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A single-point mutation in FGFR2 affects cell cycle and Tgfβ signalling in osteoblasts

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ABSTRACT

Fgf and Tgfβ are key regulators of bone development. It is not known, however, whether there is a relationship between defective Fgf signalling, resulting in a premature cranial suture fusion, and Tgfβ signalling. We used mouse calvaria osteoblasts carrying a mutation (hFGFR2-C278F) associated with Crouzon and Pfeiffer syndromes to investigate effects of this mutation on cell growth and possible mechanisms underlying it. Mutated osteoblasts displayed reduced S-phase, increased apoptosis and increased differentiation. As Tgfβ signalling appeared to be required in an autocrine/paracrine manner for osteoblast proliferation, we tested the hypothesis that reduced growth might be due, at least in part, to an altered balance between FGF and Tgfβ signalling. Tgfβ expression was indeed decreased in mutated osteoblasts, as compared to osteoblasts carrying the wild type hFGFR2. Treatment with Tgfβ, however, neither increased proliferation in mutated osteoblasts, unlike in controls, nor rescued proliferation in control osteoblasts treated with an Erk1/2 inhibitor. Significantly, Erk2, that is important for proliferation, was reduced relatively to Erk1 in mutated cells. Altogether this study suggests that the hFGFR2-C278F mutation affects the osteoblast ability to respond to Tgfβ stimulation via the Erk pathway and that the overall effect of the mutation is a loss of function.

1. Introduction

Osteoblast growth and maturation is a complex process and several signalling pathways are involved in its modulation and in maintaining the balance between proliferation and differentiation during bone development. Among key regulators of bone development are fibroblast growth factors (FGFs) and their tyrosine kinase receptors (FGFRs) [1]. Upon FGF binding, dimerisation and transphosphorylation of the receptor result in the activation of a number of signal transduction pathways including Ras/MAPK (mitogen activated protein kinase), phosphoinositol-3 kinase (PI3-K), and phospholipase C gamma (PLC-γ) [2,3].

Much interest in Fgf signalling in cranial bone development over the last few years has stemmed from the discovery that single point mutations in FGFRs represent the most common genetic cause of craniosynostosis, that is premature fusion of cranial sutures [1,4-6]. Nonetheless, the mechanisms underlying these defects are not yet fully understood.

FGFR1-3 are expressed in the developing cranial bone in characteristic patterns [7-9]. Mutations in regions involved in ligand binding and kinase activity, thought to cause a gain-of-function, have been identified in all of these receptors and linked to syndromic craniosynostosis, such as Crouzon, Pfeiffer or Apert syndromes, but the most commonly mutated receptor in patients with syndromic craniosynostosis is FGFR2 [1,10]. Several Fgfs have been implicated in cranial bone development [1,11]. *In vitro* and *in vivo* studies using a variety of models have shown diverse effects of Fgf on bone growth and differentiation and indicated that changes in the level of Fgf signalling, either due to availability of ligands or functionality of the receptor, can shift the balance between proliferation and skeletogenic differentiation both in neural crest cells and in mesenchymal cells, the two lineages from which the cranial vault osteoblasts are derived [11-20].

Besides Fgf, several other growth factors, including members of the transforming growth factor beta (Tgfβ) super-family, are known to play key roles in bone growth and differentiation, and their role depends on the stage of osteoblast maturation [2,21-23]. Of the three mammalian Tgfβ isoforms, Tgfβ1 displays the highest levels of expression in bone and most consistently induces osteoblast proliferation [24-27]. The formation of a Tgf-TβRII (Tgfβ receptor type 2) homodimer complex initiates Tgfβ signalling by promoting further binding and activation of Tgfβ receptor type 1 (TβRI, ALK5) to this complex. While Smads are key transducers of Tgfβ signalling, other signalling pathways, such as MAPK, can be activated by Tgfβ [28]. The MAPK (mitogen-activated protein kinase) pathway required in osteoblast proliferation appears to be Erk1/2 (extracellular related kinase), rather than JNK and p38 [29,30].

Cross-talking between Fgf and Tgfβ signalling has been suggested. For example, it has been shown that Fgf2 signalling may control Tgfβ1 expression, and that Fgf2 and Tgfβ1 might regulate each other [31]. In addition, a mutation in TβRI has been described to cause

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craniosynostosis [32]. Osteoprogenitors from craniosynostotic patients carrying a mutated FGFR often display limited growth and increased differentiation [33-37] and we wished to test the hypothesis that this might be due, at least in part, to an altered balance between Fgf signalling and other signalling pathways. Given the known effects of Tgfβ on bone development and of cross-talk with Fgf signalling [31,38-40], we focused our attention on this pathway.

The osteoblastic cell line MC3T3, originated from mouse calvarium, and more recently MC3T3 carrying mutated receptors, have been widely used as a model for studying bone development and osteoblast differentiation [36,41,42]. One of the mutations found in children with Crouzon or Pfeifer type of craniosynostosis is the mutation at position 278 (C278F) in the third immunoglobulin-like extracellular domain IIIa of FGFR2 [5]. MC3T3 cells carrying FGFR2-C278F have been shown to be a good model of craniosynostotic cells, and, like osteoblasts from patients with craniosynostosis, require longer time than controls to reach confluence [36]. Therefore we have used stable MC3T3 cell lines carrying human wild type FGFR2 (WT-FGFR2) or FGFR2-C278F [36] to investigate the effect of this mutation on osteoblast behaviour and on Tgfβ signalling, and to assess how Fgf and Tgfβ pathways may interact.

Our analysis of FGFR2-C278F cells shows changes in their cell cycle and reduced survival as compared to control MC3T3 and WT-FGFR2 cells, and that reduced growth appears to be due to altered Erk signalling. We also show that in the mutated osteoblasts Tgfβ expression is reduced, and that there is a reduction in Tgfβ-induced proliferation mediated by Erk, suggesting convergence of Fgf and Tgfβ signalling on Erk to induce proliferation.

2. Materials and methods

2.1. Cell culture

The osteoblastic cell line MC3T3 and previously established MC3T3 cell lines stably transfected with either human FGFR2 (FGFR2-WT) or FGFR2-C278F (FGFR2-C278F) [36] were cultured in α-MEM medium (Gibco-BRL, Paisley, UK) containing 10% fetal bovine serum (FBS), penicillin and streptomycin (100 U/ml; Gibco-BRL). Cultures were passaged every 3 days and plated at a density of 20 000 cells/cm^2. Cells were allowed to attach for 3 h before treatment with the following compounds that were used at the concentrations indicated in Results: SU5402 (572630, Calbiochem), rhTGFβ1 (240-B-010, R&D Systems), SB431542 (S4317, Sigma) and U0126 (9903, Cell Signalling Technology). Cells were grown on 12 well culture plates for FACS (Fluorescence activated cell sorting) analysis to monitor the cell cycle, and on coverslips for immunofluorescence. Live cells in culture were viewed under an inverted light microscope (Axiovert 135M, Zeiss), and photographed using a Hamamatsu ORCA-ER camera, visualised using Volocity® software (Improvision).

2.2. Assessment of cell growth

Cell growth analysis was carried out in 96 well plates using the methylene blue dye as previously reported [43]. Cells were fixed in 4 % paraformaldehyde (PFA) for 30 min before incubating with methylene blue (1% w/v methylene blue (Gurr®) in 0.01 M borate buffer (pH 8.5). After 4 washes with 0.01 M borate buffer (pH 8.5) to remove excess dye, the intracellular methylene blue was extracted using 50% v/v ethanol in 0.1 M HCl. The 96 well culture plate was stirred and absorbance was measured at 650 nm (A650) in a microplate reader (Revelation v4.21, Dynex Technologies, Inc).

2.3. Cell cycle and apoptosis analysis

Cells were detached by incubation with Trypsin-EDTA (Invitrogen) for 5 min, then pelleted and either resuspended in permeabilising solution (0.1% sodium citrate and 0.1% Triton X-100 in dH_2O) for cell cycle analysis or PBS (phosphate buffer saline) for measuring apoptosis. Cells were kept on ice for up to 2 h before analysis. The DNA was intercalated with 20 μl of 0.1 mg/ml 7 aminoactinomycin D (7AAD) just prior to flow cytometry analysis (Epics XL, Beckman Coulter). Absorption of the 488 nm argon laser by 7AAD, resulted in emission in FL3 (peaked at 647 nm), which was detected at 675 nm with a band pass filter and the data recorded, using the EXPO32 software (Beckman Coulter). Gating was used for doublet discrimination. For cell cycle analysis, the frequency versus area of 7AAD was plotted, resulting in a curve, which was mathematically analysed using MultiCycle for Windows (Phoenix flow systems, San Diego). This resulted in the G0/G1, S and G2/M phase curves, and the proportion of cells in each phase was determined by the Dean and Jett method [44]. In the apoptosis study, 7AAD fluorescence was plotted against frequency and the 7AAD positive cells gated from the baseline fluorescence, highlighting the level of apoptosis based on increased membrane permeability.

2.4. Phospho-histone H3 (pH3) immunocytochemistry

Mitotic cells cultured on coverslips were detected by incubating with rabbit polyclonal anti-phospho-histone H3 (pH3) antibody (Upstate Cell signaling solutions) dissolved in PBS containing 0.1% bovine serum albumin and 0.5% Triton X-100 at 4 °C overnight and washed using PBS. Samples where then incubated with anti-rabbit immunoglobulins-FITC conjugated secondary antibodies (Dakocytomation) dissolved in the same solution as the primary antibody, using Hoescht 33258 (Molecular probes) as a counterstain. Coverslips were mounted with Citifluor™. Cells were imaged with a ProgRes® C14 (Jenoptik) camera mounted onto a Zeiss Axiophot 2 microscope using the Openlab software (Improvision). pH3-positive cells and total number of cells per coverslip were counted and mitotic cells expressed as a percentage of the total cell number.

2.5. Protein extraction and Western blot

Protein extraction from cell pellets was performed with 50–200 μl ice-cold lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris–HCl pH 8.0) containing 1:25 proteinase inhibitor (Complete™, Roche) and 1 mM Sodium Orthovanadate. Equal amounts of protein (20–40 μg/lane) were separated by 12% SDS-PAGE. Membranes were blocked in Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween-20 (TBST) and incubated with either anti-Erk1/2 (1:1000) or anti-pErk1/2 (1:2000) antibodies (9102, 9106, Cell Signaling) at 4 °C overnight followed by washes with TBST. Bound antibodies were detected either by horseradish peroxidase (HRP)-conjugated rabbit anti-mouse (1:1000) or HRP-conjugated goat anti-mouse secondary antibodies (P0260, P0447, Dakocytomation). The enhanced chemiluminescence (ECL) method was used for immunodetection and densitometry was performed using Labimage (v2.7.1, Kapelan). The Erk1 bands were normalised to Erk2 for relative quantification of Erk1/Erk2 ratios.

2.6. RNA extraction and reverse transcription

RNA extraction was carried out using the TRI®-Reagent (Sigma) protocol. RNA was diluted to 1 ng/μl in a 10 μl volume of 2 μl of 10 pM random hexamer (pN6, Roche) in DEPC (diethylpyrocarbonate) water. Annealing was carried out at 70 °C for 10 min, followed by 5 min at 4 °C in a PTC-100 thermal cycler (MJ Research Inc.). Four μl of RT buffer (2 μl of 10 mM dNTPs, 1 μl RNAsin, 1 μl reverse transcriptase MMLV (Promega) in DEPC water) were then added to the annealed solution. Reverse transcription and MMLV denaturing were performed at 42 °C for 1 h and at 95 °C for 10 min, respectively.

2.7. Real time PCR

TaqMan® Fast Universal Master Mix (2X) with No AmpErase® UNG (Applied Biosystems) was added to cDNA. Taqman® Gene Expression assays (Applied Biosystems) used for measuring Tgfβ1 (Mm00441724_m1), Tgfβ2 (Mm004366952_m1) and Tgfβ3 (Mm00436960_m1), and normalised to Eukaryotic 18S rRNA (Part no: 4352930E). Amplification and analysis were performed using an ABI 7500 FAST real time PCR machine.

2.8. Statistical analysis

All experiments were carried out at least in triplicate ($n \geq 3$) and repeated at least three times. Data are expressed as mean ± standard error of the mean (M ± SEM) and statistical differences between FGFR2-C278F cells and each control assessed by ANOVA and independent *t*-test using SPSS (version 14, SPSS Inc.); $p < 0.05$ was taken to indicate a significant difference.

3. Results

3.1. Effects of FGFR2-C278F on proliferation and apoptosis

An initial analysis of FGFR2-C278F osteoblasts indicated that these cells needed longer time in culture to reach confluence than MC3T3 and FGFR2-WT cells, which for simplicity of presentation will be referred to as "controls." Therefore we investigated whether this was due to a defect in proliferative capability, increased apoptosis, or both. To confirm that Fgf signalling is one of the pathways involved in cell proliferation in all the cell lines used in this study, we assessed the effect of the FGFR inhibitor SU5402 on cell growth, and found that SU5402 reduced cell growth in all cell lines (Fig. 1). Further analysis of the three cell lines showed a reduced increase in cell number over time in FGFR2-C278F cells as compared to controls (Fig. 2A). In order to investigate which phase of the cell cycle may be affected in the mutated cells we carried out FACS analysis in 2-day cultures (Fig. 2). The cell cycle profile of MC3T3 and FGFR2-WT was comparable, whereas the percentage of FGFR2-C278F cells in S phase was significantly lower, and that of cells in G2/M phase higher as compared to controls (Fig. 2B). As the FACS analysis carried out did not distinguish between cells in G2 and M phase, staining with pH3 antibody both on coverslips (Fig. 2C) and in suspension (not shown) followed by FACS analysis was used to assess changes in M phase. Analysis of mitosis detected by pH3 showed that the percentage of cells in M phase was significantly lower in FGFR2-C278F than in control cell lines.

To assess whether apoptosis may also contribute to the lower cell number observed in FGFR2-C278F cells, TUNEL (not shown) and FACS analysis (Fig. 3) was carried out in 2-day cultures. Though at this time-point the extent of apoptosis was small in all cultures, the percentage of apoptotic cells in FGFR2-C278F cultures was significantly higher than in both control cell lines (Fig. 3A). This difference greatly increased with time in culture as indicated by FACS analysis at 7 days (Fig. 3B).

We also assessed cell morphology in the three cell lines at 2 and 4 days in culture (Fig. 4). A significant decrease in the percentage cells with elongated/fibroblastic morphology was observed in FGFR2-C278F cultures at 2 days, as confirmed by quantitative analysis (Fig. 4A–C, G). At 4 days in culture (Fig. 4D–F), the morphology of nearly all cells in culture was cuboidal, but FGFR2-C278F cells, unlike controls, were not fully confluent.

3.2. Control of proliferation by Tgfβ signalling

In order to assess a possible autocrine role of Tgfβ signalling in the proliferation of our cell lines, Tgfβ receptor was inhibited using different concentrations of SB431542, a TβRI inhibitor of ALK4, -5 and -7 [45,46], and changes in cell growth measured (Fig. 5). Partial inhibition of growth was induced in a dose-dependent manner in the two control cell lines (Fig. 5). SB431542 also inhibited growth of FGFR2-C278F cells, but maximal effect was already observed at the lower dose tested (Fig. 5), suggesting that Tgfβ signalling is less active in these cells. To further investigate changes in Tgfβ signalling caused by the FGFR2 mutation, we assessed expression of *Tgfβ1*, *Tgfβ2*, and *Tgfβ3* mRNA in all cell lines (Fig. 6). They all expressed *Tgfβ1* and *Tgfβ3*, and, though at very low levels, also *Tgfβ2*. In the FGFR2-C278F cells, however, *Tgfβ1* and *Tgfβ3* levels of expression were significantly lower than in the control lines (Fig. 6A–C). We therefore investigated whether exogenous Tgfβ1 could increase the proliferative activity of mutated osteoblasts (Fig. 7). Whereas Tgfβ1 did increase proliferation in the control cell lines, no significant difference was observed in FGFR2-C278F cells. This suggested that defective Fgf signalling in the mutated cells impaired Tgfβ signalling modulating proliferation.

3.3. Role of Erk in Fgf and Tgfβ signalling

As Fgf is known to mediate osteoblast proliferation via the Erk1/2 pathway we investigated Erk1/2 protein expression in the three

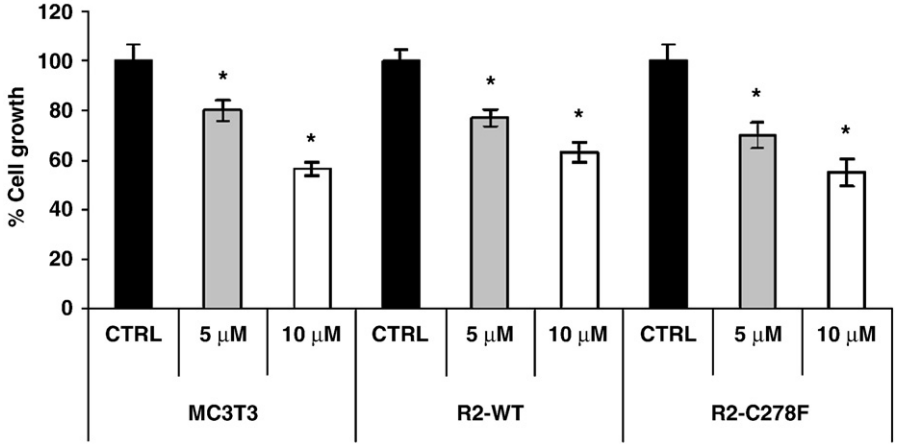

Fig. 1. Effect of FGFR inhibitor SU5402 on MC3T3, R2-WT and R2-C278F osteoblast growth. Cells were treated with no SU5402 (CTRL), 5 μM or 10 μM SU5402 every 24 h for 3 days and cell growth was assessed by the methylene blue assay. Cell growth in treated groups is expressed as percentage of growth in untreated controls. SU5402 treatment reduces growth in a dose-dependent manner ($n = 3$, $^*p < 0.05$).

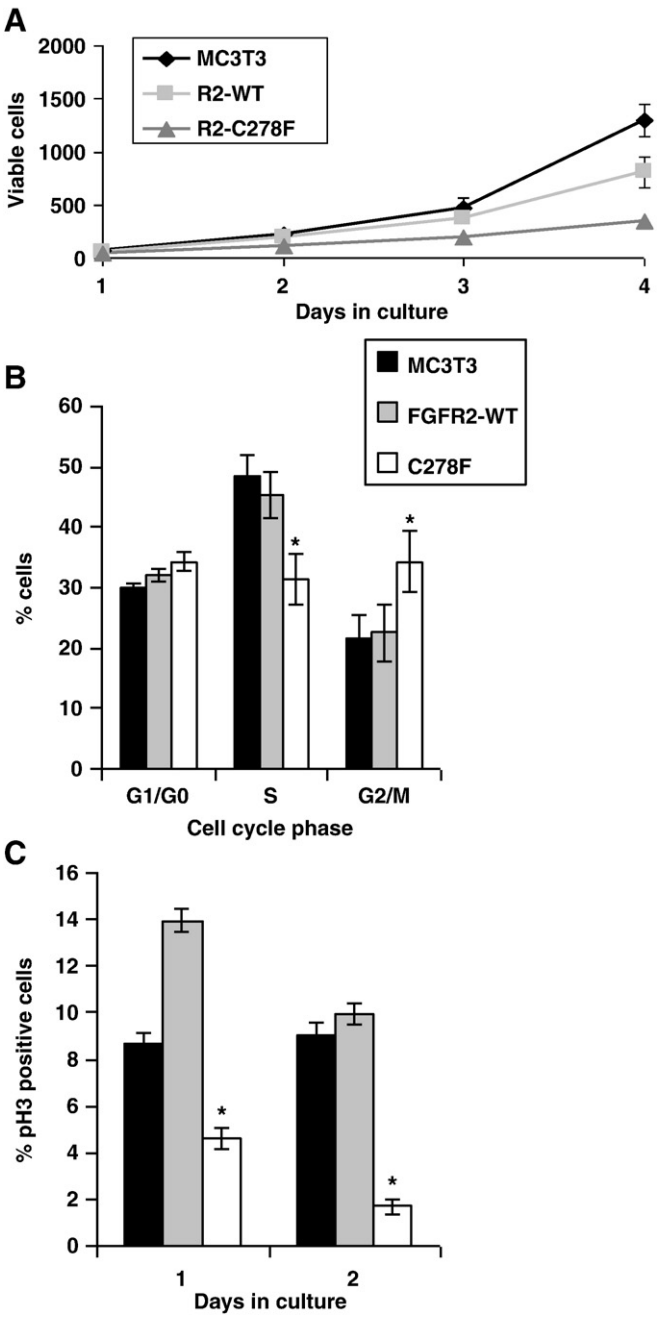

Fig. 2. Changes in cell growth and cell cycle in MC3T3, R2-WT and R2-C278F osteoblasts. (A) Curves show osteoblast growth evaluated by counting viable cells at the indicated times. Cell growth is slower in R2-C278F cells than in controls. Error bars in (A) are the standard deviation. (B) Bar charts show the percentage of 7AAD stained cells in G0/1, S and G2/M phases of cell cycle as assessed by FACS analysis at 2 days in culture. Both S-phase (decreased) and G2/M phase (increased) are significantly affected in R2-C278F compared to controls. ($n \geq 4$, $^*p<0.05$ Mann–Whitney U test). (C) Analysis of the percentage of mitotic cells in M-phase as assessed by phosphorylated histone 3 (pH3) immunocytochemistry. The percentage of R2-C278F cells in M-Phase is significantly lower than in controls ($n=3$, $^*p<0.05$).

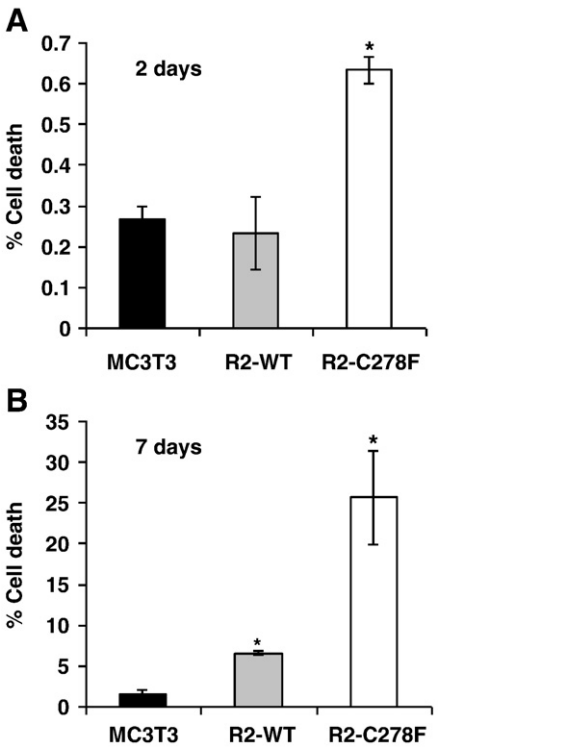

Fig. 3. Changes in cell survival analyzed by FACS. Bar charts show the percentage of cell death and apoptosis measured by DNA intercalation with 7AAD at 2 days (A) and 7 days (B) in culture. Cell survival is lower in R2-C278F cells compared to both controls and R2-WT cells ($n=3$, $^*p<0.05$).

osteoblastic cell lines. Analysis of Erk1/2 expression levels by FACS showed that Erk1/2 was significantly higher in FGFR2-C278F cells than in controls (Fig. 8A). The relative levels of Erk1 and Erk2 were further analyzed by Western blot and densitometric analysis in cells cultured for 2 and 4 days (Fig. 8B–D). At both time points the ratio between Erk1 and Erk2 was higher in the mutated cells than in controls. Furthermore, analysis of phosphorylated Erk also showed a higher pErk1/pErk2 ratio in FGFR2-C278F cells (Fig. 8E). This suggested that Erk signalling is affected in FGFR2-C278F cells and that Tgfβ1 might be unable to stimulate proliferation of the mutated cells because of defective Erk signalling.

To establish whether Tgfβ effect on proliferation was mediated via Erk1/2, we assessed the effect on cell proliferation of the Erk1/2 inhibitor U0126, alone or in combination with Tgfβ1 (Fig. 9). Treatment with U0126 significantly reduced cell growth in FGFR2-C278F cells compared to controls, both at 2 and 3 days in culture (Fig. 9A–B). We also assessed whether Tgfβ1 was able to rescue inhibition of proliferation induced by U0126 in the MC3T3 control cells (Fig. 9C). No significant increase in proliferation was observed upon Tgfβ1 treatment, suggesting that Tgfβ-induced osteoblast proliferation is mediated via Erk signalling.

4. Discussion

This study has identified novel mechanisms by which a single point mutation in the human FGFR2 affects osteoblast behaviour and their response to Tgfβ signalling.

4.1. FGFR2-C278F reduction in proliferation and survival is due to loss of function

A critical defect that we have found in MC3T3 cells carrying FGFR2-C278F concerns their decreased proliferation and survival. Reduced growth in FGFR2-C278F cells appears to result from a reduction in the number of cells entering S phase. The effect on proliferation observed seems specifically due to the C278F mutation, as expression of the normal human receptor in MC3T3 does not induce the same phenotype and changes in the cell cycle. Furthermore, cells expressing the mutated receptor behave in a fashion similar to that observed in primary human craniosynostotic cells, that is reduced cell growth and premature differentiation [36,47]. This supports the view that our mutated cells are a good model to study the molecular basis of craniosynostosis.

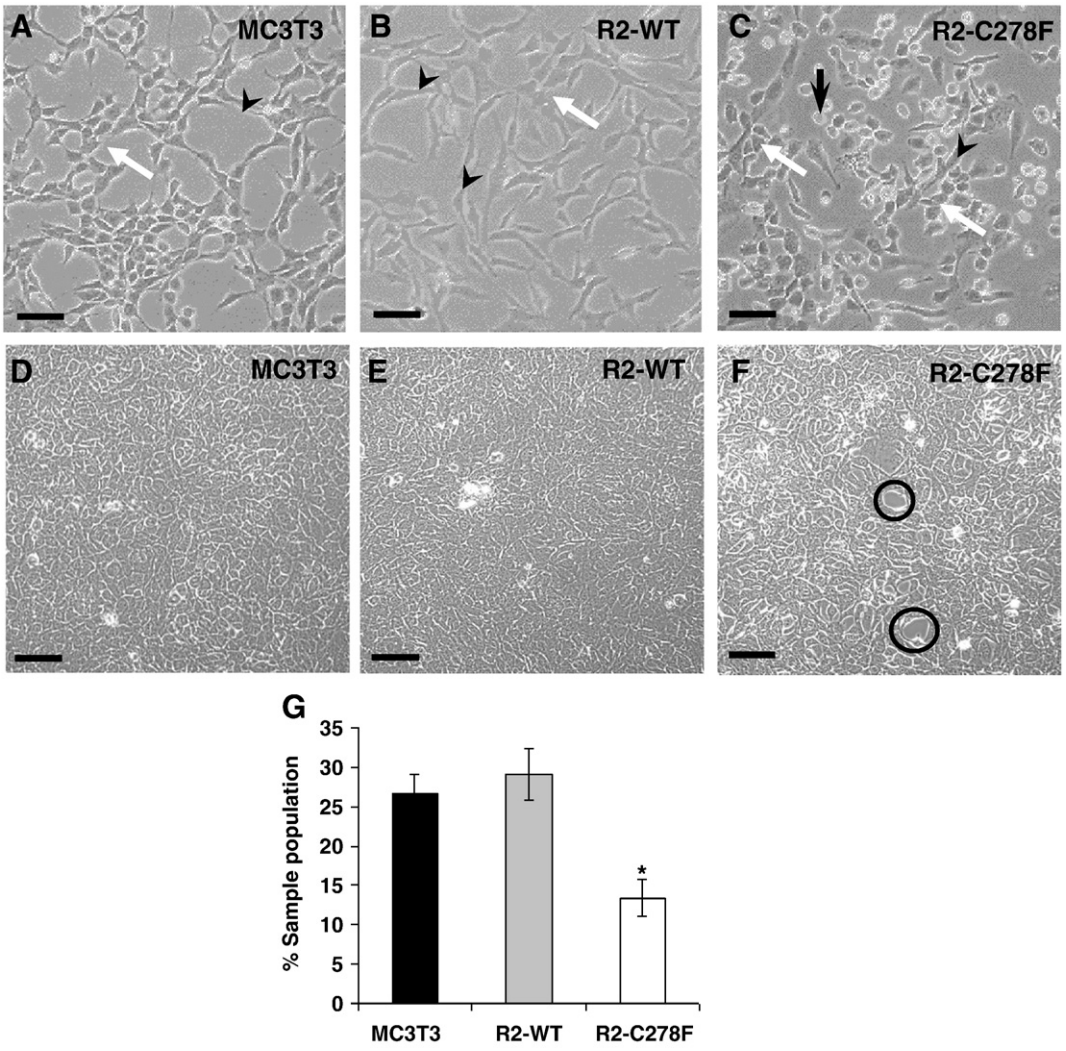

Fig. 4. Morphology of MC3T3, R2-WT and R2-C278F at 2 and 4 days in culture assessed by phase contrast microscopy. (A–C) Two-day cultures: cells with fibroblastic morphology (arrowheads) and cuboidal cells (white arrows) are present in MC3T3 cells. In R2-WT cultures most cells display a fibroblastic morphology whereas in R2-C278F cultures most cells are cuboidal and several bright round cells are present (wide arrow). (D–F) Four-day cultures: MC3T3 and R2-WT cultures are fully confluent, whereas R2-C278F cultures are not. Black circles indicate areas devoid of cells. (G) Bar chart shows quantification of cells with fibroblastic morphology in 2-day cultures. The percentage of cells with fibroblastic morphology is lower in R2-C278F cells than in control lines ($n=3$, $^*p<0.05$). Scale bar = 50 μm.

Upon ligand stimulation, C278 forms a disulfide bridge with C342 in the third immunoglobulin loop of the receptor (IgIIIa/IIIc domain) that plays a role in receptor dimerisation and its consequent activation [48-50]. Mutations in the C278 (or C342) of FGFR2 lead to covalent cross-linking of these cysteines and maintain the receptor in a dimeric active form even in the absence of ligand. Though C278F is

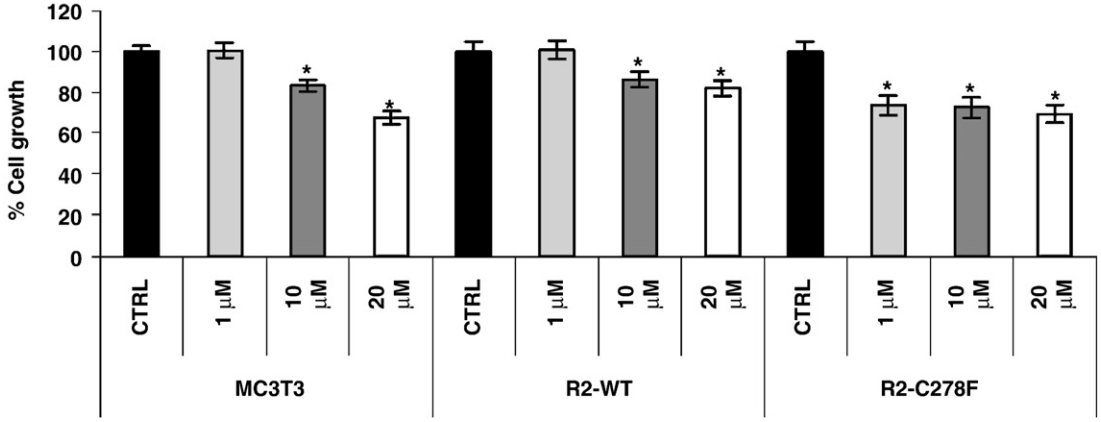

Fig. 5. Reduction in proliferation by Tgf Reduction in proliferation by Tgfβ receptor inhibition in MC3T3, R2-WT and R2-C278F osteoblasts. Cells were treated with no SB431542 (CTRL), 1, 10 or 20 μM SB431542 every 24 h and cell growth assessed 3 days later by methylene blue assay. Note that 1 μM SB431542 is sufficient to reduce cell growth significantly in R2-C278F but not in controls, and that this effect is not enhanced by increasing dose ($n=3$, $^*p<0.05$ ANOVA).

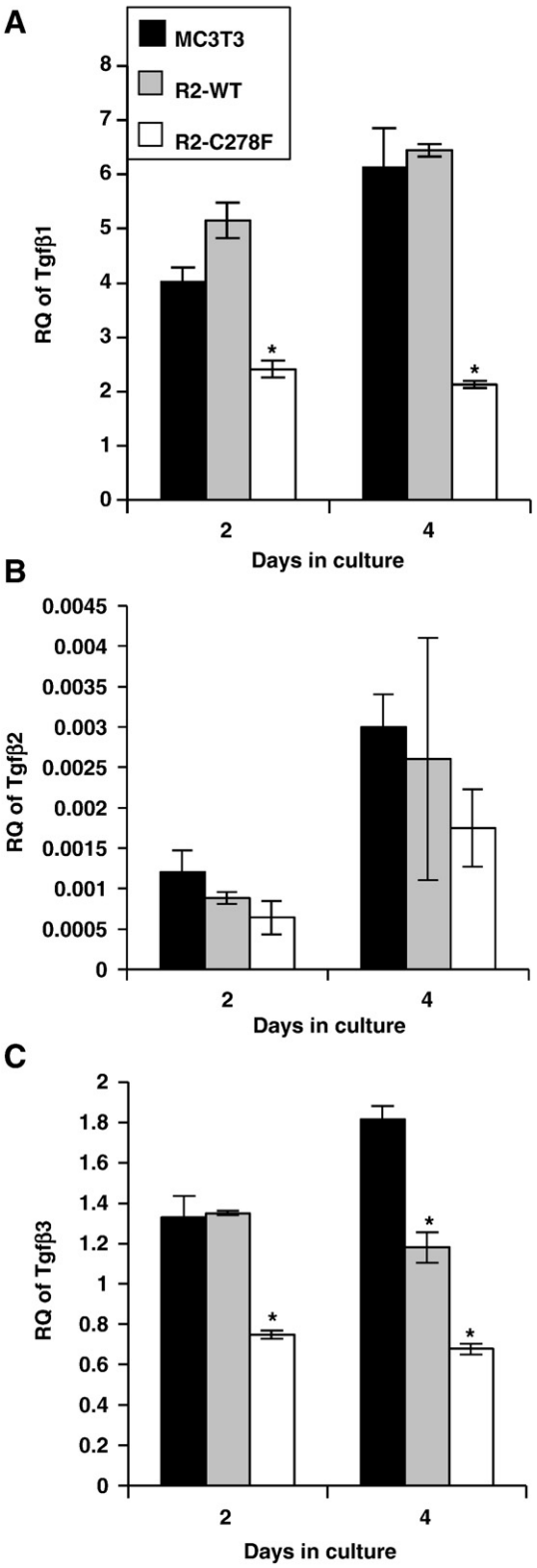

Fig. 6. Tgfβ1 and -3 gene expression is significantly reduced in cells carrying FGFR2-C278F. Cells were cultured for 2 or 4 days before relative quantification (RQ) of Tgfβ1, 2 and 3 expressions. (A) Tgfβ1 expression is significantly lower in R2-C278F cells than in controls at 2 and 4 days in culture. (B) Tgfβ2 expression is not significantly different between the three cell lines neither at 2 or 4 days. (C) Tgfβ3 expression is lower in R2-C278F cells than in both controls at 2 and 4 days ($n = 3$, $^*p < 0.05$).

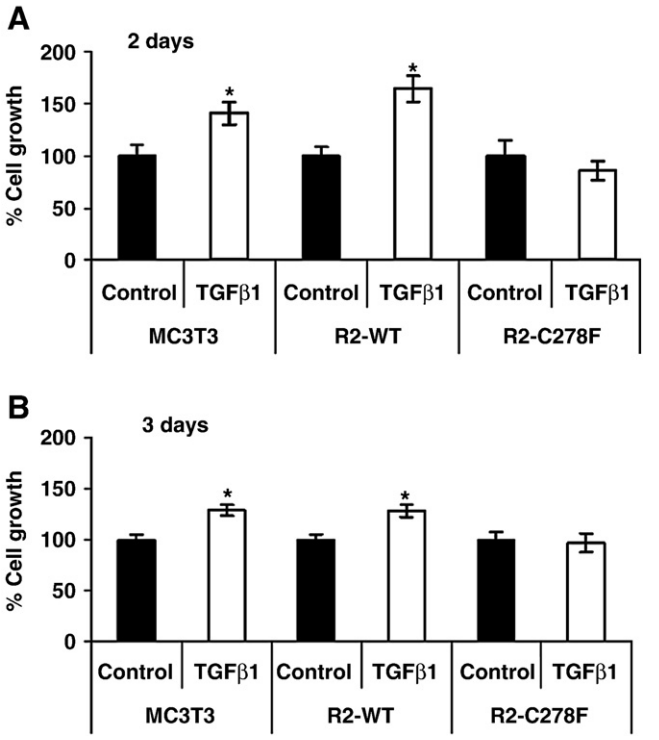

Fig. 7. TGFβ1 does not rescue proliferation in R2-C278F cells. Three hours after plating, cells were treated with 10 ng/ml Tgfβ1 every 24 h for 2 or 3 days and analysed for cell growth by methylene blue assay. (A–B) The treated groups in both MC3T3 and R2-WT cells show increased cell growth, whereas there is no difference between treated and untreated groups in R2-C278F cells ($n = 3$, $^*p < 0.05$).

a constitutively activating mutation and FGFR2's main role in developing cranial bone is osteoblast proliferation, our results suggest that the net effect of the mutation is a loss rather than a gain of function. This is consistent with a previous report showing rapid cellular degradation of FGFR2-C278F [42].

Furthermore, the mutated cells undergo apoptosis to a larger extent than controls. This is also consistent with a loss of FGFR2 function, as Fgf signalling is known to play a role in osteoblast proliferation and survival [51]. The effects of the C278F mutation on cell death parallel those reported in a study where FGFR2-C342Y (Crouzon syndrome) was introduced in OB1 cells, a cell line derived by immortalizing primary newborn mouse calvarium osteoblasts with polyoma large T antigen [49]. Also human osteoblasts from Apert patients carrying the FGFR2-S252W mutation display increased apoptosis [52], though the mechanism underlying it may be different, as the effect of that mutation appears to be loss of ligand specificity and/or extension of ligand–receptor interaction rather than constitutive activation of the receptor [53].

The effects of the C278F mutation on proliferation might seem superficially rather different from those reported in C342Y-OB1 cells, as it was suggested that the C342Y OB1 cells do not display a decrease in proliferation [49]. However, in that study the basal growth activity of the control and mutated cells was not directly compared under normal culture conditions. In low serum, a proliferative response to FGF1 was observed in OB1 cells, whereas only a small increase in DNA synthesis was induced by FGF1 in C342Y-OB1 cells [49]. This suggests that in these cells, like in FGFR2-C278F cells, the ability to proliferate may be reduced. Finally, our mutated FGFR2-C278F cells display premature differentiation and increased mineralization as compared to controls. This feature differs from the reduced mineralization reported in C342Y-OB1 cells [49], but it is in agreement with increased differentiation observed in primary cultures of osteoprogenitors from craniosynostosis patients [36,47], as well as with increased differentiation observed in FGFR2-S252W mutant cells obtained either from Apert syndrome patients or by transfecting the mutated receptor into C3H10T1/2 mouse mesenchymal cells [15,54].

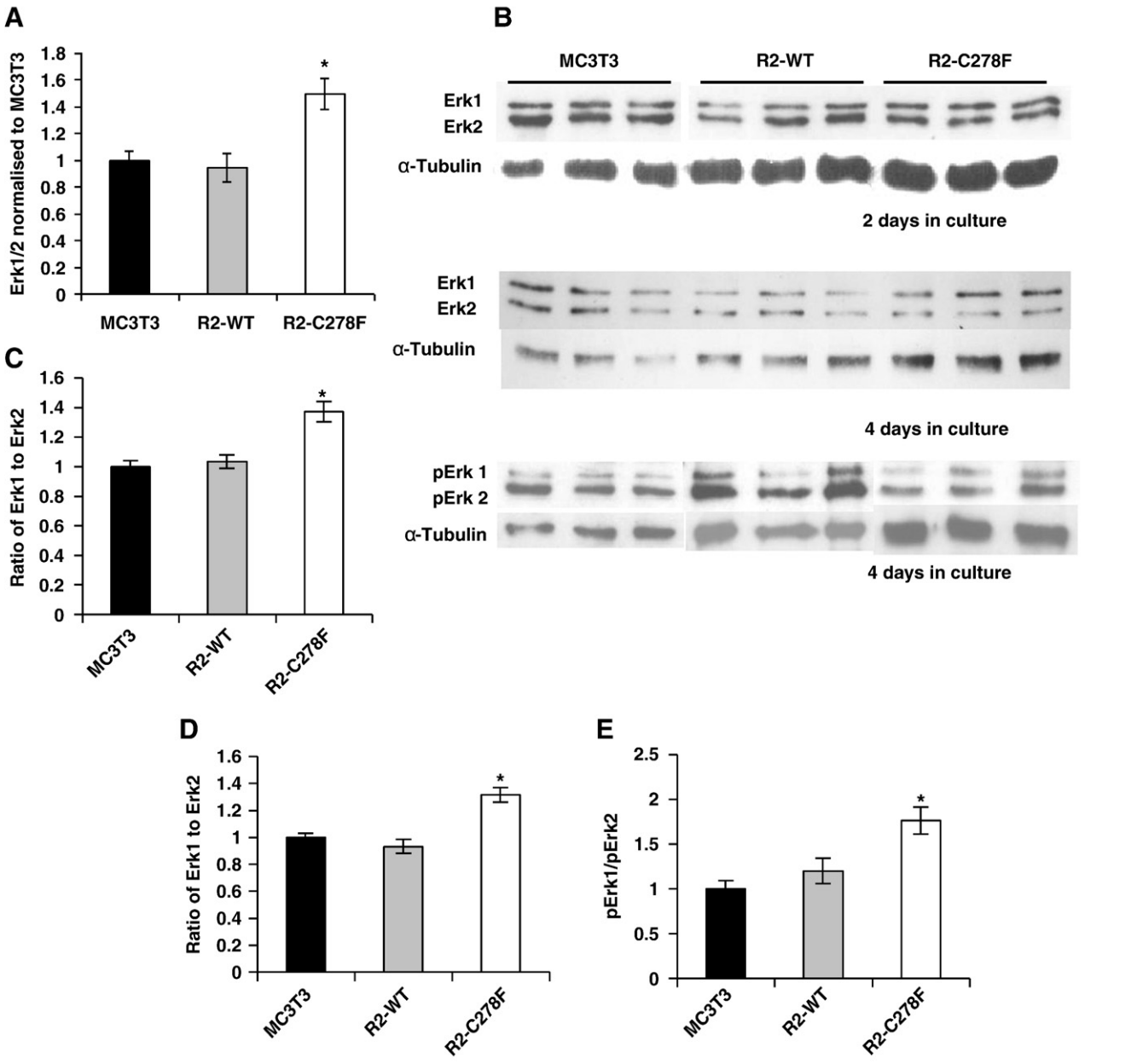

Fig. 8. Erk1/Erk2 ratio is altered in mutated cells. (A) FACS analysis of Erk1/2 expression in 2-day cultures. The level of Erk1/2 is significantly higher in R2-C278F cells than in R2-WT and MC3T3 (approximately 1.5 fold). The data represent the M ± SEM from 3 independent experiments, normalised to the MC3T3 control. (B) Cells were cultured for 2 or 4 days and analysed for Erk1/Erk2, and phosphorylated-Erk1/Erk2 (pErk1/pErk1) protein expression by Western blotting. (C–E) Ratio of Erk1/Erk2 and pErk1/Erk2 expression assessed by densitometric analysis of Western blots at 2 (C) and 4 (D–E) days and normalised to that of MC3T3 controls. The ratio of both total and phosphorylated Erk1/Erk2 is significantly higher in R2-C278F cells than in the two control cell lines (*$p<0.05$).

4.2. Impaired Tgfβ and Erk signalling underlie reduced proliferation in FGFR2-C278F osteoblasts

Both expression of Tgfβ1 and the ability of this factor to stimulate proliferation are reduced in FGFR2-C278F cells. This dependence of Tgfβ on a functional FGFR2 is consistent with previous work suggesting a complex and dynamic cross-talk between Fgf and Tgfβ signalling during cranial bone development, starting from the neural crest [39,55,56]. Inhibition of Tgfβ signalling in our cell lines resulted in a decrease in cell proliferation, suggesting cell growth modulation via an autocrine mechanism. Consistent with this proposition, Tgfβ signalling is impaired in FGFR2-C278F cells, as indicated by the lower concentration of Tgfβ receptor inhibitor required to reduce cell growth in these cells as compared to controls and the decrease in both Tgfβ1 and Tgfβ3 expression found in these cells. These two Tgfβ forms are highly expressed in non-fusing sutures *in vivo* [57,58], and their down-regulation might contribute to premature suture fusion. We focused our attention on TGFβ1 as it is the most abundant TGFβ in bone, and TGFβ3 expression in MC3T3 was much lower than that of Tgfβ1, suggesting a greater role for the latter. Furthermore, data from a parallel study in our laboratory suggested that TGFβ3 mRNA expression is regulated by Tgfβ1 in MC3T3 (Pungchanchaikul and Ferretti, unpublished data).

The Erk pathway is an important mediator of proliferation in MC3T3 cells, as inhibition of this pathway significantly reduces cell growth. Altered Erk1/2 expression and decreased proliferation is observed in FGFR2-C278F (this study), whereas cells carrying the self-activating FGFR2-S252W or FGFR2-WT activate the Erk1/2 pathway and increase proliferation [54], further supporting the view that the net effect of the C278F mutation is loss of receptor function. Altered

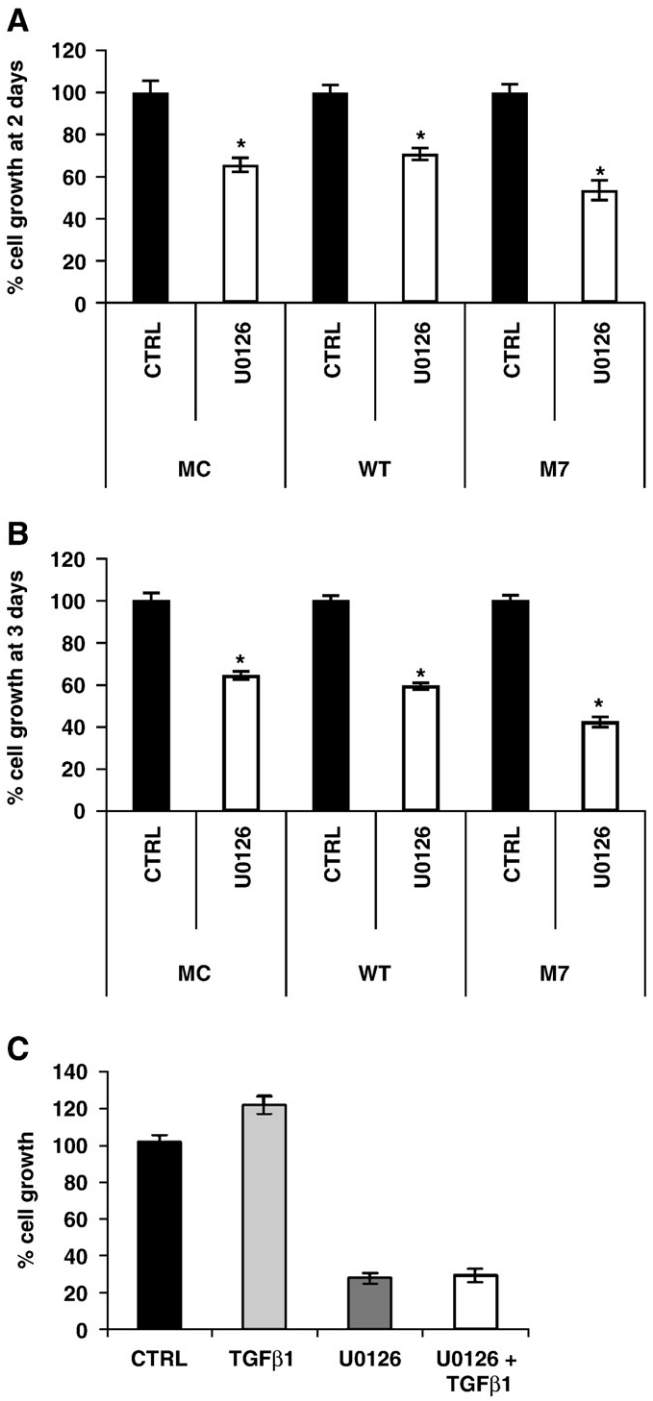

Fig. 9. Effect of Tgfβ1 and U0126 on MC3T3 cell proliferation. Cell growth was measured by methylene blue assay in cultures treated for 2 or 3 days of Erk1/2 inhibitor U0126 and/or Tgfβ1 at 24-h intervals. (A–B) Cells were grown for 2 (A) and 3 (B) days in the presence of U0126 ($n=3$, $*p<0.05$). (C) MC3T3 cells at 3 days treated with Tgfβ1 and U0126 ($n=3$, $*p<0.05$ ANOVA).

Erk1/2 signalling in our FGFR2-C278F cells might be responsible for their accumulation in G1 and G2 as it has been proposed that Erk1/2 controls G1/S and G2/M transitions [59,60]. Indeed, in serum-stimulated fibroblasts, both Erk1 and Erk2 are strongly phosphorylated in G1 phase, but only Erk2 is strongly phosphorylated in G2/M phase [61].

Altered Erk1/2 signalling also appears to affect the ability of the cells to respond to Tgfβ1 stimulation, indicating that a functional Erk pathway is required for Tgfβ-induced osteoblast growth. In addition, endogenous Tgfβ, that we speculate is required for autocrine signalling, is reduced in FGFR2-C278F cells. Therefore, both a decrease in endogenous Tgfβ and impairment of the Erk1/2 signalling pathway are likely to contribute to the decrease in cell growth observed in mutated cells. Modulation of the levels of Erk by over-expressing Erk1 and Erk2, either wild type or mutated at their phosphorylation sites, will be important to further clarify their role in proliferation and differentiation of normal and craniosynostotic osteoblasts.

4.3. Conclusions

This work supports the view that osteoblast behaviour is regulated by interaction between Fgf and Tgfβ signalling. Crucially, it suggests that normal Erk signalling is required for Tgfβ induction of osteoblast proliferation, and that this depends on a functional FGFR2 signalling. Altogether these findings are consistent with the hypothesis that the net effect of the FGFR2-C278F mutation is a loss of function.

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