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FGF9/FGFR2 increase cell proliferation by activating ERK1/2, Rb/E2F1, and cell cycle pathways in mouse Leydig tumor cells

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Abstract

Fibroblast growth factor 9 (FGF9) promotes cancer progression; however, its role in cell proliferation related to tumorigenesis remains elusive. We investigated how FGF9 affected MA-10 mouse Leydig tumor cell proliferation and found that FGF9 significantly induced cell proliferation by activating ERK1/2 and retinoblastoma (Rb) phosphorylations within 15 minutes. Subsequently, the expressions of E2F1 and the cell cycle regulators: cyclin D1, cyclin E1 and cyclin-dependent kinase 4 (CDK4) in G_1 phase and cyclin A1, CDK2 and CDK1 in S-G_2/M phases were increased at 12 hours after FGF9 treatment; and cyclin B1 in G_2/M phases were induced at 24 hours after FGF9 stimulation, whereas the phosphorylations of p53, p21 and p27 were not affected by FGF9. Moreover, FGF9-induced effects were inhibited by MEK inhibitor PD98059, indicating FGF9 activated the Rb/E2F pathway to accelerate MA-10 cell proliferation by activating ERK1/2. Immunoprecipitation assay and ChIP-quantitative PCR results showed that FGF9-induced Rb phosphorylation led to the dissociation of Rb-E2F1 complexes and thereby enhanced the transactivations of E2F1 target genes, *Cyclin D1*, *Cyclin E1* and *Cyclin A1*. Silencing of FGF receptor 2 (FGFR2) using lentiviral shRNA inhibited FGF9-induced ERK1/2 phosphorylation and cell proliferation, indicating that FGFR2 is the obligate receptor for FGF9 to bind and activate the signaling pathway in MA-10 cells. Furthermore, in a severe combined immunodeficiency mouse xenograft model, FGF9 significantly promoted MA-10 tumor growth, a consequence of increased cell proliferation and decreased apoptosis. Conclusively, FGF9 interacts with FGFR2 to activate ERK1/2, Rb/E2F1 and cell cycle pathways to induce MA-10 cell proliferation in vitro and tumor growth in vivo.

KEYWORDS
ERK1/2, FGF9, FGFR2, MA-10 Leydig tumor cell proliferation, Rb/E2F1

Ming-Min Chang and Meng-Shao Lai contributed equally to this work.

1 | INTRODUCTION

Fibroblast growth factors (FGFs), multifunctional proteins, have at least 22 members.[1] Fibroblast growth factor signaling has been reported to participate in many cell biological functions, such as proliferation, differentiation, survival and motility.[2-4] Four FGF receptors (FGFRs) have been identified in the human genome.[5] In addition to genomic difference, the mRNA of FGFRs could undergo alternative splicing to increase the protein diversity of FGFRs.[6] Thus, each FGF could interact with its specific FGFR to activate different signal pathways into cells.[7] Studies have shown that FGF/FGFR signals participate in different cancer formations[8-10]: the abnormal or overexpression of FGFR1 could cause breast cancer and pancreatic adenocarcinoma,[11,12] the isoform switch of FGFR2 and splice mutation is involved in prostate cancer and gastric cancer,[13,14] the mutation, overexpression and aberrant splicing of FGFR3 participates in thyroid cancer, cervical cancer and colorectal cancer,[15-17] and the higher expression of FGFR4 Arg388 allele in head and neck carcinoma is associated with poor survival.[18]

It is well known that FGFRs couple to tyrosine kinase receptors, which will transduce outer stimulation through PI3K, MAPK and phospholipase $C\gamma$ (PLCγ) pathways.[5,19] It was also shown that FGF could mediate FGFR activation to induce Akt phosphorylation and then to promote cell proliferation in nonsmall-cell lung cancer and nasopharyngeal carcinoma.[20,21] The MAPK signaling pathway has three main signals: ERK1/2, JNK and p38.[22] In normal chondrocytes and pancreatic cancer cells, FGFRs could be activated by FGFs to induce cell proliferation through ERK1/2 phosphorylation.[23,24] In hepatocellular carcinoma, FGFR-mediated ERK1/2 and JNK activations could increase the carcinogenesis and metastasis.[25] Activation of p38 is involved in gelsolin-induced osteosarcoma proliferation and high glucose-induced mesangial cell proliferation.[26,27] Phospholipase C could receive the signal from FGFR and then cleave the phospholipid phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-triphosphate,[28] which would activate PLCγ1 to induce cell proliferation and migration in keratinocytes and vascular smooth muscle cells.[29,30]

Defects in cell division can lead to developmental abnormalities as well as tumor development and cancerous growth. The eukaryotic cell cycle is regulated by the activities of cyclin-dependent kinases (CDKs) and cyclins.[31] In different cell cycle phase, the concentrations of cyclins and CDKs fluctuate. In the complex of cyclin D/CDK4, 6 can be activated to phosphorylate the retinoblastoma protein (Rb) promoting cells into S phase. Cyclin E and A can be upregulated in S phase to increase CDK2 and CDK1 activities to promote cells into G_2 phase. Moreover, cyclin B will be upregulated at late G_2 phase and to interact with CDK1 to promote cells into mitosis.[32] When a cell is not suitable for division, the regulatory proteins, such as p53, p21 and p27, would be activated to interact with the cyclin/CDK complex interrupting the conformational change, which is required for complex activation to cause cell cycle arrest.[33,34]

Fibroblast growth factor 9 was first isolated from the culture supernatant of a human glioma cell line.[35] Studies have reported that FGF9 participates in palate formation, sex determination and lung development in the embryonic stage and has low expression levels under normal conditions in few adult organs.[36-38] However, abnormal expression of FGF9 might cause various cancer formations,[39-42] and the mechanisms remain elusive.

Leydig cell tumors of the testis are a rare sex cord-stromal tumor, accounting 1%-3% of all testicular neoplasms and 4%-9% of testis tumors in prepubertal boys.[43–46] Leydig cell tumors usually occur in prepubertal boys and men between 30 and 60 years of age. Leydig cell tumors are benign in children but can be malignant in 10% of adults.[46,47] It has been reported that the expression of FGF9 is high in testicular cancer.[48] In the present study, FGF9-induced MA-10 mouse Leydig tumor cell proliferation is reported for the first time, and the basic mechanisms how FGF9-induced MA-10 cells are revealed, which could be applied for the treatment of testicular and related cancers.

2 | MATERIALS AND METHODS

2.1 | Cell line and treatments

The MA-10 mouse Leydig tumor cell line was a gift from Dr. Mario Ascoli (Department of Pharmacology, University of Iowa, Iowa City, IA, USA). MA-10 cells were maintained in modified Waymouth MB752/1 medium (pH7.7) containing 20 mmol/L HEPES and 1.12 g/L NaHCO$_3$, and supplemented with 10% FBS. MA-10 cells (3×10^5) were seeded in a 60-mm dish. After 18 hours of serum starvation, MA-10 cells were treated with 50 ng/mL FGF9 or PBS in the medium containing 1% FBS. In the inhibition assay, 18-hour serum-starved MA-10 cells were treated with 50 µmol/L PD98059 (a MAPK/ERK1/2 inhibitor) (Figure S1) or DMSO (vehicle control) for 1 hour and then treated with 50 ng/mL FGF9 and 50 µmol/L PD98059 or DMSO in the medium containing 1% FBS for 0.25, 12 or 24 hours. All chemicals and materials used in this study are listed in Table S1.

2.2 | Cell proliferation assay

MA-10 cells were seeded in 96-well plates at a density of 8000 cells per well. After serum-starved for 18 hours, the cells were treated with 0, 1, 10, 25 or 50 ng/mL FGF9 in medium containing 1% FBS. Then MTT (0.5 mg/mL) was added at 24, 48, or 72 hours after the FGF9 treatment and incubated at 37°C for 4 hours. The medium was discarded, and DMSO was added to dissolve the crystals by shaking the plates for 20 minutes in the dark. The OD values were determined using an ELISA reader (Molecular Devices, San Jose, CA, USA) with a 570-nm filter.

2.3 | Western blot analysis

Treated MA-10 cells were washed with PBS and lysed by ice-cold lysis buffer (20 mmol/L Tris pH7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, and 1 mmol/L sodium orthovanadate). After centrifugation at 12000 g for 12 minutes at 4°C, supernatants were collected. Total protein (25 µg) was separated by 10% SDS-PAGE and transferred onto PVDF

membranes. The PVDF membranes with transferred protein were blocked with 5% nonfat milk for 1 hour and then incubated with the primary Ab for 16-18 hours at 4°C. The signal was detected with HRP-conjugated secondary Ab and visualized with chemiluminescence HRP substrate. The proteins were quantitated by a computer-assisted image analysis system (UVP bioImage system software; UVP, San Gabriel, CA, USA). Protein level was quantitated by using ImageJ software (NIH, Bethesda, MD, USA), and β-actin was used as a loading control. The integrated optical density of the proteins were normalized to β-actin in each lane and further corrected by the control group at each time point. All Abs used in this study are listed in Table S2.

2.4 | Immunoprecipitation assay

Treated MA-10 cells were washed with PBS and lysed by ice-cold immunoprecipitation (IP) lysis buffer (50 mmol/L Tris HCl pH7.4, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L EDTA, 5 mmol/L sodium orthovanadate, and protease inhibitor cocktail). Lysates were clarified by 12 000 g for 12 minutes at 4°C and diluted with the IP lysis buffer to approximately 1 mg/mL total protein concentration. Immunoprecipitates were isolated using E2F1-conjugated protein G magnetic beads after 16 hours of incubation at 4°C, followed by immunoblot analysis.

2.5 | Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was carried out using the Magna ChIP G Kit (Table S1) according to the manufacturer's protocol. Briefly, 50 ng/mL FGF9- or PBS-treated MA-10 was fixed by 1% formaldehyde for protein-DNA cross-linking and then lysed. Nuclear extracts were collected and then sonicated to an average size of 500-1000 bp. Next, the chromatin was immunoprecipitated with 3 mg anti-E2F1 Ab or rabbit IgG (negative control) and 15 μL ChIP magnetic G beads for 16 hours. After DNA purification, 2 μL ChIP-enriched DNA was used as template for 50 cycles of quantitative PCR (qPCR) amplification with specific primers (Table S3) to amplify the E2F1 binding site in promoter regions of *Cyclin D1*, *Cyclin A1*, and *Cyclin E1* genes.

2.6 | Immunofluorescent staining

MA-10 cells were seeded on glass coverslips. Treated cells were washed with PBS and fixed with 1% paraformaldehyde at 37°C for 15 minutes. The primary Abs for immunofluorescent staining were used against FGFR1, FGFR2, FGFR3 and FGFR4. Rabbit IgG was used as a negative control (Figure S2). Goat anti-rabbit Ab conjugated with Alexa Fluor 488 was used as the secondary Ab. Finally, stained cells were fixed with 1% paraformaldehyde and mounted using the ProLong Diamond Antifade Mountant with DAPI (Table S1) for 24 hours in the dark. Samples were examined using the Olympus FluoView FV1000 confocal microscope (Olympus,

Tokyo, Japan). Images were analyzed using the Olympus FluoView FV10-ASW software (Olympus).

2.7 | Knockdown of FGFR genes using lentiviral shRNAs

The pCMV-ΔR8.91, pMD.G, and all pLKO-based shRNA clones for FGFR1-4 and nonsilencing shRNA (scrambled sequence) were obtained from the National RNAi Core Facility at Academia Sinica (Taipei, Taiwan). All shRNA plasmids used in this study are described in Table S4. Lentivirus preparation was carried out according to the supplier's protocols. MA-10 cells were infected with lentivirus in the presence of 8 μg/mL polybrene. For stable cell lines, cells were selected by 5.5 μg/mL puromycin 48 hours after infection and maintained in growth medium containing 5.5 μg/mL puromycin.

2.8 | Animals and treatments

NOD/SCID (NOD.CB17-*Prkdc*scid/NcrCrl) male mice (12 weeks old) were purchased from National Cheng Kung University Animal Center (Tainan, Taiwan). MA-10 cells (1×10^7) were s.c. injected into the right flanks. After 7 days, tumor-bearing mice were randomly assigned to 3 groups (6 mice per group): control (no treatment), FGF9 (treatment), or PBS (vehicle control). Then 50 ng/mL FGF9 in 100 μL PBS was injected into s.c. tumors once daily for 10 days. Body weight and tumor size were measured daily. Tumor volumes were calculated using the formula: length × width × width × 3.14/6. All experiments were undertaken in accordance with relevant guidelines and regulations, which were approved by the Institutional Animal Care and Use Committee of National Cheng Kung University (approval no. 103161).\

2.9 | Statistical analysis

Statistical analysis was carried out using Prism 6 software (GraphPad, La Jolla, CA, USA). $P < .05$ was considered to be statistically significant in this study.

3 | RESULTS

3.1 | FGF9 increased cell proliferation rate in MA-10 cells

In this study, MTT assay was used to confirm the effect of FGF9 on cell proliferation in MA-10 mouse Leydig tumor cells. Different doses of FGF9 (1-50 ng/mL) were used to treat MA-10 cells, and results showed that 50 ng/mL FGF9 exposure for 24, 48, and 72 hours could significantly induce cell proliferation in MA-10 cells (Figure 1A). In addition, the expression of Ki-67, a standard marker of proliferation that is commonly used to assess the growth fraction of a cell population,[49] significantly increased at 12 hours after FGF9 treatment (Figure 1B). These data highly suggested that FGF9 could induce MA-10 mouse Leydig tumor cell proliferation.
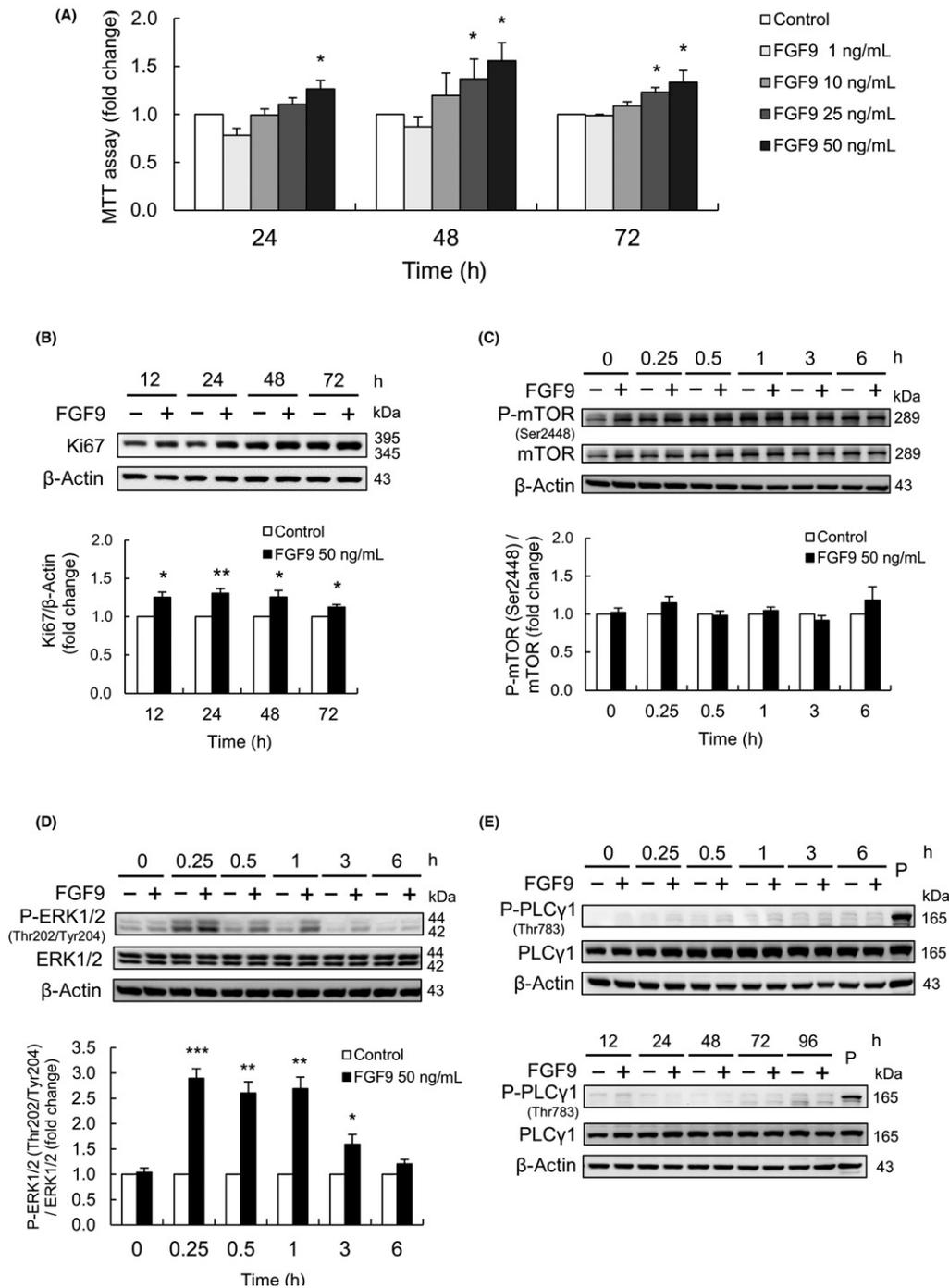
FIGURE 1 Fibroblast growth factor 9 (FGF9) induced MA-10 cell proliferation and MAPK pathway. MA-10 cells were starved for 18 hours, and then treated with 50 ng/mL FGF9 or PBS for 12, 24, 48, or 72 hours. A, MTT assay for the viability of treated MA-10 cells. B, Western blot analysis of Ki-67 expression in treated MA-10 cells. C-E, Western blot analysis for expression of (C) phosphor-mTOR and total mTOR, (D) phosphor-ERK1/2 and total ERK1/2, and (E) phosphor-phospholipase Cγ1 (PLCγ1) and total PLCγ1 in MA-10 cells treated with 50 ng/mL FGF9 or PBS for 0, 0.25, 0.5, 1, 3, or 6 hours. All values are represented as the mean ± SEM, n = 3. P values were calculated using one-way ANOVA with Tukey's multiple comparisons post-tests. *$P < .05$, **$P < .01$, ***$P < .001$ vs the control group

3.2 | FGF9 increased the MAPK pathway related to cell proliferation in MA-10 cells

Studies have shown that PI3K, MAPK, and PLCγ pathways are the downstream molecules of FGFR that FGF9 would associate to activate.[6,50,51] Previously, we have observed that FGF9 stimulated steroidogenesis by increasing the phosphorylation of Akt, JNK, and ERK1/2 in MA-10 cells.[52] Here, for studying cell cycle signaling, 18-hours serum-starved MA-10 cells were treated without or with 50 ng/ml FGF9 for 0, 0.25, 0.5, 1, 3, or 6 hours, and the protein expressions related to PI3K, MAPK, and PLCγ signaling pathways were examined. Results showed that FGF9 had no effect on the

phosphorylation of mTOR, a downstream molecule of Akt protein in MA-10 cells (Figure 1C). In the MAPK pathway, FGF9 (50 ng/mL) from 0.25 to 3 hours did significantly induce the phosphorylation of ERK1/2 (Figure 1D). However, FGF9 had no effect on the phosphorylation of PLCγ1 (Figure 1E) in MA-10 cells. Thus, FGF9 could activate ERK1/2 phosphorylation related to cell proliferation in MA-10 cells.

3.3 | FGF9 induced the expression of cell cycle-related proteins in MA-10 cell proliferation

The cell cycle control system depends on cyclically activated CDKs, and the activities of CDKs are controlled by different cyclins, which undergo a cycle of synthesis and degradation in each cell cycle.[53] Thus, changes in the expressions of cell cycle-related proteins in MA-10 cells after treatment with 50 ng/mL FGF9 were examined.

In G_1 phase-related proteins, the expressions of cyclin D1, CDK4, and cyclin E1 were increased at 12 hours after 50 ng/mL FGF9 treatment (Figure 2A-C). In $S/G_2/M$ phase, the upregulation of CDK2, cyclin A1, and CDK1 were found at 12 hours after 50 ng/mL FGF9 treatment (Figure 2D-F).

These results highly suggested that FGF9 could regulate the expression of cyclin and CDK to regulate cell cycle progression for MA-10 cell proliferation.
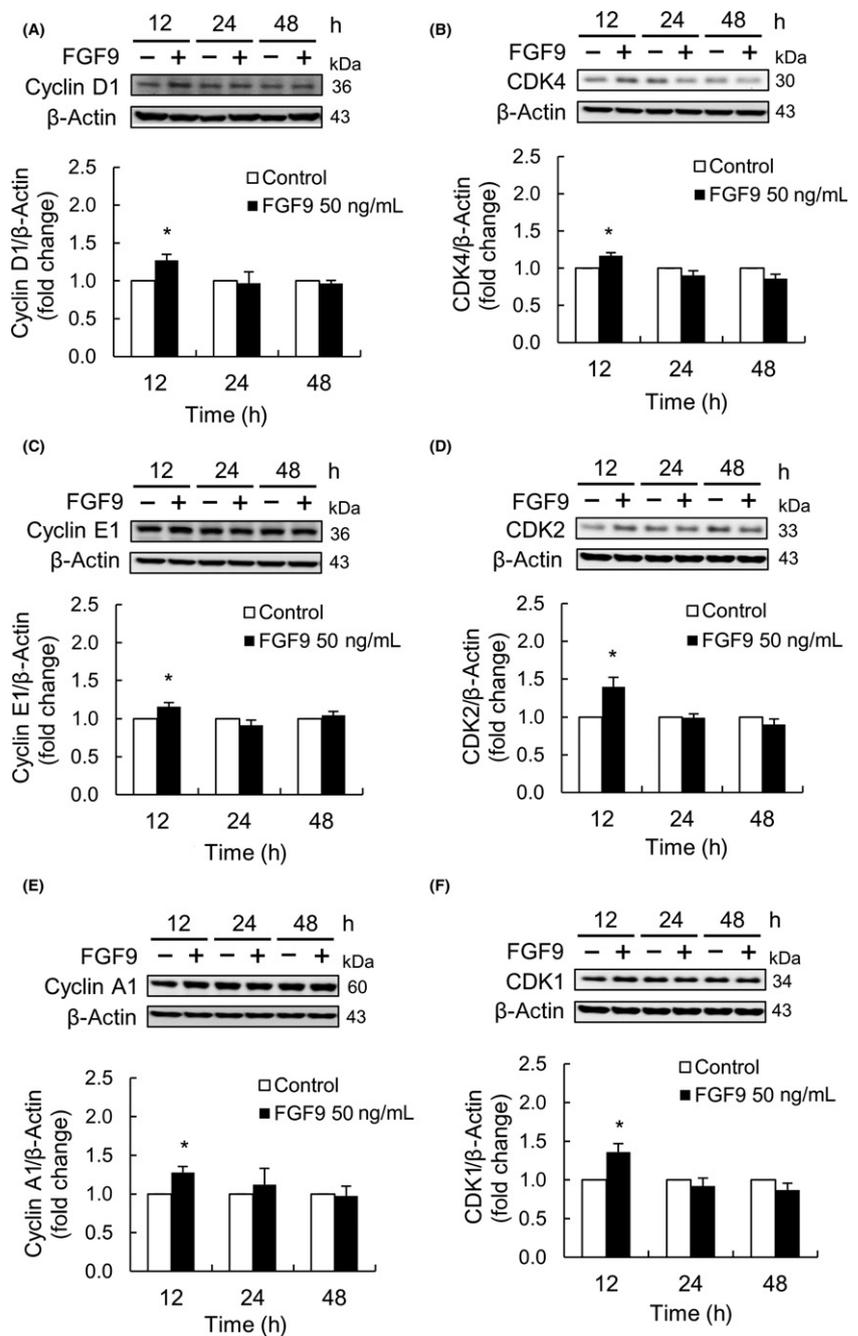
FIGURE 2 Fibroblast growth factor 9 (FGF9) induced expression of cell cycle-related proteins in MA-10 cell proliferation. Western blot analysis for the expression of (A) cyclin D1, (B) cyclin-dependent kinase 4 (CDK4), (C) cyclin E1, (D) CDK2, (E) cyclin A1, and (F) CDK1 in MA-10 cells treated with 50 ng/mL FGF9 or PBS for 12, 24, or 48 h. All values are represented as the mean ± SEM, n = 3. P values were calculated using Student's t test. *P < .05, **P < .01, ***P < .001 vs control group

3.4 | FGF9 promoted Rb phosphorylation to induce MA-10 cell proliferation

The concentration of cyclins and the activation status of CDKs directly control cell cycle progression. When it is not suitable for cell division, the regulatory proteins, such as p53, p21, and p27, are turned on to interact with the cyclin/CDK complex to suppress cell cycle progression for proliferation.[54,55] Results showed that 50 ng/mL FGF9 treatment for 12, 24, and 48 hours did not have any effect on the expression of p53, p21, or p27 in MA-10 cells (Figure 3A-C).

In addition to the p53 signal pathway, Rb was also examined. Retinoblastoma is a tumor suppressor protein, which could prevent abnormal cell growth by inhibiting cell cycle progression at the G_1/S checkpoint.[56] Retinoblastoma loses its E2F binding activity when it is phosphorylated, free E2F is then able to transactivate its cell cycle-related target genes, resulting in the cell cycle progression and cell proliferation.[57–61] Results showed that treatment with 50 ng/mL FGF9 for 12, 24, and 48 hours significantly increased the phosphorylation of Rb in MA-10 cells (Figure 3D).

These data indicate that FGF9 had no effect on the p53 pathway, but suppressed the Rb pathway to induce MA-10 cell proliferation.

3.5 | FGF9, through ERK1/2, activated the Rb/E2F1 pathway to promote cell cycle progression

To investigate whether FGF9 could induce Rb phosphorylation and cell cycle regulators by increasing ERK1/2 phosphorylation, MA-10 cells were treated with FGF9 and/or PD98059 (a MAPK/ERK1/2 inhibitor) over a time course of 15 minutes, 12 hours, or 24 hours. As early as 15 minutes after 50 ng/mL FGF9 exposure, phosphorylation of ERK1/2 and Rb was highly induced (Figure 4A,B), and these FGF9-induced phosphorylations were suppressed effectively by 50 μmol/L PD98059 in MA-10 cells (Figures 4A,B and S1), implying that FGF9-induced phosphorylation of Rb was regulated by the ERK1/2 pathway. Subsequently, the expression of E2F1, the downstream target of Rb protein, was increased by FGF9 after 12 hours of treatment (Figure 4B).

The cell cycle regulators, cyclin D1, cyclin E1, and CDK4 in G_1 phase and cyclin A1, CDK2, and CDK1 in S/G_2/M phase (Figure 4C, D) were also increased by FGF9 after 12 hours of treatment. Moreover, cyclin B1 in G_2/M was induced by FGF9 after 24 hours of treatment (Figure 4C). All of the FGF9-induced protein upregulations were suppressed effectively by 50 μmol/L PD98059 at 12 and 24 hours. These results indicate that FGF9-induced ERK1/2 phosphorylation promoted MA-10 cell proliferation by increasing the expressions of E2F1 and cell cycle-related proteins.
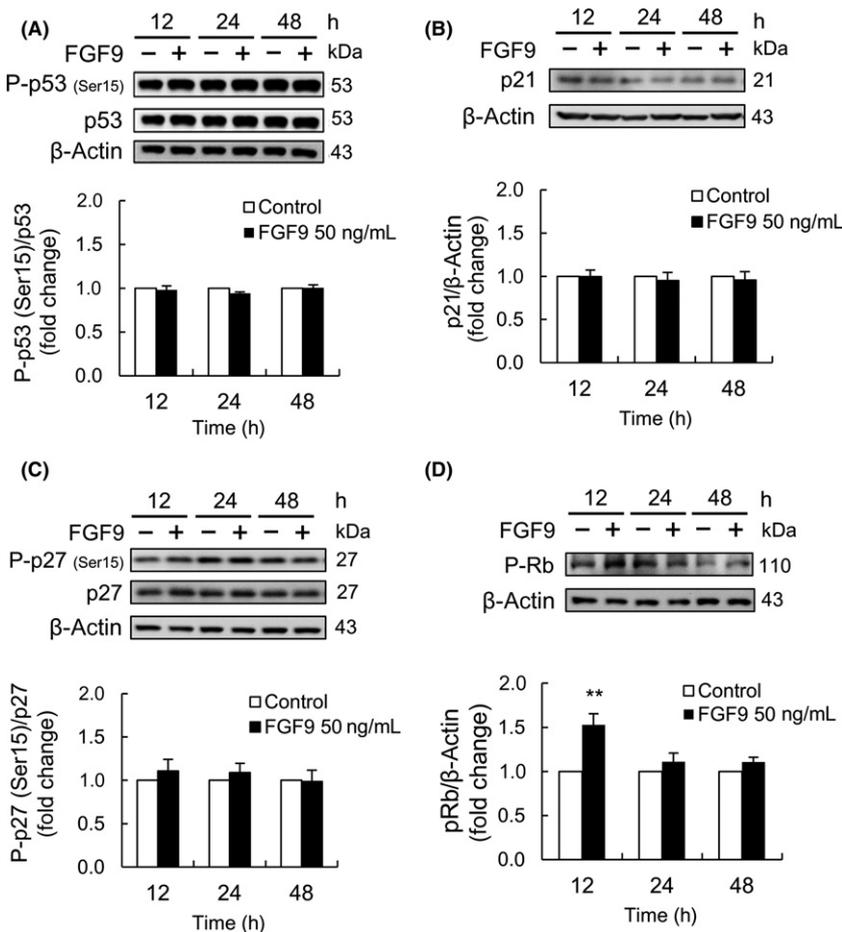
FIGURE 3 Fibroblast growth factor 9 (FGF9) increased retinoblastoma (Rb) phosphorylation, but had no effects on p53, p21, or p27 in MA-10 cells. Western blot analysis for the expression of (A) phosphor-p53 and p53, (B) p21, (C) phosphor-p27 and p27, and (D) phosphor-Rb in MA-10 ells treated with 50 ng/mL FGF9 or PBS for 12, 24, or 48 h. The integrated optical density of phosphor-proteins was normalized to that of their total protein and corrected by their control group at each time point. Quantitative values are represented as the mean ± SEM, n = 3. P values were calculated using Student's t test. *$P < .05$, **$P < .01$, ***$P < .001$ vs control group
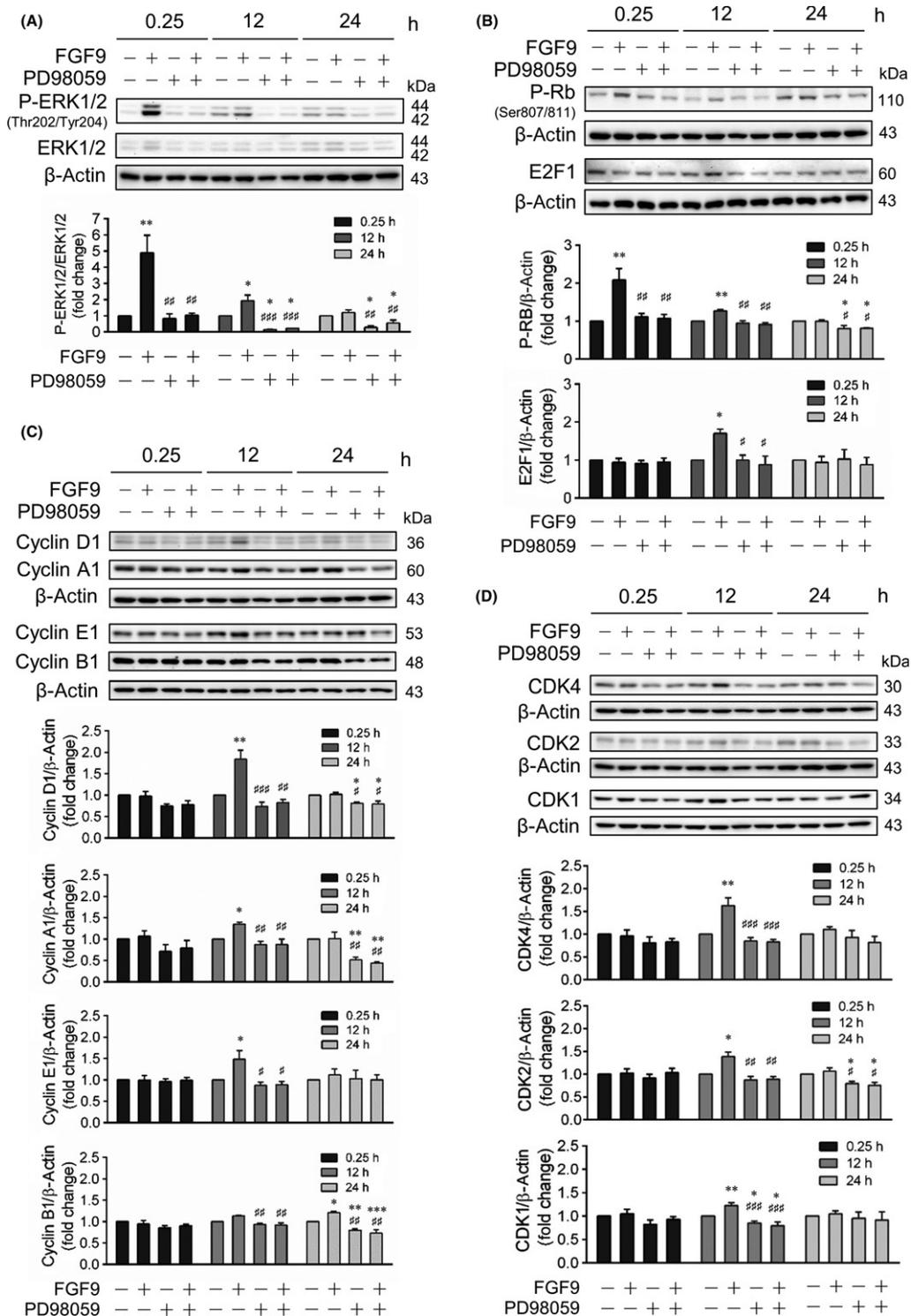
FIGURE 4 Fibroblast growth factor 9 (FGF9) enhanced retinoblastoma (Rb)/E2F and cell cycle pathways by activating ERK1/2. After 18 hours of serum starvation, MA-10 cells were treated with 50 μmol/L PD98059 for 1 hour, and then treated with 50 ng/mL FGF9 and/or PD98059 for 0.25, 12, or 24 hours. Expression of (A) phosphor-ERK1/2 and total ERK1/2, (B) phosphor-Rb and E2F1, (C) cyclin D1, E1, A1, and B1, and (D) cyclin-dependent kinase 4 (CDK4), CDK2 and CDK1 at various time periods were analyzed by western blot. Quantitative values are represented as the mean ± SEM, n = 4. P values were calculated using one-way ANOVA with Tukey's multiple comparisons post-tests. *$P < .05$, **$P < .01$, ***$P < .001$ vs control group (without FGF9 and PD98059 treatment) at each time point. #$P < .05$, ##$P < .01$, ###$P < .001$ vs FGF9 group (without PD98059 treatment) at each time point

3.6 | FGF9 stimulated dissociation of Rb-E2F1 complexes and enhanced transactivations of E2F target genes

The consequence of increased Rb phosphorylation is the dissociation of the Rb-E2F complexes, the transactivation of E2F target genes, cell cycle progression, and cell proliferation.[57-61] To test whether FGF9 could increase the release of Rb-bound E2F1 by enhancing phosphorylation of Rb at 15 minutes after treatment, IP assay was carried out. The results showed that the level of Rb-E2F1 complexes dramatically decreased at 1 hour, but did not differ significantly at 15 minutes, after FGF9 treatment (Figure 5A). These results suggest that after FGF9 stimulation, Rb was hyperphosphorylated and then subsequently lost its grip on E2F1.

A number of studies have reported that E2F1 directly transactivates its target genes involved in the G_1/S transition and S phase,

such as *Cyclin D1*, *Cyclin E1*, *Cyclin A1*, *cdc25C*, and *cdc2* genes.[58,62-65] Also, Figure 4C shows the protein levels of Cyclin D1, Cyclin E1, and Cyclin A1 were upregulated at 12 hours after FGF9 treatment, whereas Cyclin B1 were upregulated at 24 hours after FGF9 treatment. Therefore, we investigated whether FGF9 could stimulate E2F1 to transactivate the *Cyclin D1*, *Cyclin E1*, and *Cyclin A1* genes in MA-10 cells by ChIP and qPCR assays. The ChIP-qPCR results show that the fold change in the amount of *Cyclin D1*, *Cyclin E1*, and *Cyclin A1* promoter DNA pulled down with anti-E2F1 Ab significantly increased up to 5.25-, 2.26-, and 4.37-fold, respectively (Figure 5B, C), at 1 hour, but did not differ significantly at 15 minutes, after FGF9 treatment. These ChIP-qPCR and IP results imply that FGF9-induced hyperphosphorylation of Rb increased the dissociation of the Rb-E2F complexes, and free E2F1 was released and translocated to nucleus to activate the transcriptions of *Cyclin D1*, *Cyclin E1*, and *Cyclin A1* genes through the E2F1 binding site.
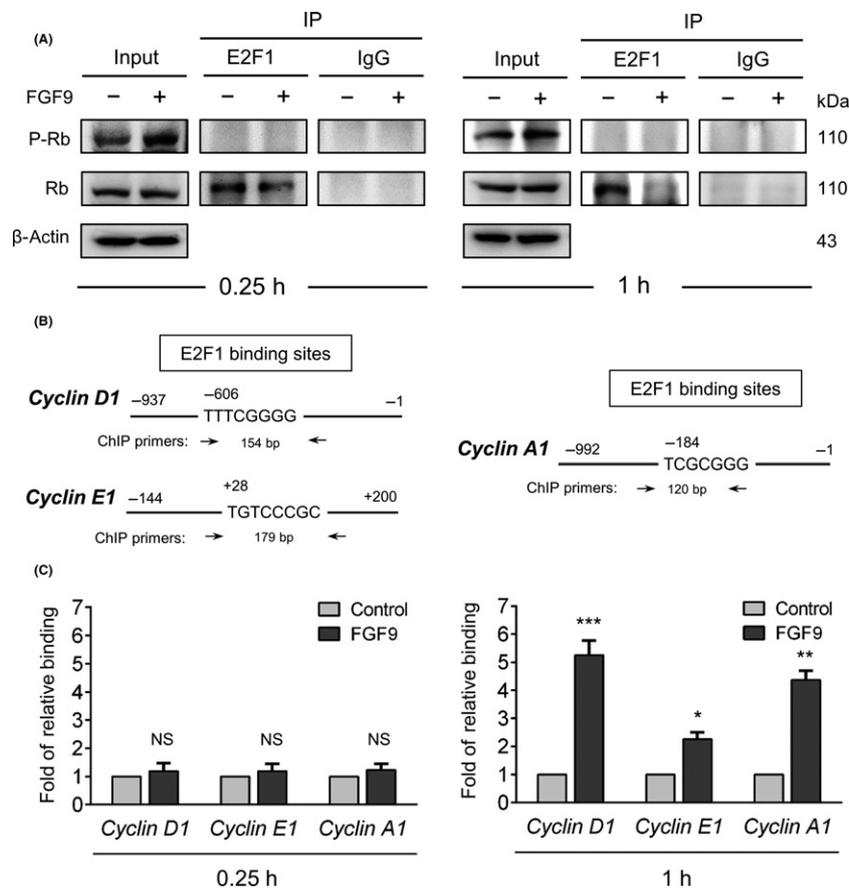
FIGURE 5 Fibroblast growth factor 9 (FGF9) increased the release of retinoblastoma (Rb)-bound E2F1 and enhanced E2F1 transcriptional activity. MA-10 cells were starved for 18 hours then treated with 50 ng/mL FGF9 or PBS for 0.25 or 1 h. A, Immunoprecipitation (IP) with anti-E2F1- or rabbit IgG (negative control)-conjugated magnetic G beads was carried out in the cell lysates from treated MA-10 cells. Western blots were probed with anti-Rb, anti-phosphor-Rb (S807/S811), and anti-β-actin. B, Schematic representation of *Cyclin D1*, *Cyclin E1*, and *Cyclin A1* promoters highlighting the E2F1 binding motif positions in relation to the transcription start site.[63-65] Horizontal arrows indicate the location of specific primers used for ChIP-quantitative PCR (qPCR) assays. Note that the figure is not drawn to scale. C, ChIP was carried out using anti-E2F1 or rabbit IgG Abs followed by qPCR analyses. ChIP-enriched DNA was amplified with the primers for E2F1 binding regions on the promoter of *Cyclin D1*, *Cyclin E1*, and *Cyclin A1* genes. The ChIP-qPCR data were represented as fold changes compared with the PBS control group after normalized to the IgG control. Values are presented as the mean ± SEM, n = 4. P values were analyzed using Student's t test. *P < .05, **P < .01, ***P ≤ .001

3.7 | FGF9 induced FGFR1-4 expressions in MA-10 cells

Each FGF could interact with its specific FGFR to activate different signals in various cells, and studies have also shown that different FGF/FGFR signals participate in different cancer formations.[7-10,66] Thus, the expressions of FGFR1-4 regulated by FGF9 in MA-10 cells were also investigated through western blot analysis. Results showed that 50 ng/mL FGF9 could significantly increase the expression of FGFR1 at 12 hours of treatment, FGR2 at 48 hours of treatment, FGFR3 at 72 hours of treatment, and FGR4 at 48 hours of treatment (Figure 6).

Immunofluorescent assay was also used to define cell-surface expressions of FGFR1-4 with 50 ng/mL FGF9 for 48 hours of treatment in MA-10 cells. Fibroblast growth factor 9 could stimulate more FGFR1-4 expression compared to control in MA-10 cells (Figures 7 and S2). Interestingly, the expression patterns between FGFR2 and FGFR3 were somewhat different in FGF9-treated groups compared to the control group; FGFR2 expression showed

a denser and more even distribution in a higher number of MA-10 cells (Figures 7B and S2C) compared to FGFR3 expression, which was lighter and appeared in clusters in fewer MA-10 cells (Figures 7C and S2D).

3.8 | FGF9 interacts with FGFR2 to activate the ERK1/2 pathway in MA-10 cell proliferation

It is well known that different FGFs can bind to different FGFRs to stimulate different intracellular signaling cascades.[6] To identify the specific receptor(s) for FGF9 signaling in MA-10 cells, further experiments were carried out by using a lentiviral vector-based shRNA system to silence FGFR1-4 (Figures 8A and S3). The results show that the FGF9-induced ERK phosphorylation in FGFR2 knockdown MA-10 cells was inhibited (Figure 8B). Also, cell proliferation was not significantly promoted by FGF9 in FGFR2 knockdown MA-10 cells (Figure 8C). In contrast, the FGF9-induced ERK phosphorylation and cell proliferation still significantly increased in FGFR1, FGFR3,
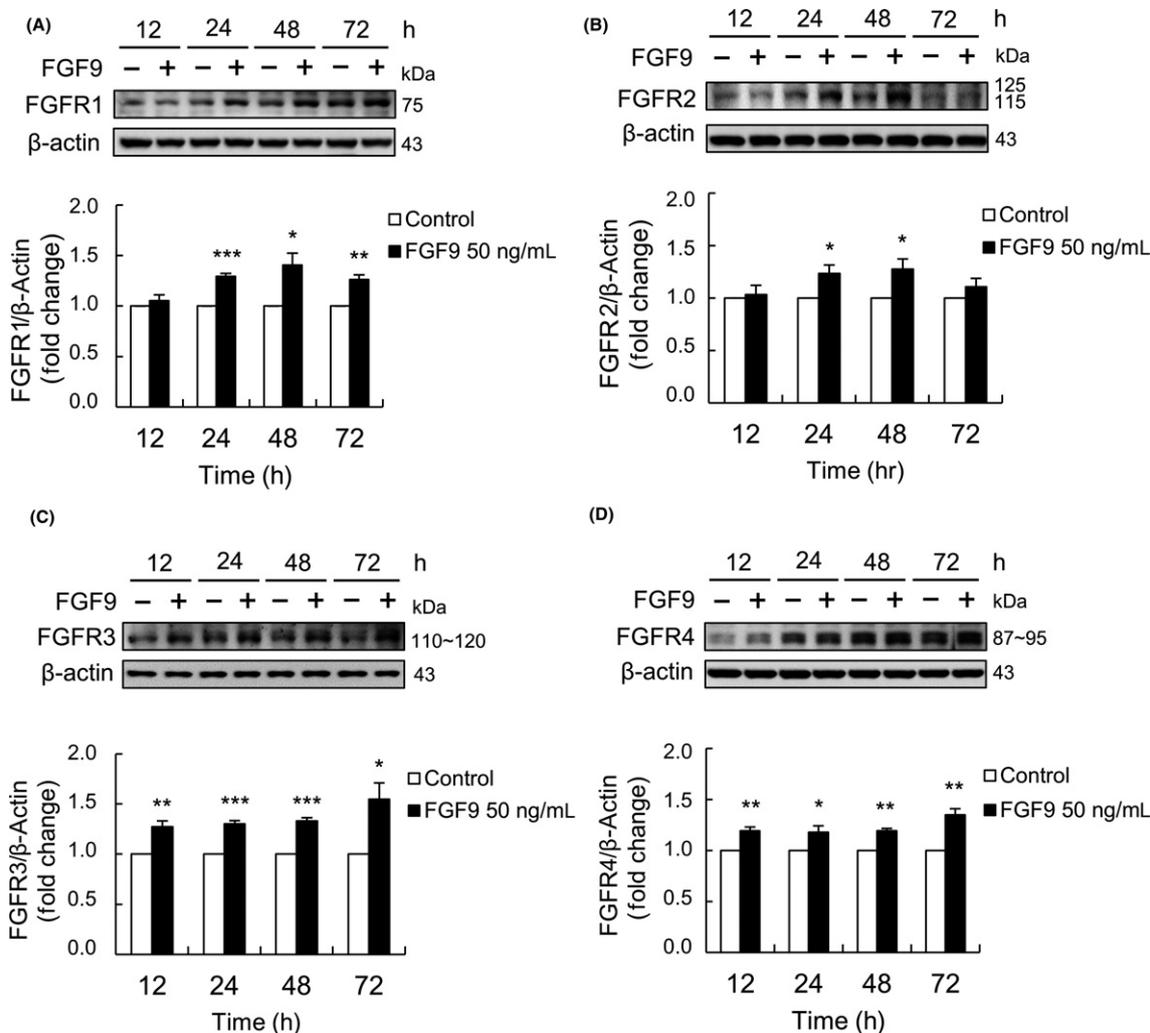
FIGURE 6 Fibroblast growth factor 9 (FGF9) regulated FGF receptor (FGFR)1-4 protein expression in MA-10 cells. Western blot analysis for the expressions of (A) FGFR1, (B) FGFR2, (C) FGFR3, and (D) FGFR4 in MA-10 cells treated with 50 ng/mL FGF9 or PBS for 12, 24, 48, or 72 h. All values are represented as the mean ± SEM, n = 3. P values were calculated using Student's t test. *P < .05, **P < .01, ***P < .001 vs control group
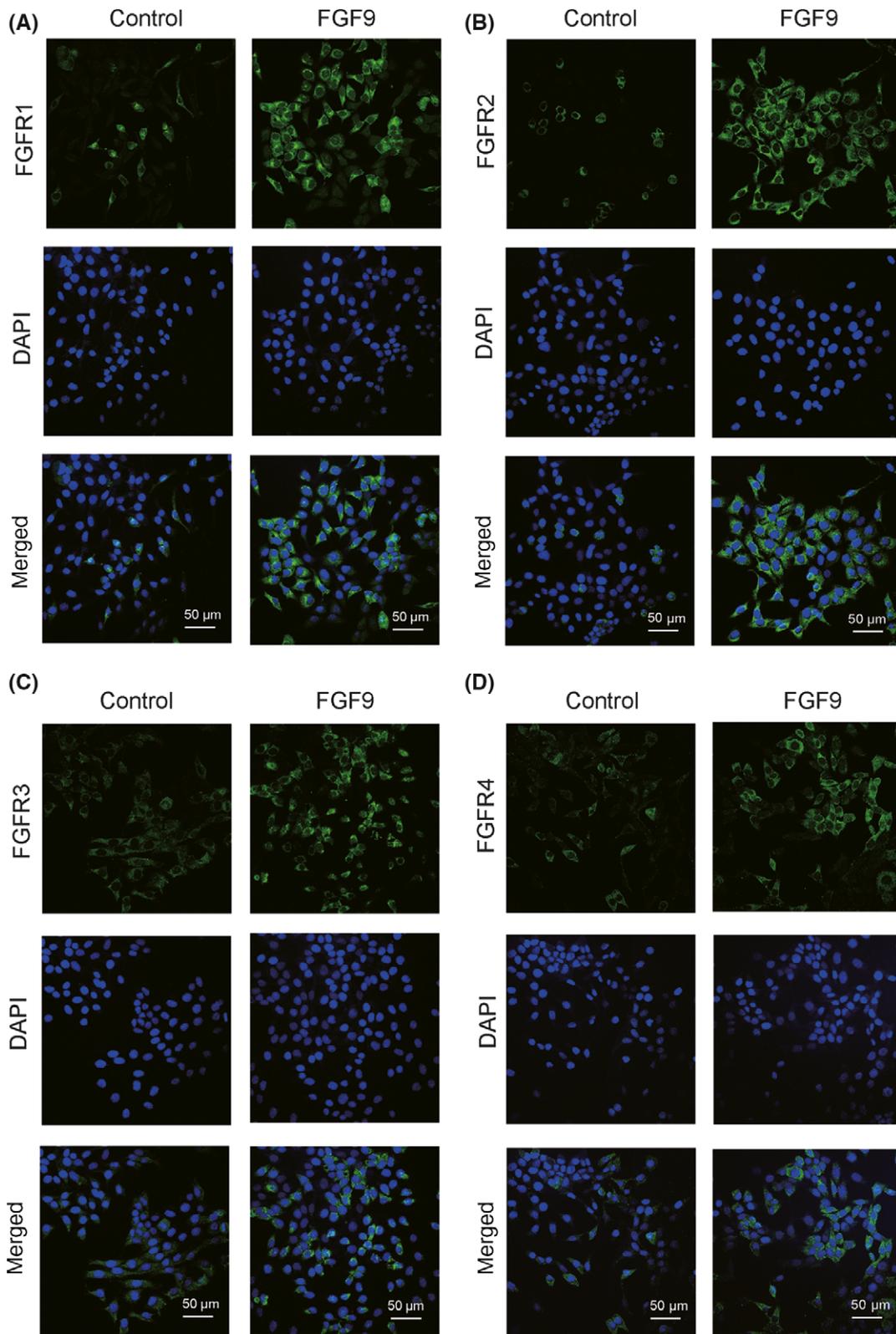
FIGURE 7 Fibroblast growth factor 9 (FGF9) regulated cell-surface FGF receptor (FGFR)1-4 protein expression in MA-10 cells. Immunofluorescent staining was used to determine the expression of (A) FGFR1, (B) FGFR2, (C) FGFR3, and (D) FGFR4 (green) on MA-10 cells treated with 50 ng/mL FGF9 or PBS for 48 hours. Nuclei were stained with DAPI (blue). Rabbit IgG was used as a negative control. All fluorescent images were taken under a fluorescent microscope with ×400 magnification. Images are representative of at least 4 separate experiments

and FGFR4 knockdown MA-10 cells (Figure 8B, C). These results indicate that FGF9 activated the ERK1/2 signaling pathway through FGFR2 to induce cell proliferation.

3.9 | FGF9 promoted tumor growth in a xenograft model of testicular cancer

As FGF9 could promote MA-10 cell proliferation in vitro, the FGF9 in vivo proliferative effect was further investigated using male NOD-SCID mice as the xenograft model. MA-10 cells were injected into the flank of the mice. After 7 days, mice were given daily injections of 5 ng FGF9 or PBS (vehicle) for 10 days. Results showed that the tumor volumes of the FGF9-treated group were significantly higher than that of vehicle and control (no treatment) groups (Figure 9A) and the tumor weights significantly increased with FGF9 treatment (vehicle group, 3.27 ± 0.17 g; control group, 3.08 ± 0.27 g; and FGF9-treated group, 4.62 ± 0.54 g) (Figure 9B). There was no significant difference in body weight between control, and PBS- and FGF9-treated mice, indicating that FGF9 treatment had an acceptable safety profile ($P > .05$; Figure 9A inset).

To further investigate the tumor growth-promoting effects of FGF9 in vivo, immunohistochemistry examinations of Ki-67, cleaved caspase-3, and FGF9, with serial sections, were carried out. Results showed that the tumor tissue expressions of Ki-67 increased and cleaved caspase-3

decreased when the expression of FGF9 increased (Figure 9C). These results suggest that, in vivo, FGF9 promoted tumor growth by increasing cell proliferation and decreasing cell death, apoptosis.

Furthermore, the immunohistochemical expression of phosphor-ERK1/2, phosphor-Rb, and E2F1 in MA-10 tumors was significantly increased in the FGF9-treated group compare to control and PBS groups (Figure 9D). These in vivo observations further support that FGF9 promoted cell proliferation by activating the ERK1/2 and RB/E2F pathways in vitro (Figure 4). In conclusion, FGF9 significantly promoted tumor growth in both an in vitro cellular system and an in vivo animal model related to tumorigenesis.

4 | DISCUSSION

In this study, we showed that FGF9, via FGFR2, significantly promoted MA-10 cell proliferation by stimulating Rb phosphorylation through activating ERK1/2 within 15 minutes of treatment. FGF9-induced Rb phosphorylation leads to the dissociation of Rb-E2F1 complexes, and thereby E2F1 was released and translocated to the nucleus to enhance the transcription of E2F1 target genes, *Cyclin D1*, *E1*, and *A1*. We also observed that FGF9 significantly enhanced the expression of E2F1 and cell cycle-related proteins, cyclins, and CDKs, to promote cell cycle progression (Figure 10). Many reports
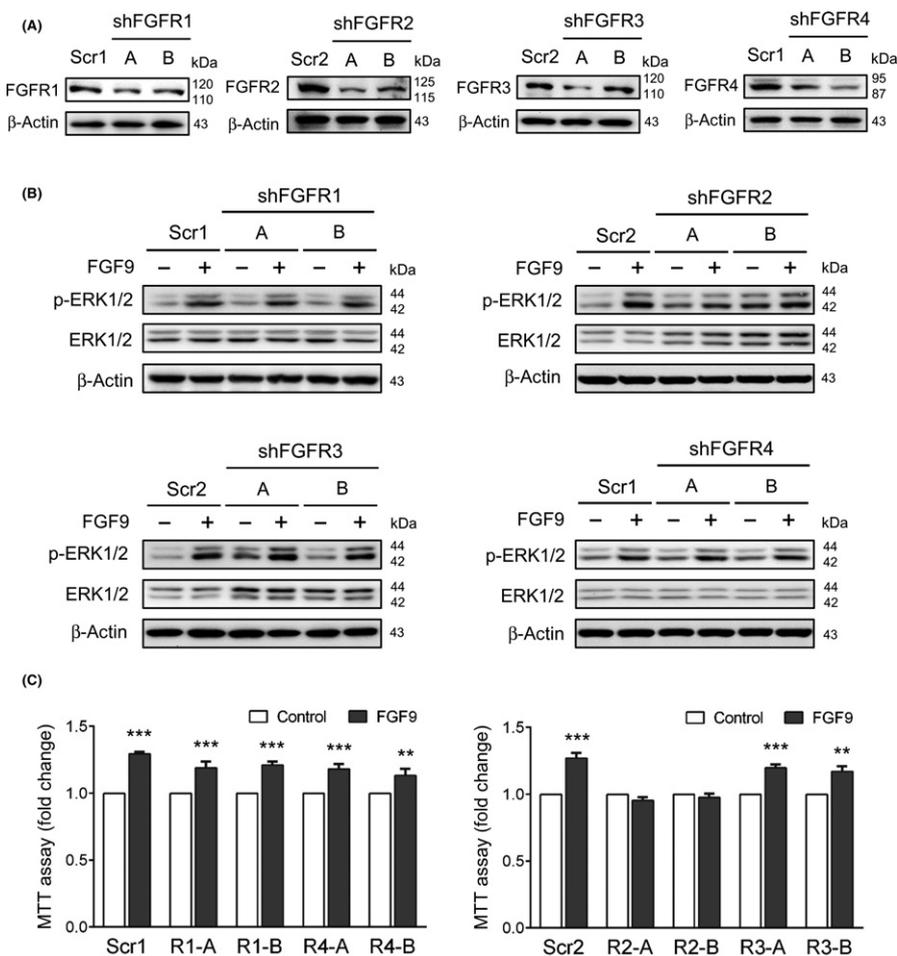
FIGURE 8 Lentiviral shRNA silencing of fibroblast growth factor receptor 2 (FGFR2) inhibited fibroblast growth factor 9 (FGF9)-induced ERK1/2 phosphorylation and MA-10 cell proliferation. A, Gene silencing verification at protein level by western blot analysis of MA-10 cells transfected with a specific shRNA against FGFR1-4. Two different shRNA targeted sequences were used for each FGFR. Cells transfected with a non-silencing shRNA sequence (scrambled sequence) in the PLKO.TRC1-puro (Scr1) or PLKO.TRC2-puro (Scr2) vectors were used as control as indicated. B, FGFR1-4 knockdown and scrambled control MA-10 cells were treated with 50 ng/mL FGF9 or PBS for 0.25 hours. Levels of ERK1/2 and phosphor-ERK1/2 expression were analyzed by western blot assay. C, MTT assay for the viability of the FGFR1-4 knockdown and scramble control MA-10 cells treated with FGF9 50 ng/mL or PBS for 48 hours. All values are represented as the mean ± SEM; n = 4. P values were calculated using Student's t test. *$P < .05$, **$P < .01$, ***$P < .001$ vs PBS control group
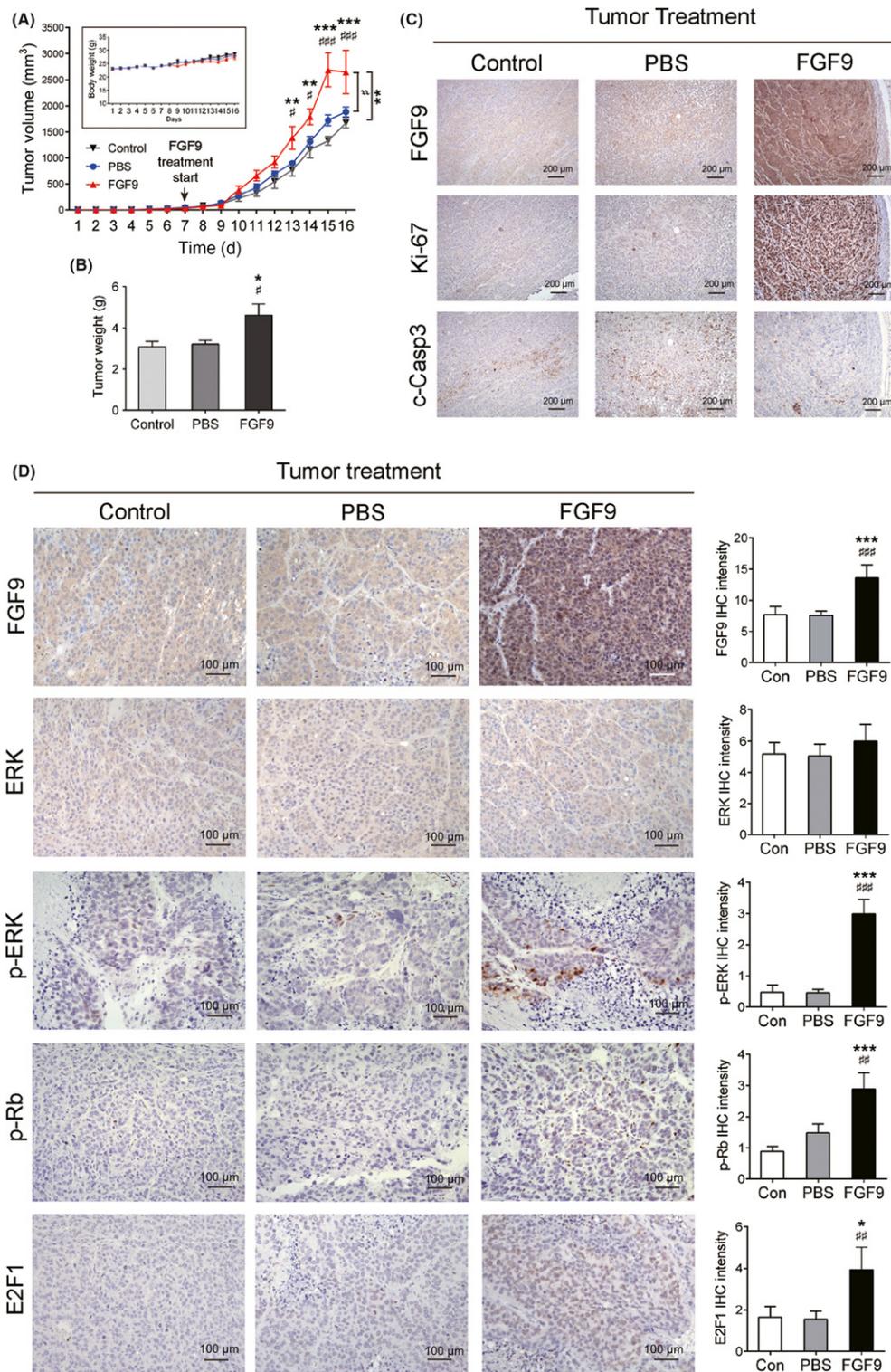
FIGURE 9 Fibroblast growth factor 9 (FGF9) stimulated tumor growth in a xenograft model with MA-10 cell induction. SCID mice s.c. injected with MA-10 cells were treated with 50 ng/mL FGF9 or PBS once daily for 10 days. Mice in the control group were inoculated with MA-10 cells and received no treatment. A, Tumor volumes (A) and body weights (A, inset) were plotted against time. B, tumor weights at the time the mice were sacrificed. Values are represented as the mean ± SEM; n = 6. P values were calculated using two-way ANOVA with Sidak's multiple comparisons post-tests (A) and one-way ANOVA with Tukey's multiple comparisons post-tests (B). C, Immunohistochemical assays of FGF9, Ki-67, and cleaved caspase-3 (c-Casp3) expression in MA-10 s.c. tumors from FGF9- and PBS-treated mice, or control mice (original magnification ×100). D, Immunohistochemical assays of FGF9, phosphor-ERK1/2 (pERK1/2), total ERK1/2 (ERK1/2), phosphor-retinoblastoma (pRb), and E2F1 expression in MA-10 s.c. tumors from FGF9- or PBS-treated mice, or control mice (original magnification ×200). Quantification values are represented as the mean ± SEM; n = 4. P values were calculated using one-way ANOVA with Tukey's multiple comparisons post-tests. *$P < .05$, **$P < .01$, ***$P < .001$ vs control group. #$P < .05$, ##$P < .01$, ###$P < .001$ vs PBS group
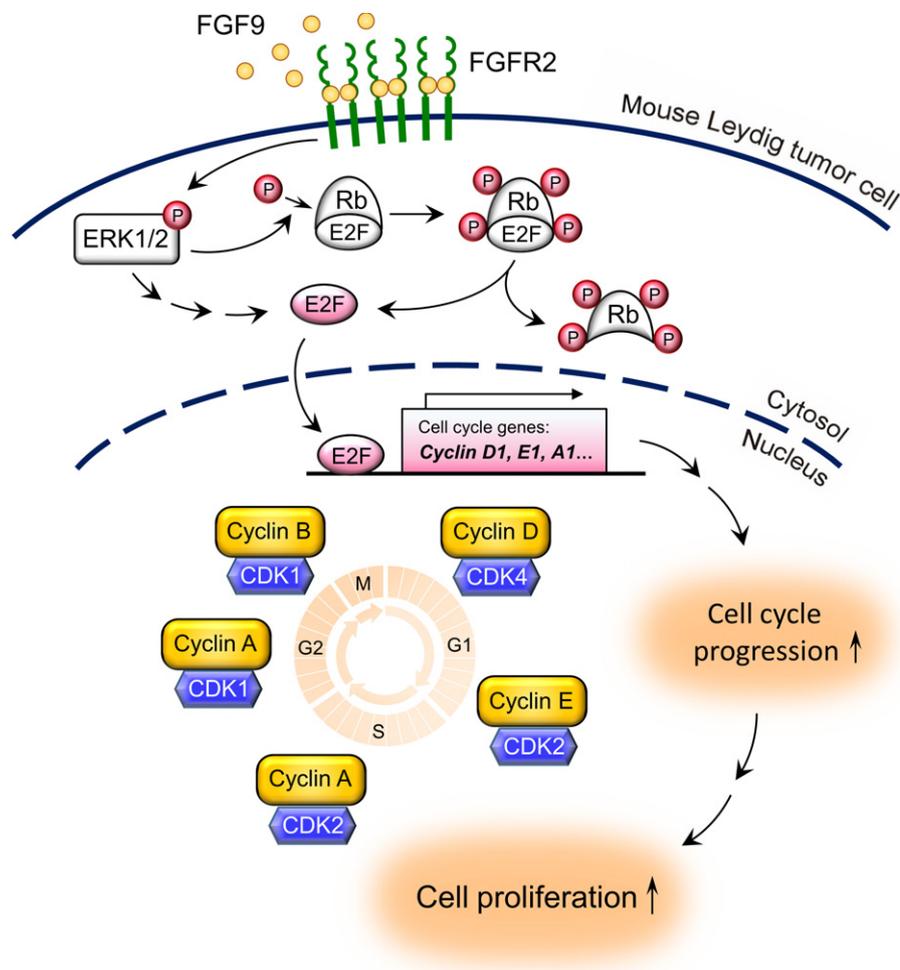
FIGURE 10 Summary scheme illustrating how fibroblast growth factor 9 (FGF9) induces MA-10 cell proliferation. FGF9 associates with FGF receptor 2 (FGFR2) to stimulate retinoblastoma (Rb) phosphorylation through ERK1/2 activation. FGF9-induced Rb phosphorylation leads to the release of Rb-bound E2F resulting in the nuclear translocation of E2F1 and transactivation of E2F target genes. FGF9 significantly increases the expression of E2F and cell cycle related proteins, cyclins and cyclin-dependent kinases (CDKs), to increase cell cycle progression, which finally promotes MA-10 cell proliferation.

have illustrated that FGF9 is essential for steroidogenesis and sex determination.[36-38] However, abnormal expression of FGF9 is involved in different types of cancers.[39-42,52,67] Studies have also shown that endogenous overexpression of FGF9 might cause lung adenocarcinoma,[68,69] and microRNA-140-5p and microRNA-26a could suppress FGF9 expression to reduce hepatocellular carcinoma and gastric cancer, respectively.[70,71] Here, we showed that FGF9 could induce cell proliferation in MA-10 mouse Leydig tumor cells. Thus, our observation that FGF9 exerted cell proliferation in MA-10 cells is not unprecedented. However, no report has described the role of FGF9 in inducing cell proliferation in testicular tumor.

To investigate the mechanism of action, the activation profile of the PI3K, MAPK, and PLCγ signaling pathways in FGF9-treated MA-10 cells was studied. Our data showed FGF9 had no effect on the AKT/mTOR and PLCγ pathways to induce MA-10 cell proliferation. In MAPK, FGF9 could only the phosphorylation of ERK1/2, but not JNK or p38,[52] in MA-10 cells. The MAPK/ERK1/2 inhibitor study further confirmed that FGF9 activated the ERK1/2 pathway in MA-10 cells. Many studies have shown that ERK1/2 signaling could promote cell proliferation in gastric cancer growth, endometrial carcinoma tumorigenesis, and breast cancer related to tumorigenesis.[72-74] Thus, our data is parallel to other studies. In fact, FGF9 activated ERK1/2 phosphorylation within 15 minutes, and significant cell proliferation occurred within 48 hours in MA-

10 cells, indicating ERK activation could last a long period of time to sustain cell proliferation. Studies have shown that short-term activation of the ERK1/2 pathway could persist for a long time on cell proliferation in dopaminergic neuron cells.[75,76] Thus, our observations are not extraordinary.

The cell cycle is important in regulating cell growth.[31] The complex CDKs and cyclins can drive cell from one stage to another during cell proliferation. Positive stimulation could promote cell progression and help cell proliferation. However, abnormal regulation of the cell cycle could drive normal cells into cancer cells.[32,77] In the present study, cyclin D1, E1 and CDK4 in G1 phase, cyclin A1, CDK2 and CDK1 in S/G2/M phase, and cyclin B1 in G2/M phase were upregulated by FGF9 in MA-10 cells. Our results strongly show that FGF9 did activate the expressions of cell cycle-related proteins to induce MA-10 cell proliferation. Many studies have shown that FGF9 and/or alternative molecules can induce cell cycle-related proteins to regulate cell progress and proliferation in different normal and cancer cells.[78] Thus, our observations are consistent with those studies.

Regarding cell cycle inhibitory proteins, p53 is a tumor suppressor that can induce the expression of p21 and p27 proteins to further induce cell cycle arrest.[79] Moreover, retinoblastoma, a tumor suppressor protein, could prevent abnormal cell growth by suppressing E2F activity and inhibiting cell cycle progression at the G_1/S

checkpoint. Retinoblastoma loses its grip on E2F when it is phosphorylated, free E2F is then able to translocate to the nuclues and transactivate its cell cycle-related target genes to promote cell cycle and cell proliferation.[56,80] In the present study, the expression of p53, p21, and p27 was not affected by FGF9, indicating the p53 pathway did not have any role in suppressing MA-10 cell proliferation. Moreover, FGF9 increased the expression of phosphor-Rb, suggesting MA-10 cell proliferation was not suppressed by the Rb and E2F pathway. Neither the p53 nor Rb inhibitory pathways regulating cell cycle progression were functioning, which would certainly cause MA-10 cell proliferation. In fact, studies have illustrated that cell proliferation in various tumors is due to the inactivation of the p53 pathway and the suppression of Rb pathway.[81] Thus, our observations are not exceptional.

In this study, the xenograft model results showed that FGF9 significantly induced in vivo MA-10 tumor growth, a result of increased cell proliferation and decreased cell death. There was no toxicity or side-effects associated with FGF9 treatment as there was no difference in body weight between control and FGF9-treated groups. To our knowledge, this is the first study to assess the proliferative efficacy of FGF9 on testicular cancer in vivo.

In conclusion, FGF9 could activate the MAPK/ERK1/2 signaling pathway through FGFR2, promote cell cycle progression with the suppression of inhibitory proteins, and upregulate expression of FGFRs to induce cell proliferation in MA-10 mouse Leydig tumor cells.

CONFLICT OF INTEREST

The authors have no conflict of interests.

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