Received: 29 July 2019 | Accepted: 25 November 2019

DOI: 10.1002/sctm.19-0234

TISSUE-SPECIFIC PROGENITOR AND STEM CELLS

STEM CELLS TRANSLATIONAL MEDICINE

The influence of fibroblast growth factor 2 on the senescence of human adipose-derived mesenchymal stem cells during long-term culture

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Funding information
National Taiwan University Hospital, Grant/Award Numbers: 108-S4129, 107-S3769; Ministry of Science and Technology, Taiwan, Grant/Award Numbers: 108-2628-B-002-020, 107-2314-B-002-176

Abstract

Adipose-derived mesenchymal stem cells (ASCs) exhibit great potential in regenerative medicine, and in vitro expansion is frequently necessary to obtain a sufficient number of ASCs for clinical use. Fibroblast growth factor 2 (FGF2) is a common supplement in the ASC culture medium to enhance cell proliferation. To achieve clinical applicability of ASC-based products, prolonged culture of ASCs is sometimes required to obtain sufficient quantity of ASCs. However, the effect of FGF2 on ASCs during prolonged culture has not been previously determined. In this study, ASCs were subjected to prolonged in vitro culture with or without FGF2. FGF2 maintained the small cell morphology and expedited proliferation kinetics in early ASC passages. After prolonged in vitro expansion, FGF2-treated ASCs exhibited increased cell size, arrested cell proliferation, and increased cellular senescence relative to the control ASCs. We observed an upregulation of *FGFR1c* and enhanced expression of downstream STAT3 in the initial passages of FGF2-treated ASCs. The application of an FGFR1 or STAT3 inhibitor effectively blocked the enhanced proliferation of ASCs induced by FGF2 treatment. *FGFR1c* upregulation and enhanced STAT3 expression were lost in the later passages of FGF2-treated ASCs, suggesting that the continuous stimulation of FGF2 becomes ineffective because of the refractory downstream FGFR1 and the STAT3 signaling pathway. In addition, no evidence of tumorigenicity was noted in vitro and in vivo after prolonged expansion of FGF2-cultured ASCs. Our data indicate that ASCs have evolved a STAT3-dependent response to continuous FGF2 stimulation which promotes the initial expansion but limits their long-term proliferation.

KEYWORDS

cell proliferation, cellular senescence, fibroblast growth factor 2, long-term culture, mesenchymal stem cell

1 | INTRODUCTION

Adipose-derived mesenchymal stem cell (ASC) represents an important source of mesenchymal stem cell (MSC). ASCs have drawn much attention in the field of regenerative medicine and tissue engineering because abundant ASCs can be easily accessed from subcutaneous adipose tissue using minimally invasive procedures such as liposuction.[1,2] Despite the abundance of adipose tissue, culture-expanded ASCs are still required to achieve a sufficient quantity for many clinical applications. Particularly, to guarantee a sustainable manufacturing process for commercialization of ASC-based products, prolonged cell expansion may be desired.[3] In addition, MSCs at later passages may exhibit different characteristics suitable for specific clinical use. For example, senescent MSCs were found to display stronger immunosuppressive properties, thus serving as a preferred treatment for host vs graft disease and other immune-relates disorders.[4] Moreover, late, but not early, passage MSCs could attenuate established pain behavior in an animal model of osteoarthritis.[5] Therefore, further investigation is necessary to understand the behavior of ASCs during prolonged in vitro expansion.

Human MSCs are commonly cultured as monolayers using conventional tissue culture techniques, but such methods may lead to reduced cell proliferation, decreased colony-forming efficiency, and decreased expression of pluripotency markers over time.[6,7] Since one of the limitations to the clinical use of ASCs is their tendency to become senescent and lose their potency for proliferation and differentiation when cultured in vitro,[8] maintaining the therapeutic efficacy of ASCs during in vitro expansion has become an important issue. Forcing the gene expression of *Nanog* or *Sox2* has been attempted to increase ASC stemness,[9] but gene transfection harbors substantial safety concerns for clinical use. Therefore, treating cells with various growth factors, including fibroblast growth factor 2 (FGF2), has become a common practice in ASC research.[10]

FGFs are key players in the proliferation and differentiation processes of a wide range of cells and tissues. In recent studies, various growth factors, such as FGFs, have been extensively investigated to elucidate how they promote the self-renewal and proliferation of MSCs.[11-13] Supplementing FGF2 in the culture medium during the in vitro ASC expansion enhances their proliferative efficiency.[7,12,14] In contrast, the senescence process of ASCs, characterized by increased doubling time, has been found to be in concordance with decreased FGF2 secretion from ASCs through autocrine signaling.[11] FGF2 also influences the differentiation capabilities of ASCs.[15-17] While FGF2 stimulates adipogenic differentiation of ASCs,[18] it has been shown to inhibit osteogenic differentiation by reducing osteocalcin expression in ASCs.[17] Although many studies have depicted the influence of FGF2 on ASCs, early passage ASCs have typically been used for the experiments.[19] The effect of FGF2 supplement on preserving the proliferative activity and senescence change of ASCs during long-term culture remains unknown.

Several studies have demonstrated the stability of human ASCs during prolonged cultivation with a low risk of tumorigenicity up to passage 20.[10,20] Although rare, spontaneous tumorigenic transformation of MSCs that are expanded in vitro has been reported, particularly when they were treated with certain carcinogens.[21,22] For example, supplementing FGF2 in the culture medium of human bone marrow-derived MSCs transfected with *TERT* (telomerase reverse transcriptase) resulted in an increased potential for neoplastic transformation.[23] Thus, cell therapy with FGF2-treated ASCs may harbor a risk of tumorigenicity, especially after long-term stimulation. Since studies conducted with FGF2 supplement have not been carefully evaluated for tumorigenic risk, it is also crucial to elucidate the tumorigenic potential during the in vitro expansion process to address the safety issue of FGF2-expanded ASCs. Therefore, prolonged in vitro expansion of human ASCs with FGF2 supplement was performed in this study, and the important changes in the biological properties, tumorigenic potential, and signaling activities at different passages of FGF2-stimulated ASCs were investigated.

Significance statement

Adipose-derived mesenchymal stem cell (ASC) is a valuable cell source for regenerative medicine, and prolonged cell expansion may be desired to develop ASC-based products. Fibroblast growth factor 2 (FGF2) is a common supplement in the culture medium to enhance ASC proliferation. However, the effect of FGF2 on the ASC aging process during prolonged culture has not been well studied. This study found that although FGF2 maintains cellular morphology and enhances cell proliferation in the early passages of ASC culture, continuous FGF2 supplement exerts adverse effects on the late passages. The information is important for large-scale ASC production for clinical use.

2 | MATERIALS AND METHODS

2.1 | Cell isolation and culture

Subcutaneous adipose tissue from the abdomen was obtained from four nonsmoking, nondiabetic females undergoing elective plastic surgery procedures (age: 32-57 years; body mass index: 21.0-26.6). The study protocol was approved by the Research Ethical Committee of National Taiwan University Hospital (No. 201303038RINB). Informed consents had been obtained from all participants in this study. The minced adipose tissue was placed in a digestion solution consisting of 1 mg/mL collagenase type I (Gibco, Carlsbad, California) at 37°C for 60 minutes. The digest was filtered, and the cells in suspension were collected by centrifugation. The cells were cultured in a basal medium consisting of Dulbecco's Modified Eagle Medium-high glucose (DMEM-HG; HyClone, Logan, Utah), 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), and 1% penicillin-streptomycin (Biological Industries) at 37°C in 5% CO_2, and the medium was changed every 2-3 days. In the experimental group, 1 ng/mL FGF2 (R&D Systems, Minneapolis, Minnesota; catalog number: 233-FB) was added to the basal medium for

ASC culture. The cells were cultured without reaching confluence, and the cells were passaged every 7 days using 0.05% trypsin-EDTA (Biological Industries). Cells were harvested at different passages for various experiments.

2.2 | Cell size analysis

The trypsinized FGF2-treated and control ASCs at P5, P10, and P15 were stained with trypan blue (Biological Industries) and photographed under an inverted phase-contrast microscope. Only cells with an aspect ratio of less than 1.5 were selected, and the periphery of cells was traced using ImageJ software for the estimation of cell diameters.

2.3 | Proliferation and senescence assay

The FGF2-treated and control ASCs were seeded at a density of 1000 cells/cm^2. Every 7 days, the cells were passaged until cells reached senescence, and the cumulative population doubling was calculated at each passage. To evaluate cellular senescence, ASCs were fixed by 4% paraformaldehyde (Sigma, St. Louis, Missouri) at room temperature for 8 minutes and then stained with senescence-associated β-galactosidase (SA-β-gal) chromogenic substrate solution (Sigma) at 37°C for 16 hours. The number of cells positively stained with β-galactosidase was calculated.

2.4 | Flow cytometry for cell surface antigen

At P5, P10, and P15, the FGF2-treated and control ASCs were trypsinized to produce single-cell suspensions. To determine the expression of cell surface antigens, the samples were incubated with the following antibodies: anti-CD31 (BD Bioscience, San Diego, California), anti-CD34, anti-CD44, anti-CD73, anti-CD90, and anti-CD166 (all from BioLegend, San Diego, California). The samples were analyzed using a flow cytometer (FACSVerse; BD Biosciences, Franklin Lakes, New Jersey) in which 30 000 cells were counted for each sample. Positive cells were defined as those with fluorescence greater than 95% of the signal of the isotype controls.

2.5 | Differentiation of human ASCs

The FGF2-treated and control ASCs at P5, P10, and P15 were harvested for differentiation assays. Adipogenic differentiation was induced in basal medium supplemented with 500 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μM insulin, and 400 μM indomethacin (all from Sigma). After 14 days, ASCs were fixed in 4% paraformaldehyde and stained with Oil Red O (Sigma) to observe lipid droplets. Osteogenic differentiation was induced by culturing ASCs in basal medium supplemented with 10 nM dexamethasone, 50 μM ascorbic acid 2-phosphate, 10 nM 1α,25-dihydroxyvitamin D3, and 10 μM β-glycerophosphate (all from Sigma). At day 21, ASCs were fixed in 4% paraformaldehyde and stained with Alizarin Red S (Sigma) to observe mineralized matrix apposition.

2.6 | Cell cycle analysis

BrdU (Sigma) was added to the cell culture medium at a final concentration of 10 μM and incubated for 1 hour. After BrdU incorporation, cells were collected, fixed in 70% iced ethanol, and incubated at 4°C for 1 day. Cells were then treated with 0.5% Triton X-100 (Sigma) for 30 minutes followed by centrifugation to remove the supernatant. The cells were resuspended in 0.1 M $NaHCO_3$ for 30 minutes, centrifuged, and stained with anti-BrdU antibody (Cell Signaling, Danvers, Massachusetts). Following staining with anti-mouse FITC-conjugated secondary antibody (Leinco Technologies, Baldwin, Missouri) for 30 minutes in the dark, the cells were then suspended in 10 μg/mL propidium iodide solution (Sigma) and analyzed by a flow cytometer (FACScan; Becton Dickinson, Franklin Lakes, New Jersey).

2.7 | Colony-forming assay

The FGF2-treated and control ASCs at P5, P10, and P15 were cultured at a density of 1000 cells per 100 mm dish. The media were changed every 2-3 days. After 14 days of culture, the cells were fixed and stained with 0.5% crystal violet solution (Sigma) for 30 minutes. The number of colonies (diameter >2 mm) was counted.

2.8 | Quantitative PCR

Total RNA from the FGF2-treated and control ASCs at P5, P10, and p15 was extracted by an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was determined by optical density at 260 nm (OD_{260}) using a spectrophotometer. Once RNA was isolated, complementary DNA (cDNA) was synthesized using a High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, California). Briefly, quantitative PCR was performed in triplicate using a StepOne Real-Time PCR system (Bio-Rad, Hercules, California). The sequences of the gene-specific primers are shown in Additional file 1: Table S1. The expression level was analyzed and normalized to GAPDH for each cDNA sample. The relative gene expression values were calculated using control ASC samples as the reference.

2.9 | FGFR and STAT3 inhibition

The FGF2-treated and control ASCs at P4 were seeded as 9000 cells/cm^2. For FGFR1 inhibition, ASCs were pretreated with 10 μM SU5402 (FGFR1 inhibitor; Sigma) for 2 hours, and 1 ng/mL FGF2 was subsequently added to the culture medium for 3 days. For signal transducer and activator of transcription 3 (STAT3) inhibition, ASCs were pretreated with 0.5 μM or 1 μM Stattic (STAT3 inhibitor; Sigma) for 2 hours before 1 ng/mL FGF2

was added to the culture medium for 3 days. The relative cell proliferation was determined by Alamar blue assay using a protocol modified from a previous study.[24] The number of viable cells was proportional to the magnitude of dye reduction.

2.10 | Western blot analysis

The cells were suspended in cell lysis buffer (Fermentas, Vilnius, Lithuania). After centrifugation, the protein content was determined in the supernatants by a bicinchoninic acid protein quantification kit (Pierce, Rockford, Illinois). Protein samples from treated ASCs were mixed with Laemmli sample buffer and boiled for 10 minutes. Subsequently, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. Western blotting was performed using anti-p21, anti-STAT3, anti-p-STAT3 (Try705), anti-extracellular signal-regulated kinase (ERK), anti-p-ERK (Thr202/Try204), and anti-GAPDH antibodies (all from Cell Signaling). After overnight incubation with the primary antibodies and extensive washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour, and then the blots were developed using an enhanced chemiluminescence detection system (Millipore, Billerica, Massachusetts).

2.11 | Chromosome count

Chromosomal spreads were performed for P12 ASCs fixed in methanol/acetic acid (3:1) solution. The fixed cells were dripped at a height of approximately 1 m from the glass slides to spread the chromosomes of metaphase cells.[25] The glass slides were stained with 4′,6-diamidino-2-phenylindole (DAPI; BioLegend) and visualized using a fluorescence microscope (Leica DMI 6000). The chromosome number was evaluated in at least 10 metaphases per sample.

2.12 | Soft agar assay for in vitro tumorigenicity

For the in vitro testing of anchorage-independent colony development, the FGF2-treated and control ASCs (P10 or P15) were suspended in 0.3% agarose (final concentration; Sigma) in DMEM-HG with 10% FBS and stratified on a bottom layer of 0.5% pre-solidified agarose in six-well plates.[8] The plates evaluated for the presence of colonies on day 21 by staining with 0.5% crystal violet (Sigma). HeLa cells (Bioresource Collection & Research Center, Hsinchu, Taiwan) were plated in the same manner as the positive control. The negative control contained no seeded cells.

2.13 | In vivo tumorigenicity assay

The FGF2-treated and control ASCs at P15 were examined for tumorigenicity by subcutaneous injection of approximately 10 million cells into the dorsal surface of anesthetized nude mice.[10] The handling and care of the animals were carried out in compliance with the animal care guidelines of National Taiwan University. After 4 months, the animals were euthanized and autopsied to determine the formation of tumors at the injection site and internal organs.

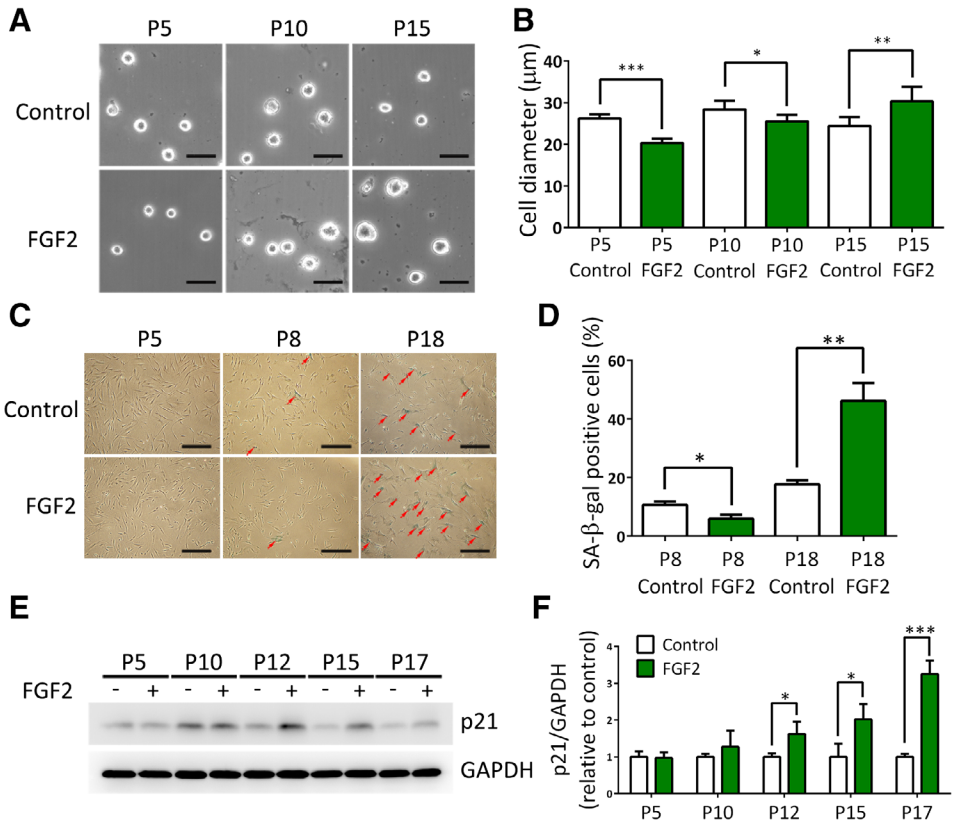

FIGURE 1 Morphology and senescence changes of adipose-derived mesenchymal stem cells (ASCs) with or without long-term FGF2 treatment. A, Microscopic images of suspended ASCs at P5, P10, and P15. Scale bar = 50 μm. B, Quantification of cell diameter revealed a smaller cell diameter of FGF2-treated ASCs in P5 and P10, but the cell size increased at P15 (n = 7). C, SA-β-gal staining of ASCs at P5, P8, and P18. Scale bar = 500 μm. D, Quantification of SA-β-gal-positive cells in 5-6 randomly selected fields (n = 3). E, Western blot analysis of the senescence marker p21 at different passages. F, Quantification of p21 protein expression was higher in FGF2-treated ASCs relative to the control after P12 (n = 3 at P17 and n = 4 at other passages). *$P < .05$, **$P < .01$, ***$P < .001$, Student's t test. FGF2, fibroblast growth factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; P, passage; SA-β-gal, senescence-associated β-galactosidase

Frozen tissue sections of dorsal skin, heart, lung, and liver were fixed with acetone and stained using anti-human nuclear antigen antibody (Millipore) to detect the transplanted human ASCs. After counterstaining with DAPI, the sections were analyzed using a fluorescence microscope.

2.14 | Statistical analysis

All measurements are presented as the means ± SD. Statistical significance was evaluated using an independent-sample Student's *t* test or ANOVA. Tukey's post hoc test was used when the group of interest

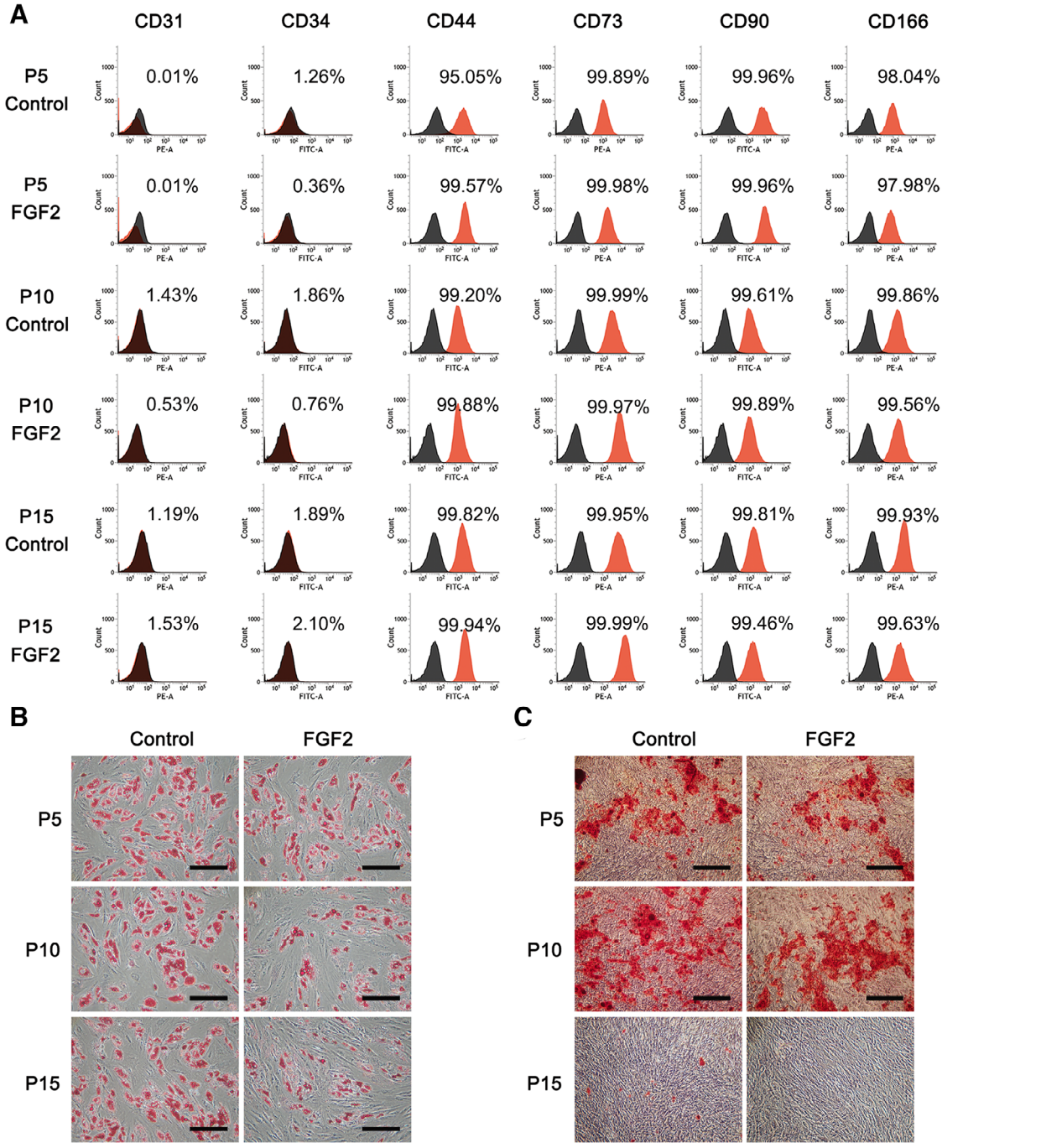

FIGURE 2 Phenotypic characterization of adipose-derived mesenchymal stem cells (ASCs) with or without long-term FGF2 treatment. A, The expression level of ASC surface markers is shown as the proportion of positively stained cells relative to the isotype control. These ASCs were negative for the hematopoietic markers CD31 and CD34 but positive for the mesenchymal stem cell-related markers CD44, CD73, CD90, and CD166. B, Microscopic images of ASCs cultured in adipogenic induction medium for 14 days. Cells were stained with Oil Red O for the detection of adipogenesis. Scale bar = 300 μm. C, Microscopic images of ASCs cultured in osteogenic induction medium for 21 days. Cells were stained with Alizarin Red for the detection of osteogenesis. Scale bar = 500 μm. FGF2, fibroblast growth factor 2; P, passage

was compared to all other groups in the experiment. All statistical analyses were performed using GraphPad Prism 7 (La Jolla, California). Statistically significant values were defined as $P < .05$.

3 | RESULTS

3.1 | Biphasic effect of FGF2 on the senescence changes of ASCs

Measuring the cell size of trypsinized ASCs at different passages revealed a significantly smaller cell diameter of the FGF2-treated ASCs relative to that of the control ASCs at P5 (20.3 ± 1.0 vs 26.3 ± 1.0 μm, $P < .001$) and P10 (25.6 ± 1.5 vs 28.4 ± 2.1 μm, $P < .05$). However, at P15, FGF2-treated ASCs exhibited a larger diameter relative to the control ASCs (30.3 ± 3.6 vs 24.4 ± 2.1 μm, $P < .01$; Figure 1A,B). Since increasing cellular size has been associated with senescence,[26] we further performed SA-β-gal staining for confirmation. The staining of SA-β-gal was virtually absent at P5 in both the FGF2-treated and the control ASCs. At P8, the ASCs treated with FGF2 exhibited a significantly smaller percentage of SA-β-gal-positive cells than the control (6.0 ± 1.5% vs 10.7 ± 1.2%, $P < .05$). In contrast, more SA-β-gal-positive ASCs were noted in the FGF2-treated group compared with the control group at P18 (46.2 ± 6.1% vs 17.7 ± 1.4%, $P < .001$; Figure 1C,D). In addition, FGF2-treated ASCs expressed significantly higher levels of senescence protein p21 relative to the control groups after p12

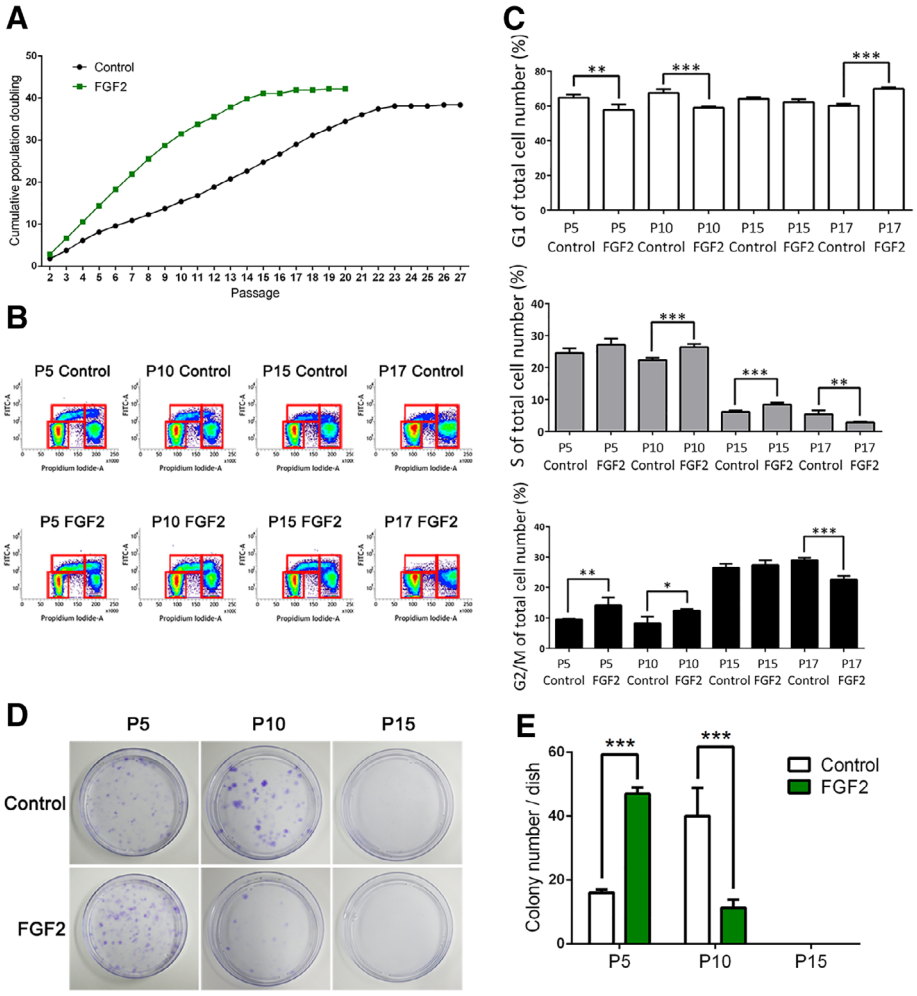

FIGURE 3 Proliferative capability, cell cycle analysis, and colony formation of adipose-derived mesenchymal stem cells (ASCs) with or without FGF2 treatment. A, Cumulative population doubling curve of ASCs. FGF2-treated ASCs expanded rapidly in early passages, but they reached senescence earlier than the control cells. B, Cell cycle analysis of ASCs at the indicated passages performed by PI staining and BrdU incorporation (with secondary FITC staining). Cells at the G1/G0, S, and G2/M phases were counted. C, Quantification of the proportion of cells at the G1/G0, S, and G2/M phases. A higher G2/M ratio and a lower G1 ratio were noted in FGF2-treated ACSs at P5 relative to control ASCs. However, FGF2-treated ASCs at P17 exhibited a reduced G1-S phase transition with a lower fraction of cells in G2/M phase and a higher G0/G1 fraction (n = 4). D, Colony-forming assay of ASCs plated at 1000 cells/dish and incubated for 14 days. Crystal violet was used to detect cell colonies greater than 2 mm in diameter. E, Quantification of the colony number per dish. The FGF2-treated group exhibited a significantly higher cell colony number at P5, whereas a significantly lower colony number was noted in the FGF2-treated group at P10 (n = 4). *$P < .05$, **$P < .01$, ***$P < .001$, Student's *t* test. FGF2, Fibroblast growth factor 2; P, passage

(P12: 1.6 ± 0.33 vs 1.0 ± 0.09, $P < .05$; P15: 2.02 ± 0.42 vs 1.0 ± 0.36, $P < .05$; P17: 3.3 ± 0.37 vs 1.0 ± 0.08, $P < .001$; Figure 1E,F).

3.2 | FGF2 maintained the immunophenotypes and differentiation capabilities of ASCs

We harvested ASCs with or without FGF2 treatment at P5, P10, and P15 to evaluate the immunophenotypes and differentiation capabilities. The surface epitopes of the FGF2-treated ASCs were similar to those of the control ASCs at the respective passages. These ASCs were negative for the hematopoietic markers CD31 and CD34 but positive for the MSC-related markers CD44, CD73, CD90, and CD166 (Figure 2A). Moreover, FGF2-treated ASCs maintained their adipogenic and osteogenic differentiation capabilities after the application of appropriate induction media, as demonstrated by the histology staining specific for oil and calcium, respectively (Figure 2B,C).

3.3 | FGF2 enhanced ASC proliferation only in the early passages

In the population doubling study, FGF2-treated ASCs expanded more rapidly through passages compared to ASCs without FGF2 treatment; however, FGF2-treated ASCs appeared to reach senescence earlier than the control cells. Eventually, ASCs in both groups reached approximately the same level of cumulative population doubling level of 40 (Figure 3A). Cell cycle analysis revealed a relatively higher G2/M ratio and a lower G1 ratio in FGF2-treated ACSs at P5 relative to control ASCs. FGF2-treated ACSs at P10 also facilitated the G1-S phase transition (S phase in FGF2-treated ASCs: 26.4 ± 1.0% vs control: 22.3 ± 0.8%, $P < .001$) with a higher ratio of cells in the G2/M phase (FGF2-treated ASCs: 12.4 ± 0.6% vs control: 8.2 ± 2.2%, $P < .05$) and a lower ratio at the G0/G1 phase (FGF2-treated ASCs: 67.5 ± 2.1% vs control: 59.1 ± 0.6%, $P < .05$). At P17, the trend reversed as the FGF2-treated ASCs exhibited a reduced G1-S phase transition (S phase, FGF2-treated ASCs: 2.8 ± 0.2% vs control: 5.4 ± 1.2%,

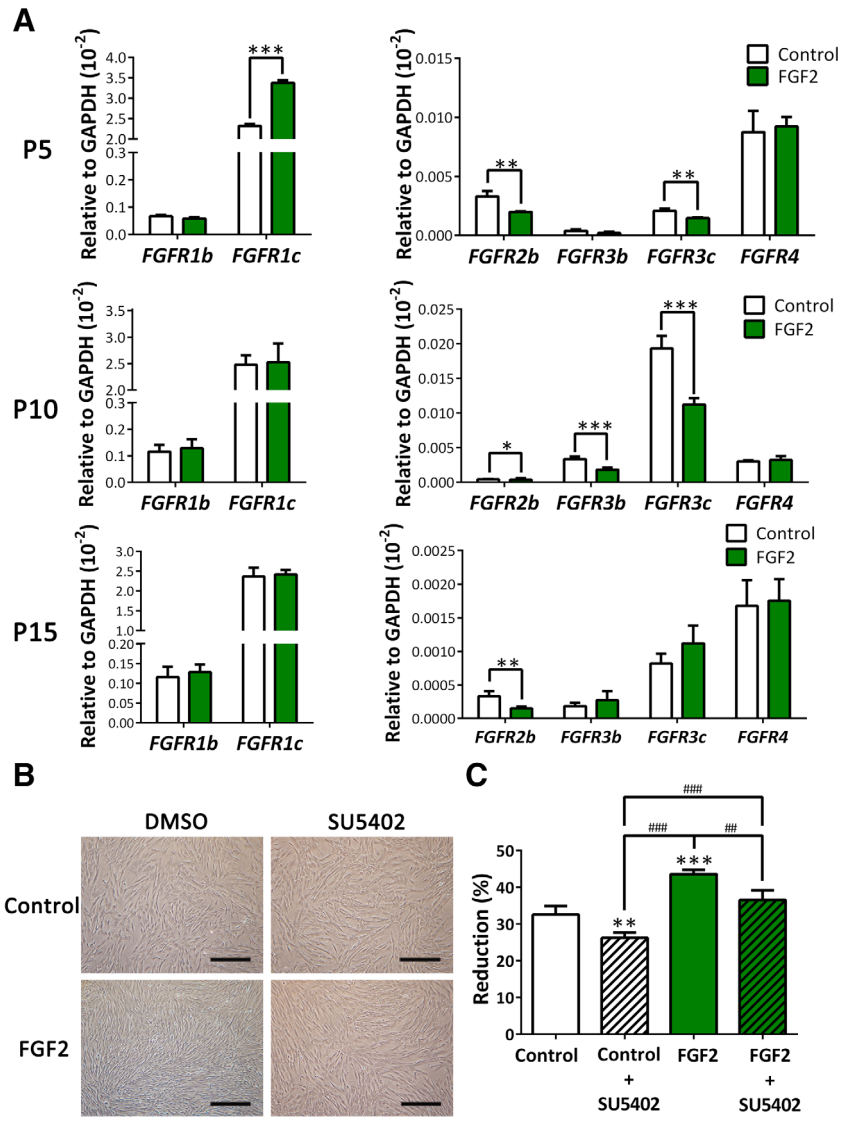

FIGURE 4 The expression of FGFR subtypes in adipose-derived mesenchymal stem cells (ASCs). A, Gene expression of FGFR subtypes in ASCs. The expression level of *FGFR1c* was remarkably higher than other FGFRs. FGF2-treated ASCs had upregulated *FGFR1c* relative to the control group at P5, but the FGF2-stimulated *FGFR1c* upregulation diminished in the later passages. Values are shown relative to each GAPDH expression level at the respective passage (n = 3). B, Microscopic images of ASCs supplemented with FGF2 and/or SU5402 pretreatment. Pretreatment of SU5402 resulted in a larger cell size and more flattened morphology in both FGF2-treated and control ASCs. Scale bar = 500 μm. C, Alamar blue assay for the evaluation of ASC proliferation in different culture conditions on day 3. SU5402 significantly inhibited ASC proliferation with or without FGF2 supplement (n = 3). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ relative to control; $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ between the indicated groups, Student's *t* test for panel (A) and ANOVA with the Tukey's post hoc test for panel (C). DMSO, dimethyl sulfoxide; FGF2, fibroblast growth factor 2; FGFR, fibroblast growth factor receptor; P, passage; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

$P < .01$) with a lower fraction of cells in G2/M phase (FGF2-treated ASCs: 22.5 ± 1.2% vs control: 28.9 ± 0.8%, $P < .001$) and a higher G0/G1 fraction (FGF2-treated ASCs: 69.9 ± 0.7% vs control: 60.2 ± 1.0%, $P < .001$; Figure 3B,C).

We employed the colony-forming assay to evaluate the effect of FGF2 on the inherent self-renewal and proliferation efficacy at different ASC passages. The FGF2-treated group exhibited a significantly higher cell colony number at P5 (47 ± 2 vs 16 ± 1 colonies per dish, $P < .001$), whereas a significantly lower colony number was noted in the FGF2-treated group at P10 (11 ± 3 vs 40 ± 9 colonies per dish, $P < .001$). At P15, neither group showed any colony formation (Figure 3D,E).

3.4 | FGF2 upregulated *FGFR1c* expression in ASCs

Our quantitative PCR data revealed a remarkably higher mRNA expression level of *FGFR1c* than other FGFRs in ASCs. FGF2 supplement further upregulated *FGFR1c* relative to the control group at P5

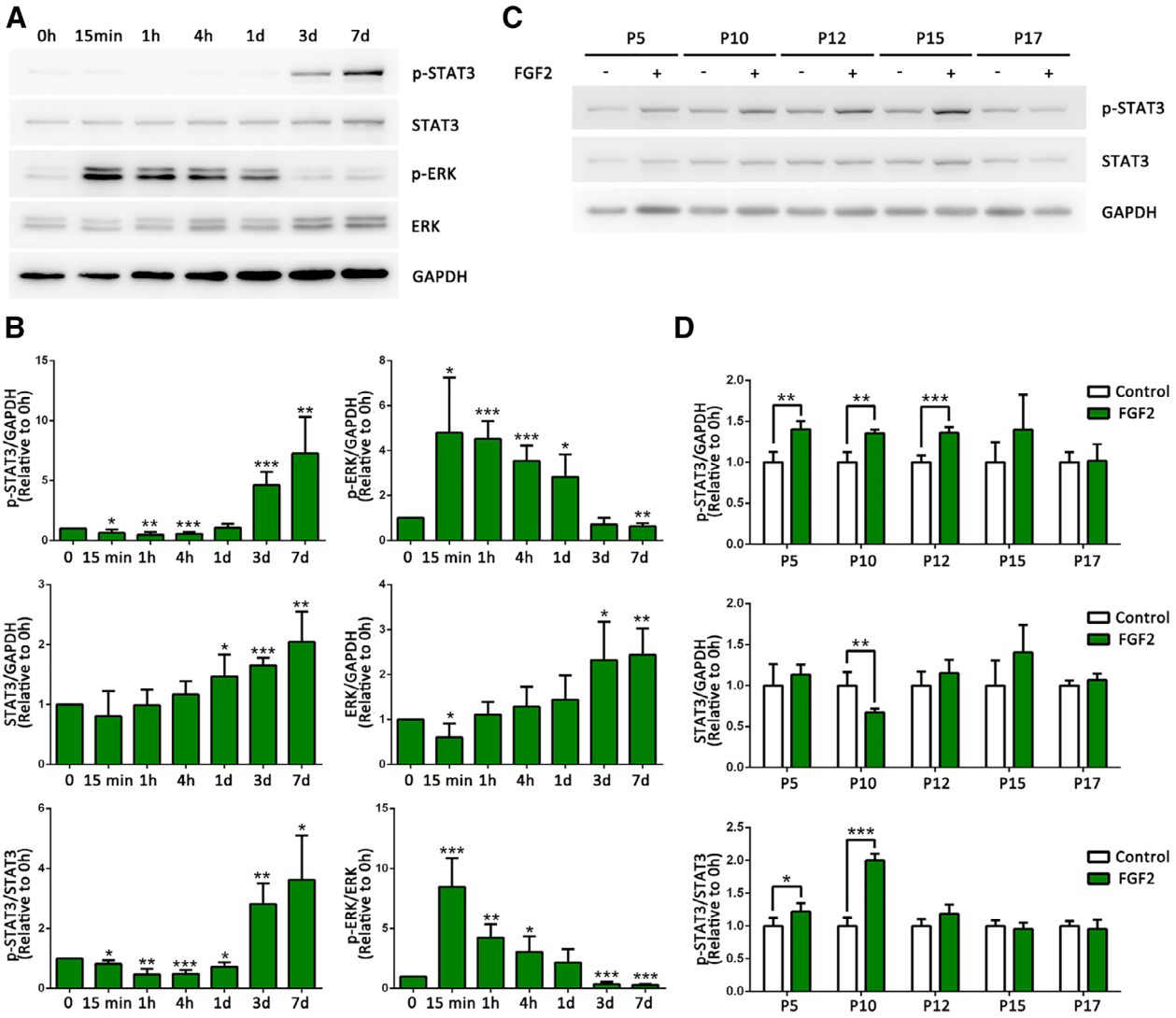

FIGURE 5 Expression and phosphorylation of STAT3 and ERK in adipose-derived mesenchymal stem cells (ASCs) with or without FGF2 treatment. A, ASCs in P4 were treated with FGF2 for 7 days, and STAT3, p-STAT3, ERK, and p-ERK expression was detected using Western blot. B, Quantification of STAT3, p-STAT3, ERK, and p-ERK protein levels in FGF2-treated ASCs in P4 for 7 days relative to the control. FGF2 triggered transient and robust phosphorylation of ERK in ASCs, which gradually faded after 24 hours. In contrast, both STAT3 expression and phosphorylation increased 24 hours after treating ASCs with FGF2, and the effect was sustained for at least 7 days (n = 4). C, STAT3 and p-STAT3 levels in ASCs during long-term FGF2 treatment were detected using Western blot. D, Quantification of STAT3 and p-STAT3 levels in FGF2-treated ASCs relative to the control in long-term culture. FGF2-treated ASCs exhibited a higher p-STAT3/STAT3 ratio until passage 10 (n = 3 at P17 and n = 4 at other passages). *$P < .05$, **$P < .01$, ***$P < .001$, Student's *t* test. d, day; ERK, extracellular signal-regulated kinase; FGF2, fibroblast growth factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; h, hour; min, minute; P, passage; p-STAT3, phosphorylated signal transducer and activator of transcription 3; STAT3, signal transducer and activator of transcription 3

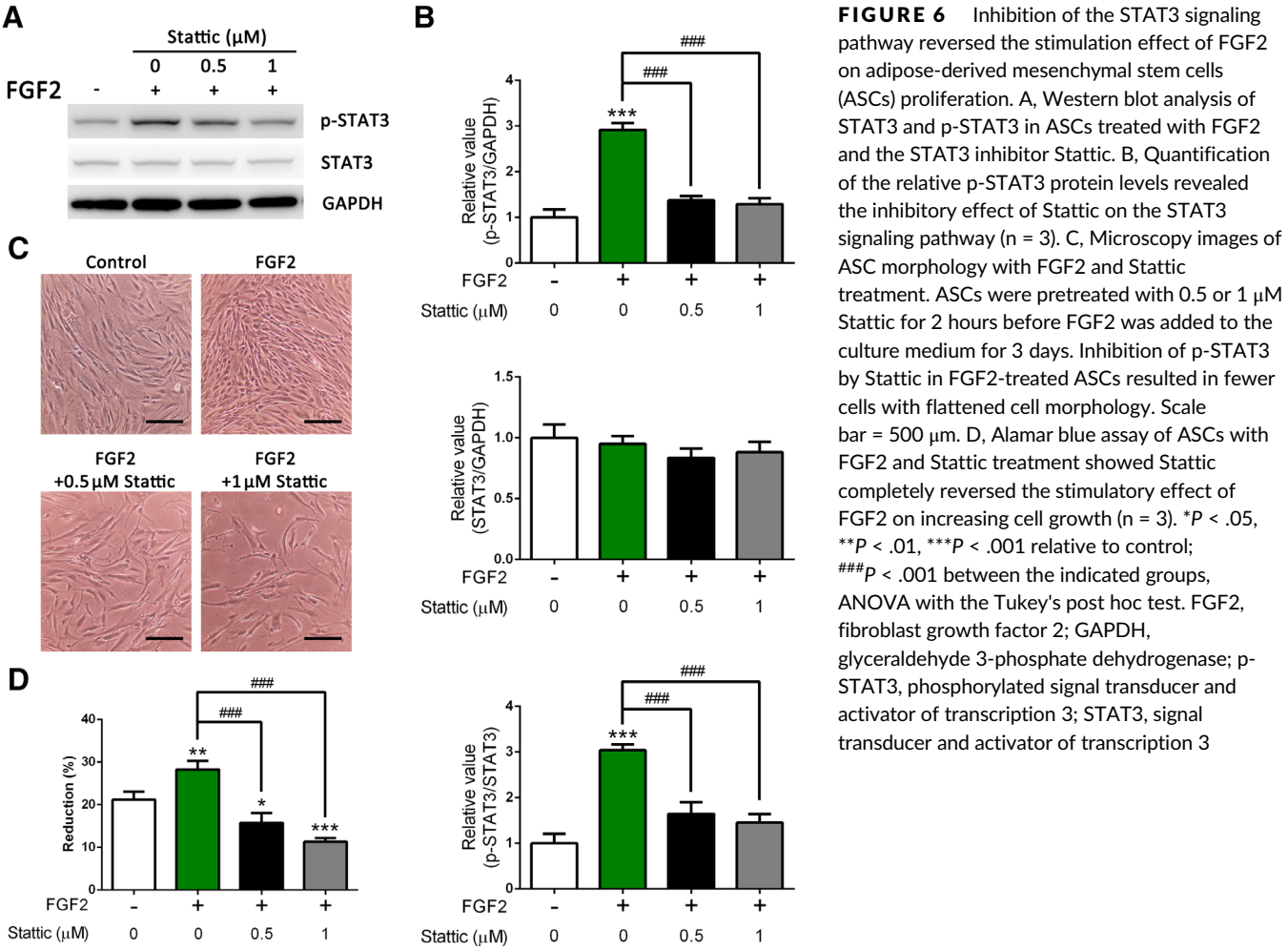

FIGURE 6 Inhibition of the STAT3 signaling pathway reversed the stimulation effect of FGF2 on adipose-derived mesenchymal stem cells (ASCs) proliferation. A, Western blot analysis of STAT3 and p-STAT3 in ASCs treated with FGF2 and the STAT3 inhibitor Stattic. B, Quantification of the relative p-STAT3 protein levels revealed the inhibitory effect of Stattic on the STAT3 signaling pathway (n = 3). C, Microscopy images of ASC morphology with FGF2 and Stattic treatment. ASCs were pretreated with 0.5 or 1 μM Stattic for 2 hours before FGF2 was added to the culture medium for 3 days. Inhibition of p-STAT3 by Stattic in FGF2-treated ASCs resulted in fewer cells with flattened cell morphology. Scale bar = 500 μm. D, Alamar blue assay of ASCs with FGF2 and Stattic treatment showed Stattic completely reversed the stimulatory effect of FGF2 on increasing cell growth (n = 3). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ relative to control; $^{###}P < .001$ between the indicated groups, ANOVA with the Tukey's post hoc test. FGF2, fibroblast growth factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; p-STAT3, phosphorylated signal transducer and activator of transcription 3; STAT3, signal transducer and activator of transcription 3

($3.37 \times 10^{-2} \pm 7 \times 10^{-4}$ vs $2.32 \times 10^{-2} \pm 5 \times 10^{-4}$ expression level relative to *GAPDH*, $P < .001$). However, the FGF2-stimulated *FGFR1c* upregulation diminished in the later passages (Figure 4A). Pretreatment with the FGFR1 inhibitor SU5402 resulted in a larger cell size and more flattened morphology in both FGF2-treated and control ASCs (Figure 4B). Alamar blue assay revealed that SU5402 significantly inhibited ASC proliferation with or without FGF2 supplement (Figure 4C). Collectively, these data suggest that FGF2 enhanced ASC proliferation in the early passages through an FGFR1-mediated pathway, particularly *FGFR1c*.

3.5 | Enhanced p-STAT3 expression induced by FGF2 was lost after long-term culture

Our Western blot results revealed that FGF2 triggered transient and robust phosphorylation of ERK in ASCs within minutes, which gradually faded after 24 hours. In contrast, the phosphorylation of STAT3 was initially suppressed upon FGF2 addition. Subsequently, both STAT3 expression and phosphorylation increased 24 hours after treating ASCs with FGF2, and the effect was sustained for at least 7 days (Figure 5A,B). During the long-term culture, FGF2-treated ASCs exhibited a higher p-STAT3/STAT3 ratio until passage 10 (P5: 1.2 ± 0.1-fold, $P < .05$; P10: 2.0 ± 0.1-fold, $P < .001$ relative to the control at the respective passage). In later passages, the phosphorylation of STAT3 exhibited no significant difference between the FGF2 treatment and the control groups (Figure 5C,D).

3.6 | STAT3 inhibition suppressed FGF2-induced cell growth in early passages

To investigate the association between ASC proliferation and STAT3 phosphorylation, we used a STAT3 inhibitor (Stattic) to inhibit the phosphorylation of STAT3. Western blot analysis showed that FGF2-induced p-STAT3 expression was reduced to a level similar to the control after the addition of Stattic (0.5 μM Stattic: 1.37 ± 0.10-fold; 1 μM Stattic: 1.29 ± 0.14-fold relative to control ASCs, both $P < .001$ compared with FGF2-treated ASCs; Figure 6A,B). Inhibition of p-STAT3 by Stattic in FGF2-treated ASCs also resulted in fewer cells with flattened cell morphology (Figure 6C). Alamar blue assay further confirmed that Stattic completely reversed the stimulatory effect of FGF2 on increasing cell growth (Figure 6D).

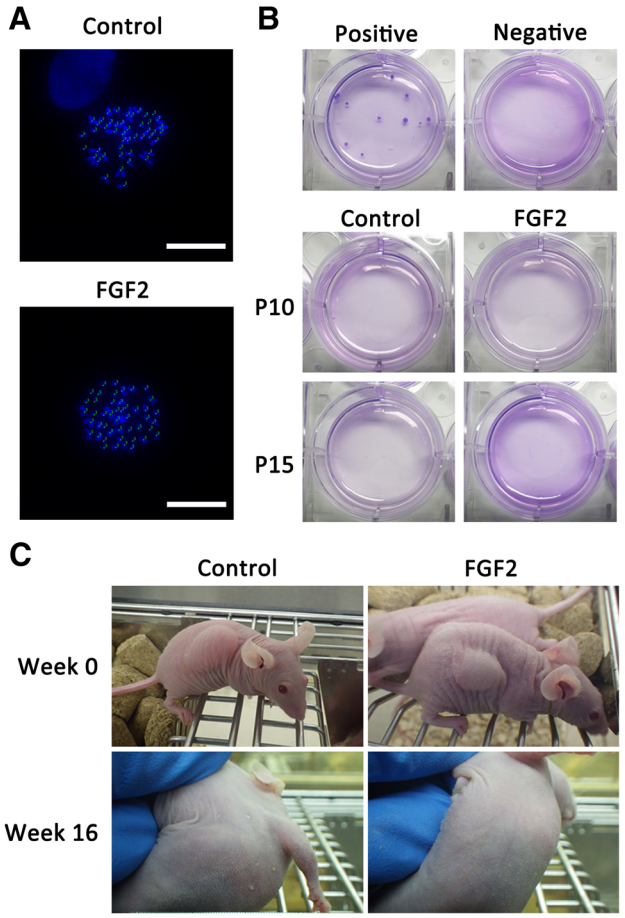

FIGURE 7 Tumorigenesis assay of adipose-derived mesenchymal stem cells (ASCs) with or without FGF2 treatment after long-term culture. A, Chromosome spread assay of ASCs with or without FGF2 treatment at P12 exhibit no chromosome anomalies in the mitotic phase. Chromosomes were detected by 4′,6-diamidino-2-phenylindole (DAPI) staining (n = 10). Scale bar = 25 μm. B, Soft agar assay of ASCs with or without FGF2 treatment at P10 and P15. ASCs were encapsulated in agarose gel for 21 days. Crystal violet was used to detect the colonies. There was no colony formation in either FGF2-treated or control ASCs, whereas the positive control HeLa cells showed obvious colony formation (n = 6). C, ASCs with or without FGF2 treatment were cultured until P15. An in vivo tumorigenesis assay was performed by subcutaneous injection of 1×10^7 ASCs into the dorsum of nude mice. No tumor formation was observed after 20 weeks (n = 5). FGF2, fibroblast growth factor 2; P, passage

3.7 | FGF2-treated ASCs exhibited minimal risk of tumorigenesis in vitro and in vivo

In the chromosome spread assay, P12 ASCs treated with FGF2 appeared to exhibit no chromosome anomalies in the mitotic phase (Figure 7A). We also assessed anchorage-independent growth with the soft agar colony formation assay and observed no colony formation in either FGF2-treated or control ASCs, whereas the positive control HeLa cells showed obvious colony formation (Figure 7B). After long-term in vitro culture, we further evaluated the tumorigenicity of FGF2-treated and control ASCs in vivo. A large quantity of FGF2-treated or control P15 ASCs were injected subcutaneously on the backs of nude mice, and no tumor formation was noted after 16 weeks (Figure 7C). Frozen tissue sections of dorsal skin, heart, lung, and liver also showed no immunolabelled ASCs, suggesting no ASC retention in the subcutaneous tissue or internal organs.

4 | DISCUSSION

In vitro expansion of ASCs is required to obtain a sufficient quantity of cells for certain therapeutic purposes, and supplementing growth factors in the culture medium is usually necessary to expedite the expansion process. Moreover, depletion of growth factors during serial passage of ASCs has been shown to induce autophagy and senescence while downregulating stemness genes.[27] Since the FGF2 pathway plays an important role in ASC self-renewal via both autocrine and paracrine effects, supplementing FGF2 for ASC expansion has become a common practice.[11] Our study showed that FGF2 enhanced ASC proliferation in the early passages, but the proliferative activity decreased to a level lower than that of the control group after extensive passaging. This observation is consistent with the cell cycle analysis, which revealed that FGF2 treatment significantly increased G2/M cells in P5 ASCs. ASCs in long-term culture were shown to exhibit increased proliferation indicated by the increased percentage of cells in S and G2/M phases, which decreased in later passages.[20] Our study further revealed a relatively higher G2/M ratio in FGF2-treated ACSs at P5 and P10 relative to the control ASCs, and the proportion of G2/M phase ASCs in the FGF2-treated group became significantly lower than that in the control group at P17. Overall, the data collectively indicate that FGF2-treated ASCs reached senescence earlier than non-treated cells during long-term cell culture.

The first evidence of cell senescence is morphological changes. During in vitro expansion, ASC morphology changed from a spindle shape to a more flattened form and was accompanied by a decrease in cell proliferation ability and a loss of differentiation potential. Consistent with a previous study,[28] we showed that FGF2-treated ASCs maintained a slender morphology with a smaller cell size in the early passages. Subpopulations of small and rapidly self-renewing MSCs have been shown to exhibit higher proliferative activity than their larger and more spread-out counterparts.[29,30] One common cause of premature senescence is the accumulation of DNA damage during long-term in vitro culture and the subsequent checkpoint activation.[31] FGF2 has been shown to suppress the cellular senescence of human MSCs through the suppression of p21, p53, and p16 mRNA expression.[32] Consistently, our findings showed that FGF2 maintained a smaller cytoplasmic volume of ASCs with a shorter doubling time in early passages. However, after long-term culture with FGF2, the ASCs became liable to senescence with a trend towards a larger cell size, increased SA-β-Gal staining, and enhanced p21 expression. We noted that at different passages during in vitro culture, FGF2-treated ASCs appeared to express a higher level of the senescence marker p21. This

finding may be associated with the results of a study showing that adult stem cells have evolved a unique p21-activation response to DNA damage that leads to their immediate expansion and limits their long-term survival.[33]

In the colony-forming assay, the superior colony-forming capability of FGF2-treated ASCs at P5 was reversed at P10. Because cells were seeded at low density in the colony-forming assay to determine the inherent self-renewal and proliferation efficacy of cells, the stem cells in this assay had the characteristics of isolated cells. In contrast, an optimal cell density was used in the proliferation assay, and intercellular communication via paracrine signaling and direct contact could influence cell growth and related behaviors.[34] Hence, it is possible that the FGF2-treated ASCs had been losing their stemness before reaching P10, as suggested by the result of our data, whereas enhanced proliferation was still observed for FGF2-treated ASCs compared with the control at P10. In contrast, for stem cells isolated from human exfoliated deciduous teeth, FGF2 supplement was shown to enhance the colony-forming capability, but it did not enhance cell proliferation.[34] Therefore, MSCs from different sources may respond differently to FGF2 stimulation.

Although FGF2 is widely used to maintain stem cell properties in culture, the mechanism of FGF-regulated self-renewal is not entirely clear. Dombrowski et al demonstrated that blocking FGFR1 signaling inhibited the proliferation and G1-S phase transition of the cell cycle in human bone marrow-derived MSCs.[35] FGFR1 promotes stem cell proliferation through multiple mechanisms that together antagonize cyclin-dependent kinase inhibitors. Our data also showed a higher level of FGFR1 mRNA expression, particularly *FGFR1c*, in FGF2-treated ASCs throughout different passages. At P5, the FGF2-treated ASCs exhibited a significant upregulation of *FGFR1c*. However, the gene expression of *FGFR1c* diminished to a level comparable to that of the control at P10 and P15, in concordance with the decreased proliferative activity in later passages. Moreover, the use of the FGFR1 inhibitor SU5402 suppressed the effect of FGF2 on stimulating ASC proliferation. Therefore, FGFR1 may be a target of FGF2 in modulating the proliferation activity of ASCs.

The FGF-FGFR signaling pathway is regulated at multiple levels, and the way in which different downstream pathways are activated to mediate the cellular response to FGF2 treatment has not been well established.[36] FGF2 likely acts as a mitogen via ERK activation, a multipotency factor through Sox2 induction, and an inhibitor of cellular senescence through the PI3K-AKT pathway.[6] The involvement of JAK/STAT signaling has been implicated in coordinating the processes of intestinal stem cell proliferation, ensuring a robust cellular output in the lineage.[37] STAT3 plays a role in the self-renewal of human embryonic stem cells[38] as a downstream transcription factor of the cytokine leukemia inhibitory factor.[39,40] Activated STAT3 has been reported to promote self-renewal of rodent hematopoietic stem cells in vivo,[41] and leptin seemed to increase ASC proliferation through activating STAT3 protein and downstream gene expression.[42] In line with our study, Dong et al. also revealed that FGF2 regulates melanocytes viability through the STAT3-transactivated PAX3 transcription.[43]

Our data revealed transient but robust ERK phosphorylation within minutes of FGF2 supplement of the ASC culture, followed by the emergence of p-STAT3, which was maintained at least until day 7. Zaragosi et al also reported transient activation of ERK pathway in ASCs within 24 hours upon FGF2 stimulation.[11] These results corresponded to a previous study showing that FGF-2 or FGF-4 promoted the proliferation of bone marrow MSCs with upregulated pERK1/2 and pAKT within minutes, but without a significant change in STAT3 or p-STAT3 level.[44] In this study, we also observed enhanced STAT3 activation by FGF2 treatment throughout the earlier passages, which diminished in the later passages. Moreover, the blockage of STAT3 resulted in the reduced cell growth of ASCs. Therefore, JAK/STAT signaling may mediate the effect of FGF2 on enhancing the proliferation and self-renewal of ASCs in early passages, whereas further FGF2 supplement induces senescence and suppresses proliferation due to refractory STAT3 expression.

A common concern regarding the use of stem cell therapies is the risk of tumorigenicity.[45-47] Previous studies have shown that in vitro-expanded ASCs exhibited a low level of telomerase activity after passage 10,[20] without evidence of alternative mechanisms for telomere lengthening.[48] Hence, ASCs were considered to exhibit a low risk of tumorigenicity up to P20.[10] However, a recent study found that the FGF2-FGFR1 pathway is critical for the adipose tissue-stimulated transformation of skin and mammary epithelial cells.[49] Despite the establishment of many safeguard mechanisms to prevent tumorigenesis in human cells, this finding raised the concern of possible transformation when extensively culturing ASCs with FGF2. Our in vitro and in vivo studies revealed a minimal tumorigenicity risk from FGF2-treated ASCs after long-term culture. These findings confirm that FGF2-treated ASCs are unlikely to develop one of the hallmarks of malignant transformation and can be considered amenable for cell therapy purposes.

FGF2 supplement in prolonged culture of ASC possibly alters not only the proliferative, self-renewal ability, but also the genetic and epigenetic changes related to apoptosis, DNA damage repair, or tumorigenicity. In a study conducting long-term ASC culture, elevated expression of tumor suppression gene *p53* with decreased *CDK1* (required for entry into M phase) and *Bcl-2* (anti-apoptotic gene) expression in later passages was noted, indicating that ASCs might undergo apoptosis to inhibit malignant transformation.[20] According to Gharibi et al, FGF2 supplement upregulated stemness gene *Oct4* only at initial passages of ASC culture, and increased expression of senescence gene *p16* and DNA repair gene *Pold3* was noted at later passages.[13] Therefore, FGF2 supplement in long-term ASC culture may increase cell senescence, apoptosis, and DNA damage repair to prevent tumorigenicity. The underlying mechanism awaits further investigation.

5 | CONCLUSIONS

In the present study, we demonstrated that FGF2 exerts positive effects in the early passages of ASC culture, resulting in the maintenance of cellular morphology and the expedition of proliferative kinetics. However,

after prolonged in vitro expansion of ASCs with FGF2, negative effects of increased cell size, arrested proliferation, and increased senescence are noted. This phenomenon may be mediated through *FGFR1c* and the downstream STAT3 signaling pathway. Despite the low risk of tumorigenicity of FGF2-treated ASCs, the continuous supplement of FGF2 during long-term ASC culture may adversely affect the cells in later passages. In conclusion, FGF2 supplement for ASC expansion is desirable for limited passages to expeditiously obtain ASCs with efficacy for therapeutic purposes.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of the staff at the Eighth Core Lab of National Taiwan University Hospital. This research was supported by the Ministry of Science and Technology, Taiwan (MOST 107-2314-B-002-176, 108-2628-B-002-020), and National Taiwan University Hospital (107-S3769, 108-S4129).

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Y.C.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; K.H.L.: collection and assembly of data, data analysis; T.H.Y.: provision of study material and administrative support; N.C.C.: conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

DATA AVAILABILITY STATEMENT

The data sets used or analyzed during the current study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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How to cite this article: Cheng Y, Lin K-H, Young T-H, Cheng N-C. The influence of fibroblast growth factor 2 on the senescence of human adipose-derived mesenchymal stem cells during long-term culture. *STEM CELLS Transl Med*. 2020;9:518–530. https://doi.org/10.1002/sctm.19-0234