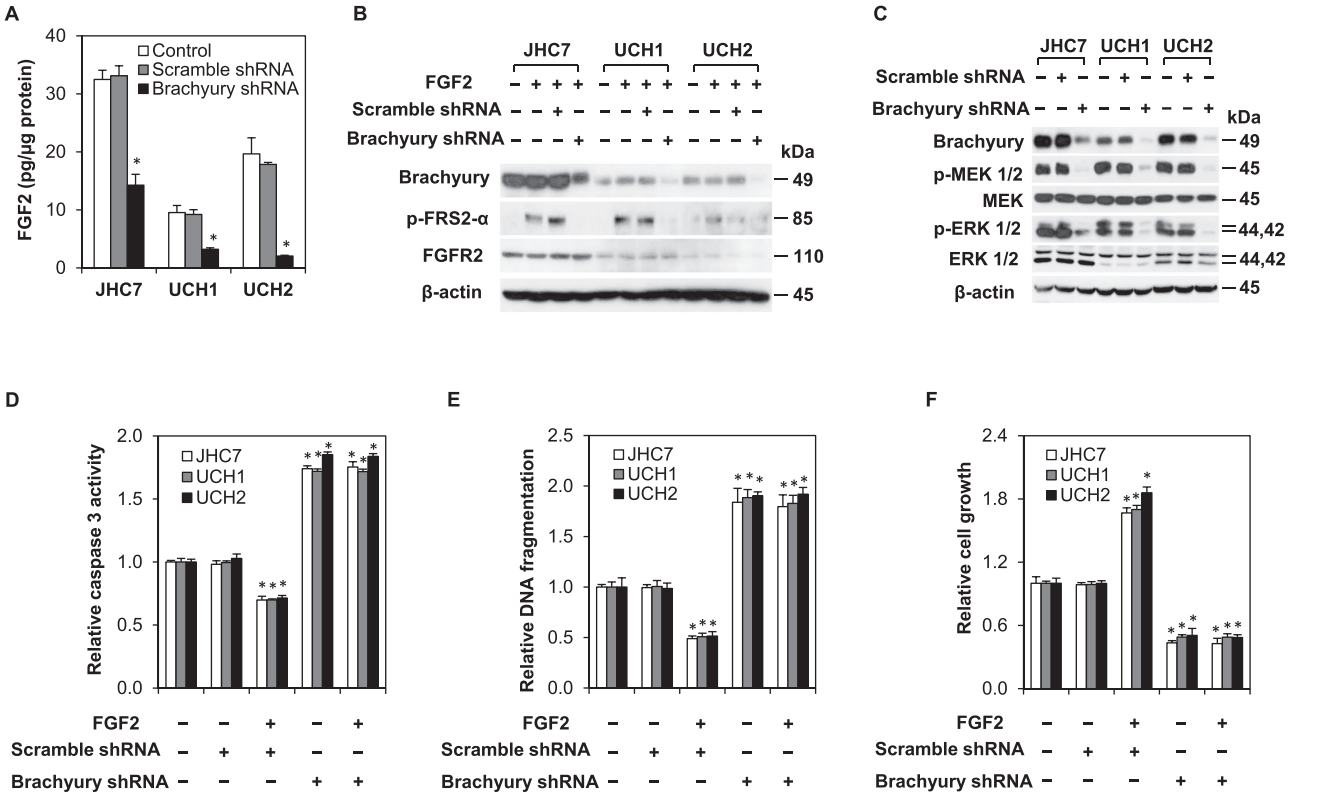

Fig. 5. Brachyury knockdown inhibited FGFR/MEK/ERK signaling and blocked the effects of FGF2 on apoptosis and growth of chordoma cells. (**A**) JHC7, UCH1 and UCH2 cells were cultured in triplicate in FBS-free medium and transfected with scramble shRNA or brachyury shRNA for 48 h. FGF2 secreted into the medium was measured by ELISA and normalized to protein measured in cell lysates. Values represent means ± SD ($n = 3$). (**B**) JHC7, UCH1 and UCH2 cells were transfected with scramble shRNA or brachyury shRNA for 24 h, then were starved overnight and treated with FGF2 (100 ng/ml) for 10 min. Cell protein extracts were used for western blot analysis of brachyury, FGFR2 and p-FRS2-α. Representative bands from three independent experiments are shown. (**C**) JHC7, UCH1 and UCH2 cells were transfected with scramble shRNA or brachyury shRNA for 48 h. Cell protein extracts were used for western blot analysis of brachyury, p-MEK 1/2, MEK, p-ERK 1/2 and ERK 1/2. Representative bands from three independent experiments are shown. (**D**, **E** and **F**) JHC7, UCH1 and UCH2 cells were seeded at 96-well plate and transfected with scramble shRNA or brachyury shRNA for 24 h, then were starved overnight and treated with FGF2 (100 ng/ml) for 48 h (D and E) or 3 days (F). Caspase 3 activity (D), DNA fragmentation (E) and cell growth (F) were measured by the Caspase-GloR 3/7 assay, a DNA Cell Death Detection ELISA PLUS Kit and MTS assay. Values represent means ± SD ($n = 3$). *$P < 0.05$, compared with control group.

involved in different stages of EMT-mediated mesoderm formation (21,27). Furthermore, brachyury is essential during EMT-mediated mesodermal formation in vertebrate embryos (11,18). The role of brachyury-mediated EMT in chordoma pathophysiology is unclear. To elucidate whether FGF signaling plays a role in brachyury-mediated EMT in chordoma, we examined expressions of E-cadherin (epithelial marker), Snail and Slug (mesenchymal markers) in JHC7, UCH1 and UCH2 cells transfected with brachyury shRNA or scramble shRNA followed by treatment with FGF2. The messages of E-cadherin, Snail and Slug were measured by real-time PCR. As shown in Figure 6A, brachyury messenger RNA levels of JHC7, UCH1 and UCH2 cells transfected with brachyury shRNA for 44 h were reduced by 27%, 51% and 67% compared with controls (no treatment), respectively (Figure 6A). Brachyury knockdown blocked the effects of FGF2 on EMT by upregulating E-cadherin expression (Figure 6B) and down-regulating Snail (Figure 6C) and Slug expression (Figure 6D) in all chordoma cell lines. These results suggest that brachyury mediates the FGF-initiated EMT in chordoma cells.

Discussion

We have demonstrated that the coordinate regulation of FGF/FGFR signaling and brachyury plays an important role in the growth and survival of chordoma cells. Chordoma cells produce FGF2. Neutralization of FGF2 inhibits MEK/ERK phosphorylation and decreases brachyury expression, which results in an induced apoptosis and cell growth suppression. In addition, exogenous human recombinant FGF2 reduces apoptosis while promoting chordoma cell growth and EMT by activating the FGFR/MEK/ERK/brachyury pathway. Inhibition of FGFR/MEK/ERK phosphorylation reduces brachyury expression, induces apoptosis and inhibits chordoma cell growth. Our data suggest that FGF2 and FGFR signaling play a role in the growth stimulation and prevention of apoptosis of chordoma cells. We have also shown that FGFR/MEK/ERK pathway is positively regulated by brachyury. Knockdown of brachyury by shRNA reduces FGF2 secretion, inhibits FGF2-induced phosphorylation of FRS2-α and blocks the FGF2-induced effects on FGFR/MEK/ERK-dependent growth, apoptosis and EMT. Taken together, these results indicate that FGF2-activated FGFR/MEK/ERK/brachyury pathway interactively controls cell growth, survival and EMT in chordoma (Figure 7).

In humans, the FGF family consisting of FGF1–FGF22, which binds to four high-affinity FGF receptors (FGFR1–FGFR4), is involved in a broad range of biological processes such as migration, proliferation and differentiation of various cell types (28,29). The important role of FGF and FGFR in regulating cancer cell proliferation, survival and/or tumor angiogenesis is emerging in multiple cancers including prostate (30), breast (31,32), thyroid (33), skin (34), lung (35), liver (36), bladder (37,38), and head and neck cancers (39). Previous descriptive data from immunohistochemical analysis of chordomas have found that FGF2 expression is linked to chordoma development (40,41), but the functional role of FGF2 signaling in chordoma cell growth is not yet understood. In this study, we have demonstrated that FGF2 is secreted by chordoma in multiple cell lines. Furthermore, we have used complementary strategies, including FGF2 immunoneutralization and pharmacologic inhibition of the tyrosine kinase activity of

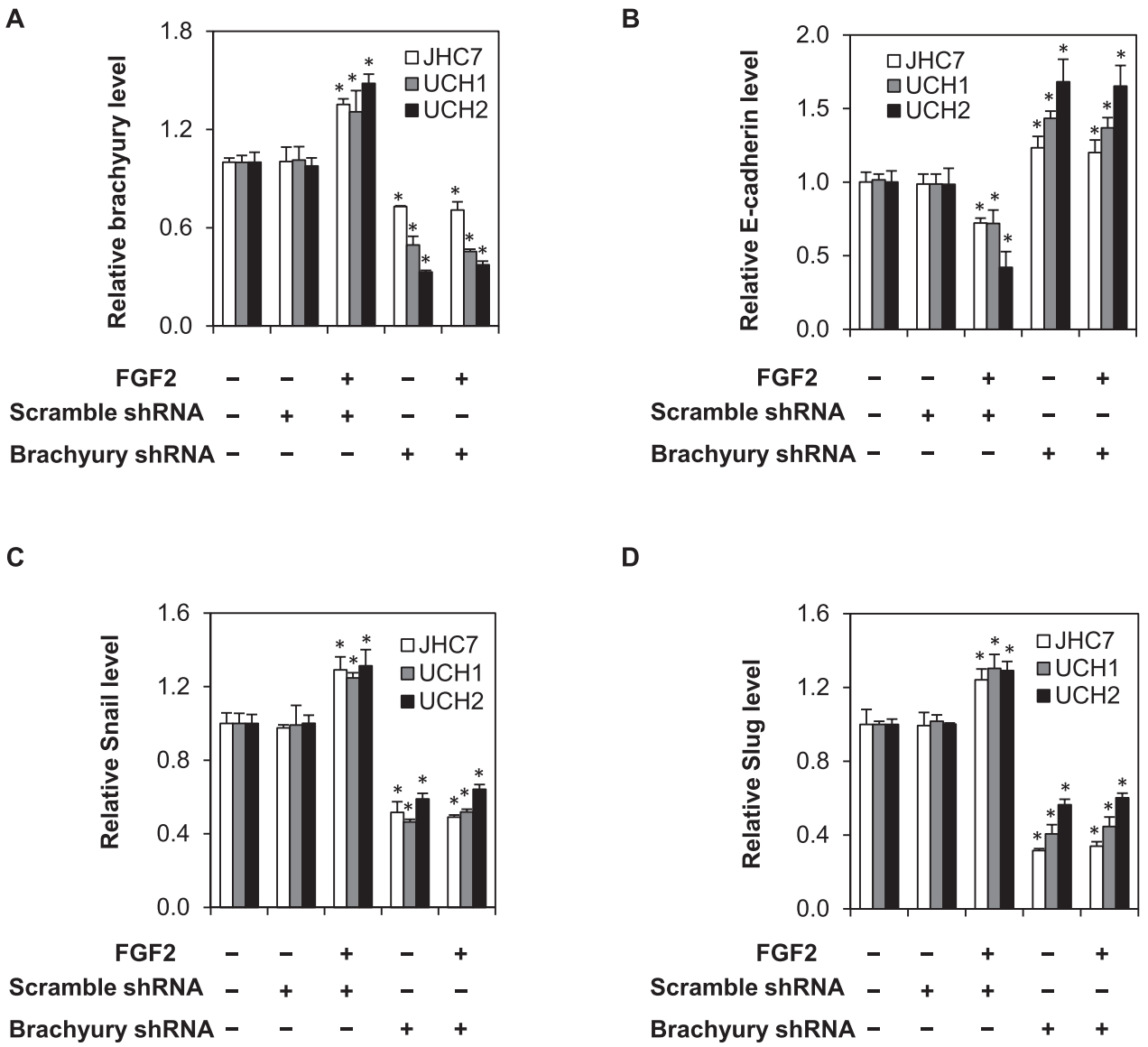

Fig. 6. Brachyury knockdown blocked the effects of FGF2 on EMT in chordoma cells. JHC7, UCH1 and UCH2 were cultured in triplicate for 24 h and transfected with scramble shRNA or brachyury shRNA for 24 h, then were starved overnight and treated with FGF2 (100 ng/ml) for 8 h. Total RNA was isolated and messenger RNA messages of brachyury, E-cadherin, Slug and Snail were determined by real-time PCR. Data shown are the levels of brachyury (**A**), E-cadherin (**B**), Slug (**C**) and Snail (**D**) gene expressions relative to control ($n = 3$). *$P < 0.05$, compared with control group.

FGFR, and disclosed that the constitutive MEK/ERK/brachyury cascade is an autocrine mediator of FGF2-induced chordoma cell growth and antiapoptosis.

FGFR is involved in oncogenesis and is currently under investigation for its potential as drug targets. The expressions of FGFR, MEK and ERK in chordoma have been described at a clinical level (42). However, a relationship between these potential biomarkers and clinical outcome is unclear. In this study, we have found that all three chordoma cell lines express FGFR2 and FGFR3, and activation by FGF2 or inhibition of FGFR by PD173074 impacts chordoma cell growth and survival. Therefore, it seems that this FGFR2 or FGFR3 signaling is common and important to chordoma.

The classical MEK/ERK cascade is an essential component of the well-characterized FGF signaling pathway for induction of mesoderm in *Xenopus* embryos and mammals (23,43). This pathway is often upregulated in human cancers and represents an attractive target for mechanism-based approaches to cancer treatment. In this study, we have found that FGF2 promotes chordoma cell growth and rapidly induces MEK/ERK phosphorylation and upregulates brachyury expression, whereas disruption of this FGFR/MAPK/brachyury signaling by specific inhibitors of FGFR, MEK, ERK or brachyury shRNA leads to significant growth inhibition of chordoma cells. These data indicate that the growth stimulatory effect of FGF2 is immediately transmitted through MEK1/2/ERK1/2/brachyury in chordoma cells. Double immunofluorescence staining demonstrated that FGF2 activates ERK phosphorylation to recruit it into the nucleus with subsequent transcriptional activation of brachyury, implying that the FGF-driven translocation of ERK phosphorylation to the nucleus may impact the transcriptional regulation of brachyury in chordoma cells. In addition, brachyury knockdown blocks the effects of FGF signaling on chordoma cell growth and survival, suggesting that brachyury may play a critical regulatory role in chordoma pathophysiology.

EMT is emerging as a critical factor for cancer growth, metastasis and drug resistance. Brachyury has been recently characterized as a driver of EMT in human cancer cells (10,11). Overexpression of brachyury in human lung cancer cells induces characteristic changes of EMT, including upregulation of N-cadherin and vimentin (mesenchymal markers)

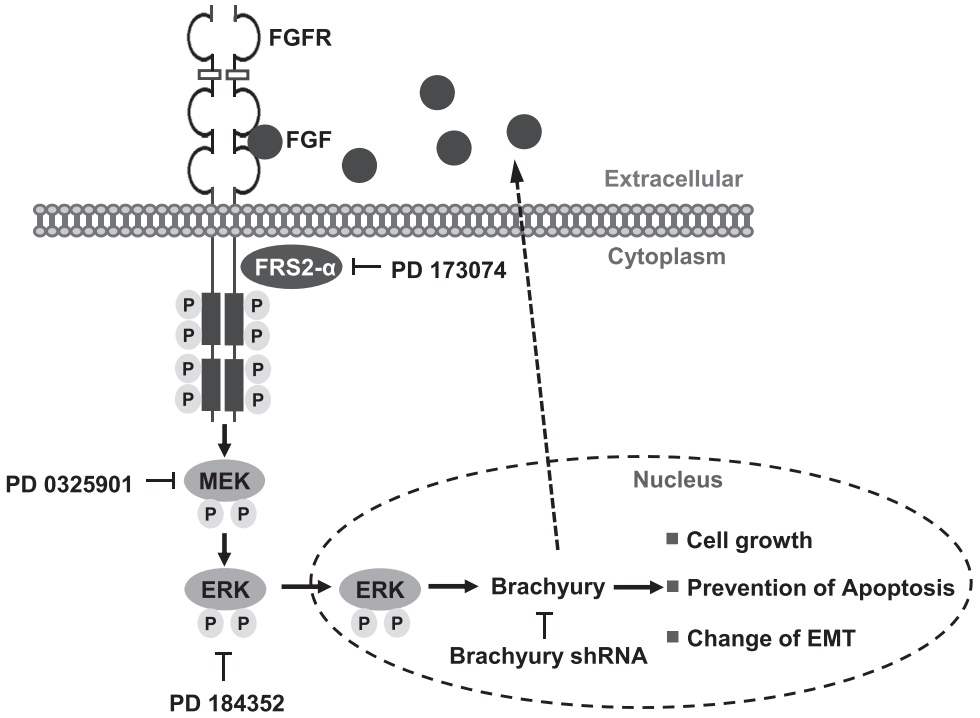

Fig. 7. Schematic diagram illustrating the feedback loop of FGF/FGFR signaling and brachyury. FGF binds to FGFR to induce FGFR phosphorylation and further activate MEK/ERK, which results in an increased brachyury expression. Brachyury in turn promotes the secretion of FGF and enhances FGF/FGFR signaling and, subsequently, MEK and ERK. This feedback loop of FGF/FGFR signaling and brachyury may be critical for chordoma cell growth, survival and EMT.

and downregulation of E-cadherin (epithelial marker) (18). Conversely, inhibition of brachyury results in upregulation of plakoglobin (epithelial marker) and downregulation of Snail, Slug and fibronectin (mesenchymal markers) (18). A recent retrospective cohort study from 152 patients with oral squamous cell carcinoma has showed that brachyury expression is associated with lymph node and distant metastasis (9). In chordoma, loss of brachyury results in a significant decrease in mesenchymal markers N-cadherin and Slug (17). In this study, we further highlight the potential role of brachyury in the EMT process by showing downregulation of factors implicated in EMT promotion (Snail and Slug) after specific knockdown of brachyury using shRNA. Furthermore, the upregulation of Snail and Slug by FGF2 is blocked by brachyury knockdown, suggesting that brachyury may be a critical regulator of EMT in chordoma, if not other tumor types.

Previous studies have showed that FGF can activate the brachyury promoter and maintains brachyury expression to control a diverse array of developmental events including mesodermal induction, patterning and body length (44–46). In addition, ectopic expression of brachyury activates transcription of the embryonic FGF (44), suggesting that the regulatory loop of FGF and brachyury plays an important role in development. Because chordoma is thought to arise from remnants of the embryonic notochord, it raises the possibility that the interplay of FGF and brachyury could modulate the progression of chordoma. In this study, we found the regulation of brachyury by FGF/FGFRs signaling via MAPK pathway. Further, brachyury had a feedback effect on FGF/FGFRs signaling. These results imply that a positive feedback loop of FGF/FGFR and brachyury could be required for promoting chordoma cell growth and survival (Figure 7).

In conclusion, our data underscore the importance of FGF-activated FGFR/MEK/ERK/brachyury signaling in chordoma cell growth, survival and EMT. Our study provides a biological basis for novel chemotherapeutic strategies against chordoma that target the FGF-activated FGFR/MEK/ERK/brachyury pathway. Newly developed animal models of chordoma provide an opportunity to test chemotherapeutic strategies targeting this pathway, which may lead to clinical trials in human patients in the near future.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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