

LncRNA STK4 antisense RNA 1(STK4-AS1) promoted osteosarcoma by inhibiting p53 expression

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Research Article

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Abstract

Background: LncRNA STK4 antisense RNA 1 (STK4-AS1) has been identified as a potential biomarker associated with multiple cancers. We proposed that STK4-AS1 plays a role in the proliferation of osteosarcoma by regulating the cell cycle.

Methods: We compared the expression of STK4-AS1, p53, and p21 in osteosarcoma vs normal samples in clinical tissues and cell lines. We determined the effect of overexpression and knockdown of STK4-AS1 in p53 expressing osteosarcoma cells U2OS, p53 muted osteosarcoma cells MG63, and osteoblast cells hFOB on p53 and p21 expression and the cell viability. For U2OS and MG63, the cell cycle was analyzed and the expression of cyclin proteins was determined. We overexpressed p53 or p21 in STK4-AS1 overexpressed cells to explore the association of STK4-AS1 and p53 in U2OS.

Results: The STK4-AS1 expression was higher and p53 and p21 expression were lower in osteosarcoma tissue and cells than in their non-cancer counterparts. The expression of STK4-AS1 was negatively correlated with the expression of p53 or p21. Knockdown of STK4-AS1 in U2OS decreased the cell viability, increased cells in the G0/G1 phase, decreased cells in the S and G2/M phase, decreased expression of cyclin A and B, increased p53 and p21, and had no effect on cyclin D and cyclin E, while overexpression of STK4-AS1 did the opposites. Overexpression of p53 or p21 recovered some changes caused by STK4-AS1 overexpression in U2OS. MG63 expressed no p53 and the expression of p21, cyclin A, and cyclin B, cell viability, and cell cycle were not affected by altered STK4-AS1 levels. In hFOB cells, the expression of p53 and p21 was decreased and the cell viability was increased when STK4-AS1 was overexpressed, but they were not affected when STK4-AS1 was knocked down.

Conclusion: LncRNA STK4-AS1 promoted the cell cycle of osteosarcoma cells by inhibiting p53 expression.

1. Introduction

Osteosarcoma is the most common primary bone malignancy [1]. The incidence of osteosarcoma is 5.4 cases per year per million in males and 4 cases per year per million in females [1]. Although the mortality for osteosarcoma has steadily declined by approximately 1.3% per year, the 5-year overall survival rate is still lower than 70% [1]. Studies have revealed multiple regulation pathways of bone metabolism [2–6], and many effective therapeutic approaches to bone disease have been developed [7, 8]. Although many preclinical studies have contributed to clinical cancer treatment [9–12], to date, the understanding of the pathology of osteosarcoma is insufficient [13]. Hence, the study of osteosarcoma is necessary.

Long non-coding RNAs (lncRNAs) are RNAs whose lengths are over 200 bp but are not translated into protein [14]. lncRNAs function as transcriptional regulators for the expression of many genes [15]. Recently, more and more ncRNAs were found to be involved in bone cell regulations [16–19]. Many lncRNAs were identified to be associated with human diseases including cancers [20–22]. Studies revealed that the lncRNAs expressed in bone play a critical role in regulating bone formation [23] and

differentiation [24–26]. Osteosarcoma derives from primitive bone-forming mesenchymal cells [1] and it is also suggested to be regulated by lncRNAs [27]. Recently popular cancer bioinformatics studies [28] showed that an increasing number of lncRNAs were identified as the prognostic biomarkers or potential therapeutic targets of cancer by bioinformatics study [29, 30] and the role of many of them in osteosarcoma has been validated in vivo or in vitro [31–34]. As the number of lncRNAs is large and the regulation network of lncRNAs is complex, more works are required in this field.

A lncRNA, STK4-AS1, has previously been identified as a cancer biomarker associated with the progression of breast cancer[35], lung cancer [30], and colon cancer [29]. STK4-AS1 orientates from the minus strand and is located at 20q13.12 in the human genome with a size of 2,665 bases [36]. It is one of the antisense RNAs of the STK4 gene. STK4 protein was reported to regulate many types of cancers, including liver cancer [37], prostate cancer [38], and thyroid carcinoma [39]. However, the effect of its antisense RNA was still unclear. Some lncRNAs can regulate cell growth by regulating the cell cycle [40]. We proposed that STK4-AS1 plays a role in the proliferation of osteosarcoma by regulating its cell cycle. In this study, we investigated the expression of lncRNA STK4-AS1 in osteosarcoma, both in clinical samples and cell models, and study its effect on the osteosarcoma cell cycle. Our study aims to deepen the understanding of the role of STK4-AS1 in osteosarcoma and develop it as a potential novel target for the treatment of osteosarcoma.

2. Materials And Methods

2.1. Clinical tissue samples

Clinical osteosarcoma and adjacent normal bone tissues were collected from osteosarcoma patients who underwent surgery from 2016 to 2019 in the Affiliated Tumor Hospital of Zhengzhou University (Hunan, China). This work was approved by the Ethical Committee of Zhengzhou University and all of the patients consented to participate in this study. Samples were immediately snap-frozen in liquid nitrogen for further study.

2.2. Cell lines and cell culture

Human osteosarcoma cell lines MG-63 (ATCC® CRL-1427™), Saos-2 (ATCC® HTB-85™), U-2 OS (ATCC® HTB-96™), and human osteoblast cell line hFOB 1.19 (ATCC® CRL-11372™) was obtained from ATCC (Washington, USA). Human primary osteoblast cell line HOB was obtained from PromoCell (Heidelberg, Germany). Cell lines were cultured in Osteoblast Growth Medium C-27001 (Heidelberg, Germany) in a humidified atmosphere of 5% CO₂ at 37°C.

2.3. MTT assay

The MTT assay was first reported by Mossmann in 1983 [41]. It is commonly used for both cancer cell and non-cancer cell viability determination [42, 43]. Briefly, cells were grown in 96 well plates and at the endpoint of the culture time, the culture medium was removed and 0.8 mg/ml of MTT working solution (dissolved in serum-free medium) was added to the wells. After 4 hours, DMSO was added to the wells,

and absorbance was recorded at 560 nm using the microplate spectrophotometer system (Spectra max190-Molecular Devices).

2.4. EdU assay

The EdU assay was conducted using EdU Staining Proliferation Kit (iFluor 488) (ab219801) with BD FACSCalibur was used to acquire cell cycle data. FlowJo Version 10 was used to analyze the data. Briefly, EdU solution was added to cells and the cells were incubated for 4 hrs. Then cells were fixed and permeabilized. The cells were analyzed by flow cytometry.

2.5. RNA extraction and QPCR

The expression of STK4-AS1 was determined by QPCR assay which was described previously [44]. Total RNA from tissue or cell samples was extracted by using Trizol reagent (Invitrogen, Calsbad, CA, USA). Then the total RNA was reversely transcribed into cDNA using a reverse transcription kit (Thermo, USA). Quantitative real time-PCR was done with PowerUp™ SYBR™ Green Master Mix (Thermo, USA) using the ViiA 7 Real-Time PCR System (Applied Biosystems). The protocol for PCR: 95°C for 3 min, 40 cycles of 95°C for 30 s, 58°C for 15 s, and 72°C for 30 s. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method. The primers used for RT-PCR were designed by Eurofine Genomic primer design online tools and synthesized by Sigma-Aldrich, Inc. (St. Louis, MO, USA). The sequences are as follows: GAPDH forward: 5'-AATGGGCAGCCGTTAGGAAA-3' and reverse: 5'-TGAAGGGGTCATTGATGGCA-3'. STK4-AS1 forward: 5'-CGGAGCGCACAAAATACTCG-3' and reverse: 5'-CCACAAATACCTCCAGGCGT-3'.

2.6. Cell transfection

The STK4-AS1 knockdown and overexpression cells and p53 and p21 rescue cells were conducted by transfection of siRNA or expression vector. The cell lines were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The expression levels of STK4-AS1 were validated by qPCR. The expression of p53 and p21 was validated by western blotting. The cell transfection method was described previously [45].

The experimental details are as follows: (1) STK4-AS1 knockdown: STK4-AS1 small interfering (si)RNA and negative control siRNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sequences were as follows: STK4-AS1 siRNA, 5'-ACCTCGGCCAGAGAGGTGAAGTATCTTCAAGAGAGATACTTCACCTCTCTGGCCTT-3'. A scramble siRNA (siN05815122147) was used as the NC siRNA. The transfection concentration of siRNA was 60 nM. (2) STK4-AS1 overexpression: The entire sequence of human STK4-AS1 was amplified from hFOB cell lines using PCR and cloned into the pcDNA3.1 vector. The negative control empty vector, which was purchased from Sigma-Aldrich (St. Louis, MO, USA). The transfection concentration of the plasmid was 2 µg/ml. (3) P53 or p21 rescue: p53 or p21 expressing vectors, TR200pa-ppGF-p53(or p21)-mCMV-EF1α-Puro (plasmid), and negative vectors were purchased from System Biosciences (Palo Alto, CA, USA). The transfection concentration of the plasmid was 2 µg/ml.

The control of cell transfection was set as follows: Ctrl1: cells without treatment; Ctrl2: cells with NC siRNA transfection; Ctrl3: cells with empty pcDNA3.1 vector transfection.

2.7. Cell cycle analysis

The cell cycle was analyzed using flow cytometry with propidium iodide (PI) staining, which was described previously [46]. Briefly, cells were washed with PBS and resuspended at a concentration of 1×10^6 /mL. Cells were fixed with 100% ethanol for three hours at 4°C. Then suspended cells were washed with PBS two times and incubated with propidium iodide staining solution (0.1% Triton X-100, 0.2 mg/mL DNase-free RNase A, 0.02 mg/mL in cold PBS) at 37°C for 15 min. BD FACSCalibur was used to acquire cell cycle data. FlowJo Version 10 was used to analyze the data.

2.8. Western blotting

The western blotting method was described previously [47]. Briefly, proteins were extracted from cells using RIPA buffer with protease inhibitor (Sigma-Aldrich, USA). Total protein concentrations were determined using a BCA protein assay kit to control the loading amount (25 µg). SDS gel electrophoresis was performed to separate proteins. Afterward, the proteins were transferred to 0.2-µm polyvinylidene difluoride membranes which were subsequently blocked with 5% skim milk in Tris-buffered saline with 0.5% Tween-20 (TBST). Membranes were then reacted with primary (1:1000 dilution of 5% skim milk in TBST) and secondary antibodies (1:3000 dilution of 5% skim milk in TBST) subsequently. The band intensities were photographed using a super-sensitive gel imaging system after reacted with ECL reagents (Bio-Rad, Hercules, CA, USA).

The primary antibodies used in the experiment are as follow: Anti-cyclin A antibody (sc-271682), Anti-Cyclin B antibody (ab72), Anti-p53 antibody (ab26), Anti-p21 antibody (ab109520), Cyclin D1 Antibody (#2922), and Anti-Cyclin E1 antibody (ab33911). The secondary antibodies are all purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.9. Interaction analysis of STK4-AS1 and TP53.

All the analyses were conducted using LncRRlsearch [48] (<http://rtools.cbrc.jp/LncRRlsearch/index.cgi>).

2.10. Statistics

T-test or one-way ANOVA and Dunnett's post hoc tests were used to compare the significant difference between the control and experimental group ($p < 0.01$). GraphPad Prism (version 8) was used to plot the figures and calculate statistical significance.

3. Results

3.1. STK4-AS1 was overexpressed in osteosarcoma tissues

This study started with clinical osteosarcoma samples. We collected clinical osteosarcoma and adjacent normal tissues from patients who underwent surgery and compared the STK4-AS1, p53, and p21

expression in these samples. Results showed that STK4-AS1 expression in osteosarcoma samples was significantly higher than that in the adjacent normal tissues (Fig. 1A), suggesting that STK4-AS1 might be a cancer biomarker associated with the development of osteosarcoma. Results also showed that p53 and p21 expression was lower in osteosarcoma compared with the adjacent normal tissues both in mRNA and protein levels (Fig. 1B-F).

3.2. STK4-AS1 was associated with p53/p21 regulation

Based on these results, we suspected that STK4-AS1 might be associated with p53/p21 regulation, therefore, a correlation analysis between STK4-AS1 and p53 or p21 expression was conducted with these expressions data. Interestingly, both analyses revealed significant negative slopes with a p-value less than 0.05 (Fig. 1 GH), which strongly suggested an association between STK4-AS1 and p53/p21 regulation. We hypothesized that STK4-AS1 can inhibit the expression of p53 and p21.

3.3. STK4-AS1 was overexpressed in osteosarcoma cells

To further test this hypothesis, we also compared the expression of STK4-AS1 in osteosarcoma cells vs normal osteoblast cells. Results showed that the expression of STK4-AS1 in osteosarcoma cells U2OS, MG63, and SAOS-2 was much higher than in normal osteoblast cells hFOB and HOB (Fig. 2A). Although all three osteosarcoma cell lines tested expressed STK4-AS1, only U2OS expressed p53 while MG63 and SAOS-2 expressed almost no detectable level of p53 (Fig. 2B). According to previous studies, MG63 and SAOS-2 cells have inactivated p53 while U2OS have wild-type p53 [49, 50]. In addition, results showed that normal osteoblast cells hFOB and HOB expressed higher p53 and p21 compared to osteosarcoma cells (Fig. 2B-D).

3.4. STK4-AS1 regulated p21 through p53.

In order to get further insight into the effects of STK4-AS1 on osteosarcoma, we selected three typical cells with different p53 expression for the subsequent study, including p53 expressing osteosarcoma cells U2OS, p53 muted osteosarcoma cells MG63, and osteoblast cells hFOB. Cell transfection was used to knock down or overexpress STK4-AS1 in these three cell lines, which were validated by QPCR assay (Fig. 2E-G1). In p53 expressing U2Os, p53 was increased when STK4-AS1 was knocked down, while p53 was reduced when STK4-AS1 was overexpressed (Fig. 2F2-3). But in hFOB cells, p53 was not changed when STK4-AS1 was knocked down, while p53 was reduced when STK4-AS1 was overexpressed (Fig. 2G2-3). We suggested that in hFOB, STK4-AS1 was already low that unable to inhibit p53 expression, thus the knockdown did not eliminate the inhibition of p53, while the overexpression can suppress p53 expression. In U2O2 and hFOB cells, the alteration of p21 expression was similar to the changing trend of p51 (Fig. 2E2-3). This suggested that the STK4-AS1 inhibited p21 via reducing p53 signals. In addition, in p53 defected MG63 cells, the change of STK4-AS1 had no effect on p21 (Fig. 2G2-3), which also supported that p53 is critical for the regulation of STK4-AS1 toward p21 (Fig. 2E-G 2-3).

3.5 STK4-AS1 regulated cell viability through p53

As p53 and p21 are key regulators for cell viability, we determined the cell viability using MTT assay with our cell models. Results showed that knockdown of STK4-AS1 in osteosarcoma cells U2OS decreased cell proliferation while overexpression of STK4-AS1 increased cell proliferation (Fig. 2E4). Similar results were shown in the MTT assay of hFOB (Fig. 2G4). However, artificially altered levels of STK4-AS1 failed to affect the cell viability of p53 muted cell line MG63 (Fig. 2F4). This indicated that p53 might mediate the effects of STK4-AS1 on the viability of osteosarcoma and osteoblast cells. To confirm the effect of STK4-AS1 on cell viability and proliferation, we conducted the EdU assay. Results showed similar results as the MTT assay (Fig. 3). Therefore, we suggested that STK4-AS1 could regulate cell proliferation.

3.5. Effect of STK4-AS1 on the cell cycle

To further explore the exact effect of STK4-AS1 on cell growth, we measured its effect on the U2OS cell cycle. Results showed that, compared with the control, the knockdown of STK4-AS1 increased cells in G0/G1 phase and caused a significant decrease in the S phase and G2/M phase cells. On the other hand, the overexpression of STK4-AS1 decreased cells in G0/G1 phase but caused an increase in S phase cells and G2/M phase cells (Fig. 4 AB). This indicated that the expression of STK4-AS1 might facilitate cell division. Hence, we suggested that STK4-AS1 promotes osteosarcoma by regulating the cell cycle.

3.6. Effect of STK4-AS1 on the expression of cyclin proteins

This suggestion was confirmed by the determination of key regulators of the cell cycle, cyclin proteins. Western blotting experiments revealed that the knockdown of STK4-AS1 decreased the expression of cyclin A and cyclin B while the overexpression of STK4-AS1 did the opposite (Fig. 4 CD). Cyclin A is a critical factor to initiate and complete DNA replication [51, 52] and Cyclin B regulates the G2 checkpoint [53]. The alteration of these two critical factors further confirmed that STK4-AS1 promotes osteosarcoma by regulating the cell cycle. These results could account for the changes in cell numbers in the S and G2 phases. However, although cell in G1 phase was affected, G1 regulators cyclin D and cyclin E [54] were not affected (Fig. 4 CD).

3.7. P53 mediated the regulation of STK4-AS1 on cell cycle

Cancer suppressor p53 has long been reported to function as a cell cycle controlling protein in osteosarcomas [55]. It has been proved to regulate the G2 phase by affecting cyclin B [56]. Here we tested it as a potential target underlying the effect of STK4-AS1 on osteosarcomas cells. Results showed that the knockdown of STK4-AS1 decreased the expressions of both p53 and p21 while the overexpression increased the expression of p53 and p21 (Fig. 5AB). Previously, the p53/p21 pathway was reported to regulate G2/M cell cycle genes [57]. Our results provided evidence that p53 and p21 are potential downstream targets of STK4-AS1.

3.8. Rescue experiments of p53 and p21 proteins in U2OS

A rescue experiment of p53 and p21 in STK4-AS1 overexpressed U2OS cells was conducted to demonstrate the involvement of p53 and p21 in the effect of STK4-AS1 on U2OS cells. The western blotting validated that most of the p53 or p21 expression was successfully rescued in STK4-AS1

overexpressed U2OS cells. Western blotting results showed that the rescue of p53 also upregulated the expressions of p21, cyclin A, and cyclin B, while the rescue of p21 did not increase p53 level, indicating that p21 was the downstream target of the STK4-AS1/p53 pathway. (Fig. 5CD). The recovery of p53 or p21 expressions also decreased the viability of STK4-AS1 overexpressed U2OS cells (Fig. 5E), indicating that p53 and p21 might mediate STK4-AS1 cell viability regulations. However, as p53 and p21 have many regulatory effects on cell viability, these results were not enough to confirm that p53/p21 was involved in U2OS cell viability regulations.

3.9. STK4-AS1 did not affect p53 muted MG63 cells

To confirm that p53 is essential for STK4-AS1 cell cycle regulations, we repeated the cell cycle analysis in MG63 cells. As MG63 cells naturally mute the p53 expression (As we confirmed in western blotting, Fig. 2F2-3), it can be a proper negative control for our hypothesis. Results showed that both knockdown and overexpression of STK4-AS1 failed to make any difference in the cell cycle of MG63 cells (Fig. 6 AB) and the expression of cyclin A and cyclin B (Fig. 6 CD). These results supported that p53 is essential for STK4-AS1 cell cycle regulations.

3.10. STK4-AS1 regulated cell cycle in osteoblast cells hFOB

We have previously shown that STK4-AS1 overexpression in osteoblast cells hFOB brings down p53 and p21 levels while promoting proliferation. To explore whether p53/p21 mechanism was also presented in the cell cycle of hFOB cells, we repeated the cell cycle analysis in hFOB cells. Results showed that the knockdown of STK4-AS1 failed to alter the cell cycle and cell cyclinA and cyclinB. However, the overexpression of STK4-AS1 resulted in a decrease in G0/G1 and an increase in S/G2/M. In addition, the overexpression of STK4-AS1 also promoted the expression of cyclin A and cyclinB (Fig. 7). These results supported that upregulation of STK4-AS1 in normal osteoblasts would mimic malignant phenotype.

3.11. Rescue experiments of p53 and p21 proteins in hFOB

To further confirm that the p53/p21 mechanism was involved in the cell proliferation, a rescue experiment of p53 and p21 in STK4-AS1 overexpressed hFOB cells was conducted. The western blotting validated that p53 and p21 expression was successfully rescued in STK4-AS1 overexpressed hFOB cells (Fig. 8AB). Western blotting results showed that the rescue of p53 also upregulated the expressions of p21, cyclin A, and cyclin B, while the rescue of p21 did not increase p53 level, which is consistent with the results in U2OS (Fig. 8CD). The rescue of p53 or p21 or p53/p21 co-rescue also decreased the viability of STK4-AS1 overexpressed hFOB cells (Fig. 8E), indicating that p53 and p21 might also mediate STK4-AS1 cell viability regulations in hFOB cells.

3.12. Interaction of STK4-AS1 and TP53.

To explore whether STK4-AS1 directly interacted with TP53, we analyzed the subcellular localization (subcytoplasm and subnucleus compartments) of STK4-AS1 and TP53 and predicted the potential base-pairing between STK4-AS1 and TP53 mRNA. Results showed that STK4-AS1 was mostly presented at membrane and cytosol sub, and it was also found at insoluble, nucleolus, nucleoplasm, and nucleus sub. Results also suggested that STK4-AS1 might have sufficient assessable of TP53 mRNA at these subcellular localizations. The base-pairing interaction analysis revealed two interactions between STK4-AS1 and TP53 mRNA. The total local base-pairing interaction energy between STK4-AS1 and TP53 mRNA was -49.17 kcal/mol (Fig. 9). Therefore, we believed STK4-AS1 can directedly interact with the TP53 mRNA, thereby regulating TP53 protein.

4. Discussion

This study revealed the role of one lncRNA STK4-AS1 in the proliferation of osteosarcoma. From clinical samples, we found an association between the expression of STK4-AS1 and osteosarcoma: the cancer bone tissue expressed a higher level of STK4-AS1 over noncancer bone tissues. Thus, STK4-AS1 can be developed as a potential clinical prognostic biomarker for osteosarcoma. We also found that the expression of STK4-AS1 in osteosarcoma is correlated with p53 and p21 expression in tissue samples. As p53 and p21 are critical regulators for cancers, this study revealed that STK4-AS1 can be a potential pharmacological target for osteosarcoma treatment. In addition, we suggested that STK4-AS1 might have different expression profiles in a certain fraction of cells within one sample. For example, cancer stem cells [58] might express a different level of STK4-AS1 from the other cancer cells.

Among the three osteosarcoma cell lines tested in this study, MG63 expressed most STK4-AS1. However, MG63 had no p53 expression, thus, it is a good negative control cell line for us to explore the role of p53 in STK4-AS1 regulation. We found that the actions of STK4-AS1 are different in U2OS and MG63. In U2OS, STK4-AS1 regulated the cell cycle while in MG63 STK4-AS1 showed not regulatory effects on cell viability or cell cycle. We suggested that the difference of p53 expression in U2OS and MG63 accounts for these differences. We also discovered that the viability of osteoblast cells fHOB only altered by overexpression of STK4-AS1 but not the knockdown. We suggested that this is because STK4-AS1 expression in fHOB is too low to regulate cell viability.

STK4-AS1 was found to be the “booster” in breast cancer [35], lung cancer[30], and colon cancer[29]. In this study, we demonstrated that it promoted the progression of osteosarcoma by inhibiting the p53/p21 pathway. MTT assay with transfected cells revealed that the expression of STK4-AS1 level was associated with cell viability. Many pharmacological targets were discovered because they can regulate cell viability [59, 60]. We analyzed the effect of STK4-AS1 on the cell cycle of the p53 expressing osteosarcoma cell line U2OS and found that STK4-AS1 affected cell viability by regulating the cell cycle. Although some studies showed that the arresting of the cell cycle at the S or G2 phase can also lead to suppression of proliferation [61, 62], based on our viability results, we suggested that the increased cells in S and G2 phase resulted in more proliferation of U2OS cells.

In western blotting experiments, we observed the effect of STK4-AS1 on cyclin A, a regulator of the S phase regulation [63], cyclin B, a regulator of the G2 phase [53], and cyclin D/cyclin E, regulators of the G1 phase [54]. Furthermore, we also determined p53/p21 pathway. P53 has long been reported to regulate the cell cycle in osteosarcomas [55]. Here we tested it as a potential target underlying the effect of STK4-AS1 on osteosarcomas cells. The p53/p21 pathway is responsible for the negative regulation of cyclin B and cdk1, regulating activities of the G2 phase in cancer cells[53]. The accumulation of p53 can increase the expression of its transcriptional target gene p21 which can potentially inactivate the cyclin B/cdk1 complex [64]. Our results showed that the knockdown or overexpression of STK4-AS1 affects the expression of p53, and the p53 subsequently affected p21 and negatively regulates activities in the G2 phase. P53 rescue experiment showed that the abnormal cell viability, p21 expression, cyclin A expression, and cyclin B expression were largely recovered. Besides, p21 can also potentially binds and inactivate the cyclin E/CDK2 complex. Interestingly, although cyclin D and cyclin E have been found regulated by the p53/p21 pathway [65, 66], in this study the cyclin D and cyclin E levels were not affected even with p53/p21 level altered. We suggested that STK4-AS1 might have other impacts on cyclin D and cyclin E besides the p53/p21 pathway that subset the effect of the p53/p21 pathway (Fig. 10). We supposed that this unidentified regulation might involve other cancer-related mechanisms such as ion channel regulations [67, 68]. On the other hand, in the p53 negative control, MG63 cells, STK-AS1 failed to affect cyclin A and cyclin B, which was consistent with our hypothesis.

As STK4 has known roles in regulating cancer progression, it might be helpful to distinguish the impact of STK4-AS1 from the potential effects of STK4 upregulation. Although STK4-AS1 is transcribed from the non-coding region of the STK4 gene, we determined the levels of STK4 protein in tumor tissues and cell lines, as well as in cell lines involving knockdown and overexpression of STK4-AS1. Results showed that there is no correlation between STK4-AS1 and STK4, and the expression of STK4 was not affected by the alteration of STK4-AS1 expression in the knockdown and overexpression experiments (SFig.1). In addition, we suggested that the study might greatly benefit from the data on patient prognosis. Thus, we analyzed the available survival data in this study (SFig.2). We were also interested in the effect of STK4-AS1 on therapy response. However, the case number in this study was low and patients were treated with different therapy, including traditional medicine that was complex in their components, hence, we did not have enough cases to conclude the association of STK4-AS1 levels and patient survival or therapy response.

This study demonstrated that lncRNA STK4-AS1 is overexpressed in osteosarcoma and plays a role in the proliferation of p53 expressing osteosarcoma cells by affecting the cell cycle, which is mediated by the p53/p21 pathway. Given the potential value of STK4-AS1 for clinical osteosarcoma therapy and diagnosis, our study is conducive to the development of STK4-AS1 as a novel treatment target and diagnostic biomarker for osteosarcoma. In future clinical settings, STK4-AS1 can be determined using PCR or tested as one of the screening items in microarray probe assays. Potential gene therapy to reduce STK4-AS1 is also promising.

Declarations

Ethics approval and consent to participate

This work was approved by the Ethical Committee of Affiliated Tumor Hospital of Zhengzhou University.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and the supplementary materials.

Competing interests

There is no conflict of interest in this paper.

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Authors' contributions

CONCEPTION: Chuchu Wang; INTERPRETATION OR ANALYSIS OF DATA: Weitao Yao and Jingyu Hou; PREPARATION OF THE MANUSCRIPT: Jingyu Hou, Guoqing Liu, Fangxing Wu, and Qiang Yan; REVISION FOR IMPORTANT INTELLECTUAL CONTENT: Liangyu Guo and Chuchu Wang; SUPERVISION: Liangyu Guo and Chuchu Wang.

Acknowledgments

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Abbreviations

STK4-AS1

LncRNA STK4 antisense RNA 1

lncRNAs

Long non-coding RNAs

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Figures

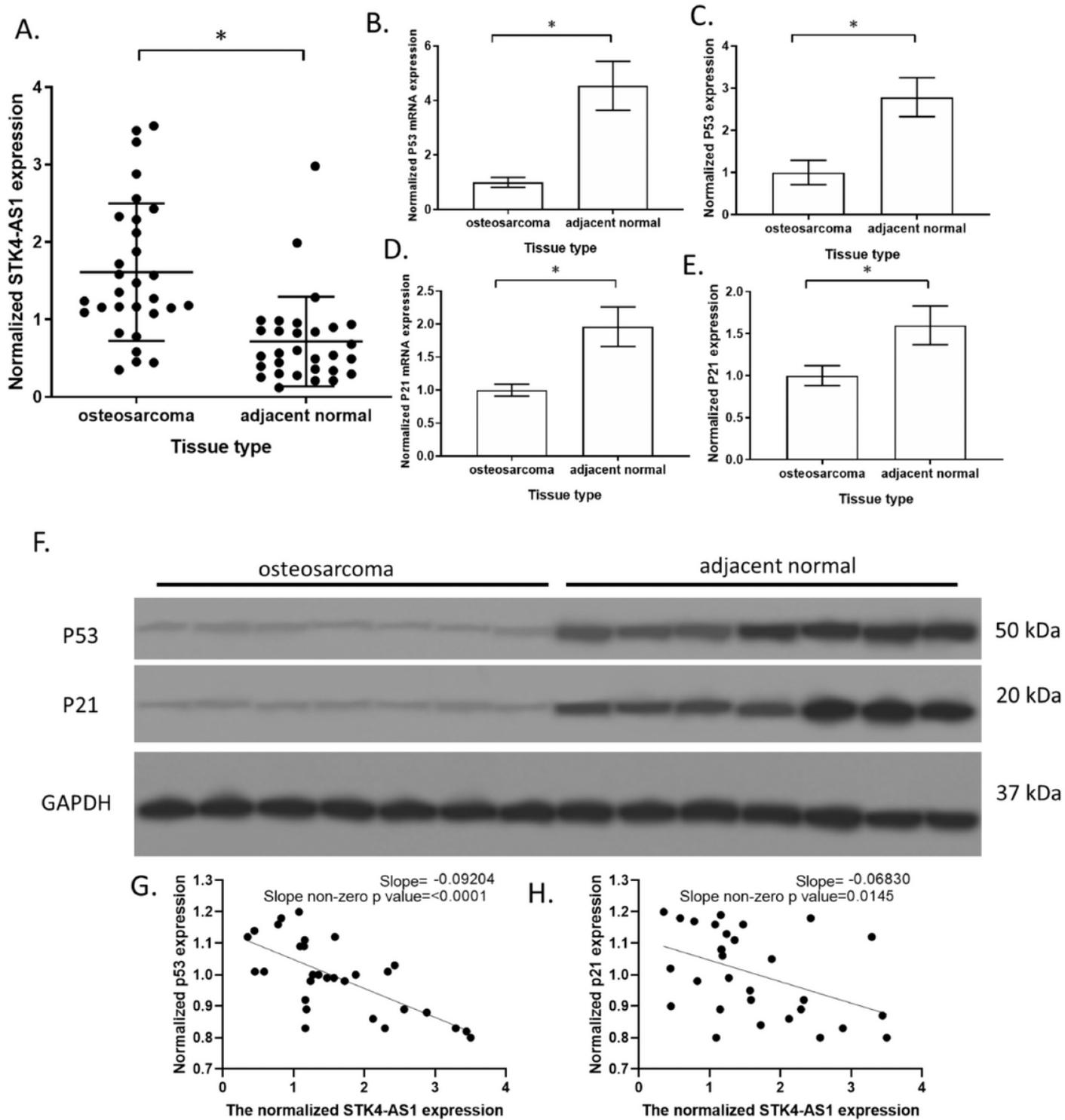


Figure 1

STK4-AS1, p53, and p21 expression in osteosarcoma and osteoblast. The STK4-AS1 and p53 and p21 mRNA expression were determined using QPCR assay. The p53 and p21 protein expressions were determined using western blotting. (A) STK4-AS1 expression in human osteosarcoma and adjacent normal tissues from 30 patients with osteosarcoma resection. (B) p53 mRNA expression in human osteosarcoma and adjacent normal tissues. (C) p53 protein expression in human osteosarcoma and adjacent normal tissues. (D) p21 mRNA expression in human osteosarcoma and adjacent normal

tissues. (E) p21 protein expression in human osteosarcoma and adjacent normal tissues. (F) Representative images of western blotting assay of tissues. (G) Correlation between STK4-AS1 and p53 expression in osteosarcoma tissues. (H) Correlation between STK4-AS1 and p21 expression in osteosarcoma tissues. (* $p < 0.01$.)

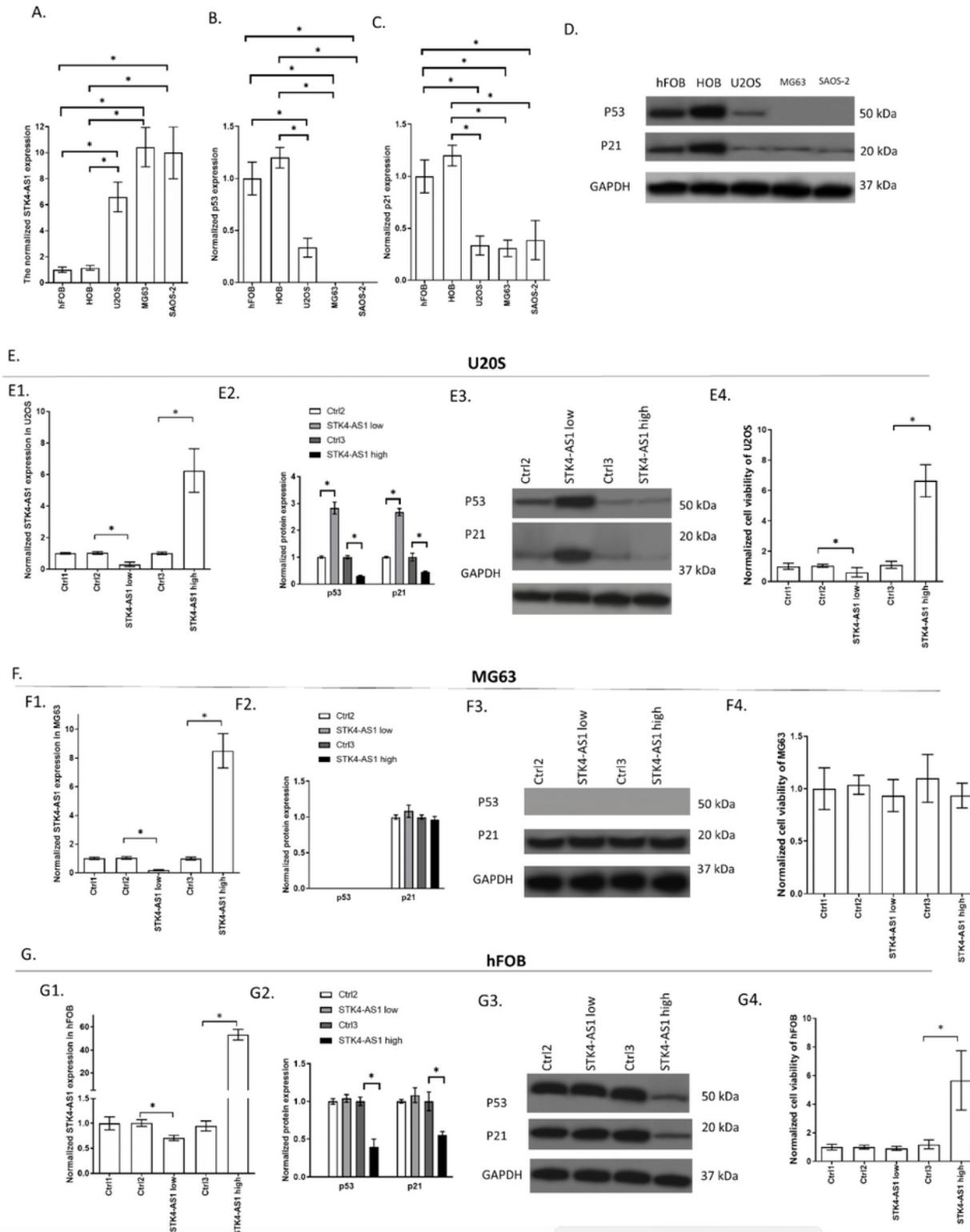


Figure 2

STK4-AS1 in osteosarcoma and osteoblast cells. (A) STK4-AS1 expression in human osteosarcoma and osteoblast cells. (B) p53 protein expression in human osteosarcoma and osteoblast cells. (C) p21 protein expression in human osteosarcoma and osteoblast cells. (D) Representative images of western blotting assay of human osteosarcoma and osteoblast cells. The STK4-AS1 and mRNA expression were determined using QPCR and the protein expression was determined using western blotting. The cell viability was determined using the MTT assay. (E) Knockdown or overexpression of STK4-AS1 in osteosarcoma cells U2OS. E1. STK4-AS1 expression in U2OS. E2. Protein expression of p53 and p21 in U2OS. E3. Representative image of western blotting of p53 and p21 in U2OS. E4. The cell viability of U2OS. (F) Knockdown or overexpression of STK4-AS1 in osteosarcoma cells MG63. F1. STK4-AS1 expression in MG63. F2. Protein expression of p53 and p21 in MG63. F3. Representative image of western blotting of p53 and p21 in MG63. F4. The cell viability of MG63. (G) Knockdown or overexpression of STK4-AS1 in osteoblast cells hFOB. G1. STK4-AS1 expression in hFOB. G2. Protein expression of p53 and p21 in hFOB. G3. Representative image of western blotting of p53 and p21 in hFOB. G4. The cell viability of hFOB. (* $p < 0.01$. Ctrl 1: cells without treatment; ctrl 2: cells with negative control siRNA; ctrl 3: cells with negative control empty vector.)

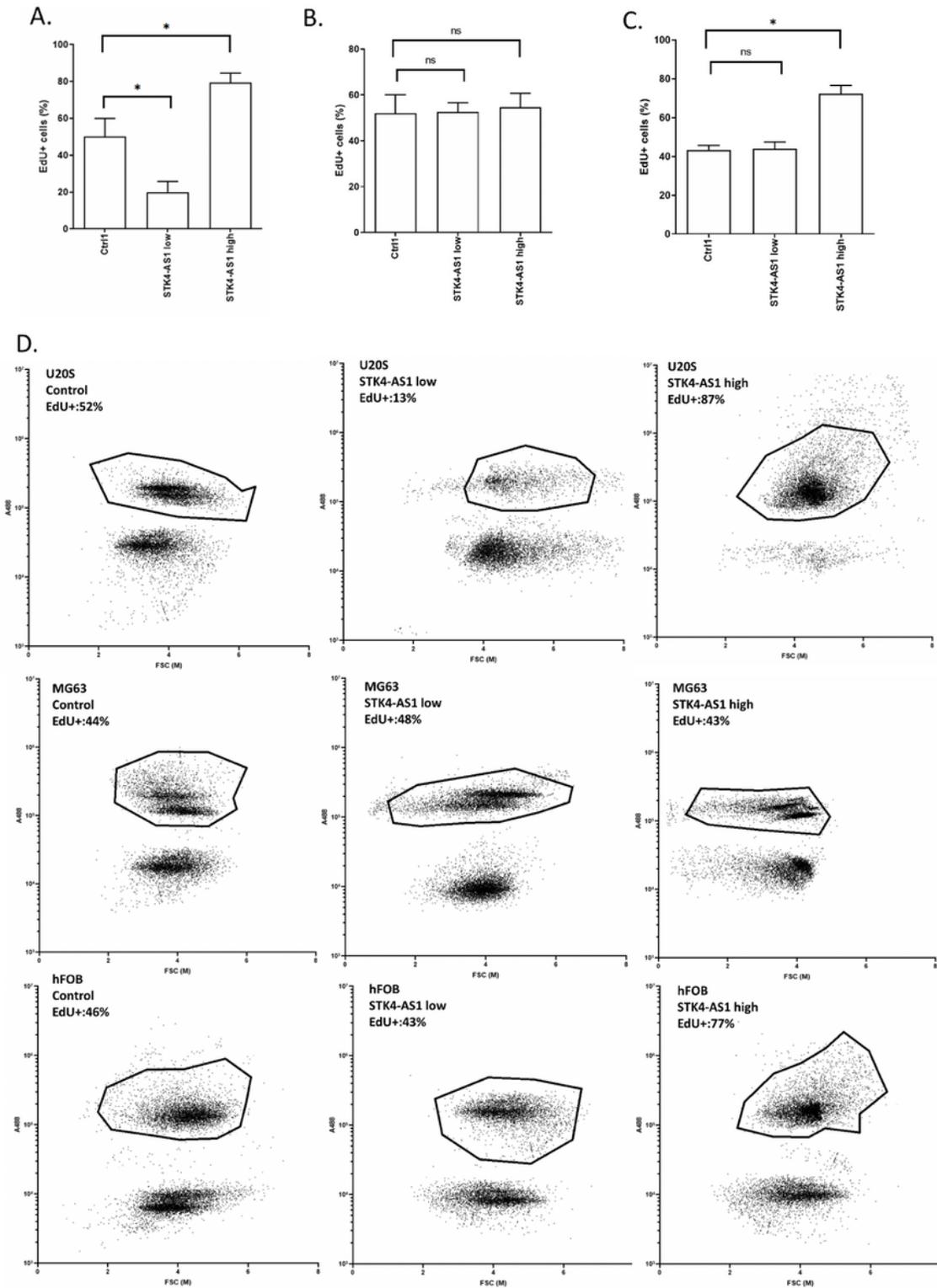


Figure 3

Effects of knockdown or overexpression of STK4-AS1 on cells proliferation. Cells proliferation levels were analyzed using EdU assay. (A) Effects of knockdown or overexpression of STK4-AS1 on U2OS cells proliferation. (B) Effects of knockdown or overexpression of STK4-AS1 on MG63 cells proliferation. (C) Effects of knockdown or overexpression of STK4-AS1 on hFOB cells proliferation. (D) Representative

image of EdU cells in flow cytometry. (* $p < 0.01$. Ctrl 1: cells without treatment; ctrl 2: cells with negative control siRNA; ctrl 3: cells with negative control empty vector.)

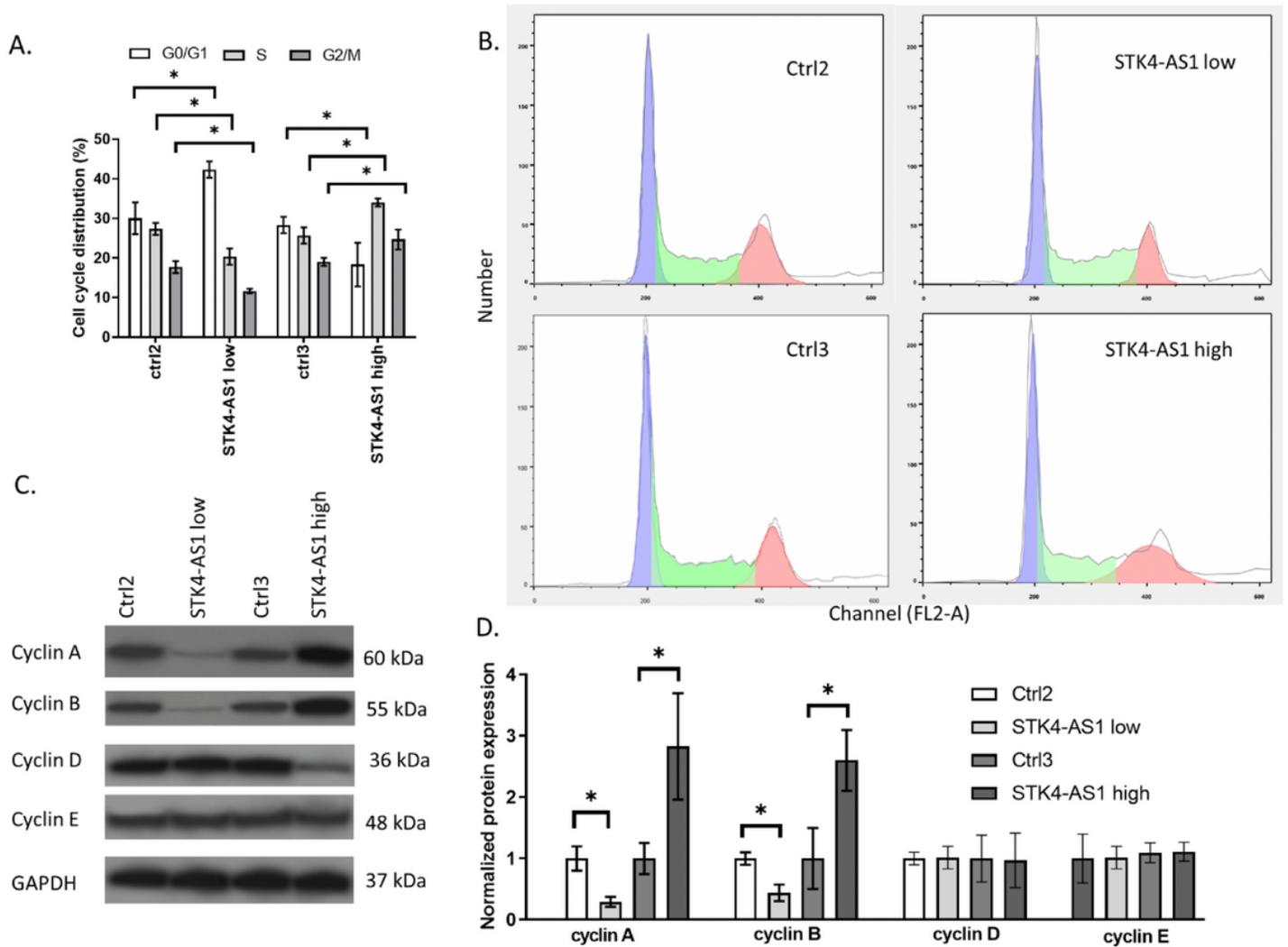


Figure 4

Effect of STK4-AS1 on the U2OS cell cycle. (A) Effect of knockdown and overexpression of STK4-AS1 on U2OS cell cycle. (B) Representative images of cell cycle analysis. (C) Representative images of western blotting for cyclin proteins. (D) Effect of knockdown and overexpression of STK4-AS1 on the level of cyclin proteins in U2OS. (* $p < 0.01$. Ctrl 1: cells without treatment; ctrl 2: cells with negative control siRNA; ctrl 3: cells with negative control empty vector.)

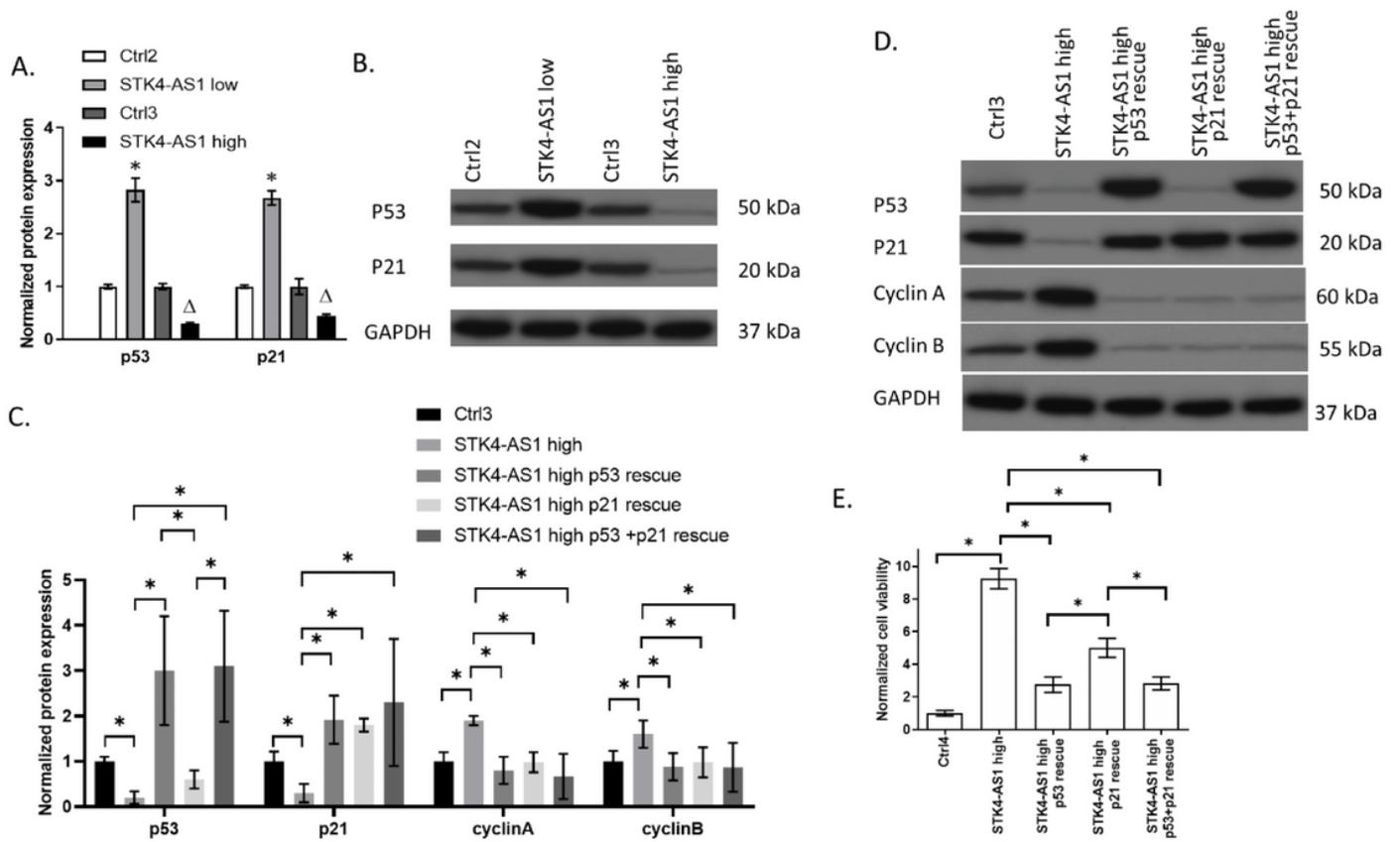


Figure 5

The effect of STK4-AS1 on p53/p21 regulation in U2OS cells. Protein expression was determined using western blotting and the cell viability was determined using the MTT assay. (A) Effect of STK4-AS1 on the expression of p53 and p21. (B) Representative images of p53/p21 western blotting in U2OS. (C) Protein expression of STK4-AS1 overexpressed U2OS cells with p53 or p21 rescue. (D) Representative images of p53/p21 western blotting in U2OS rescue experiments. (E) The cell viability of STK4-AS1 overexpressed U2OS cells with p53 or p21 rescue. (* $p < 0.01$. Ctrl 1: cells without treatment; ctrl 2: cells with negative control siRNA; ctrl 3: cells with negative control empty vector.)

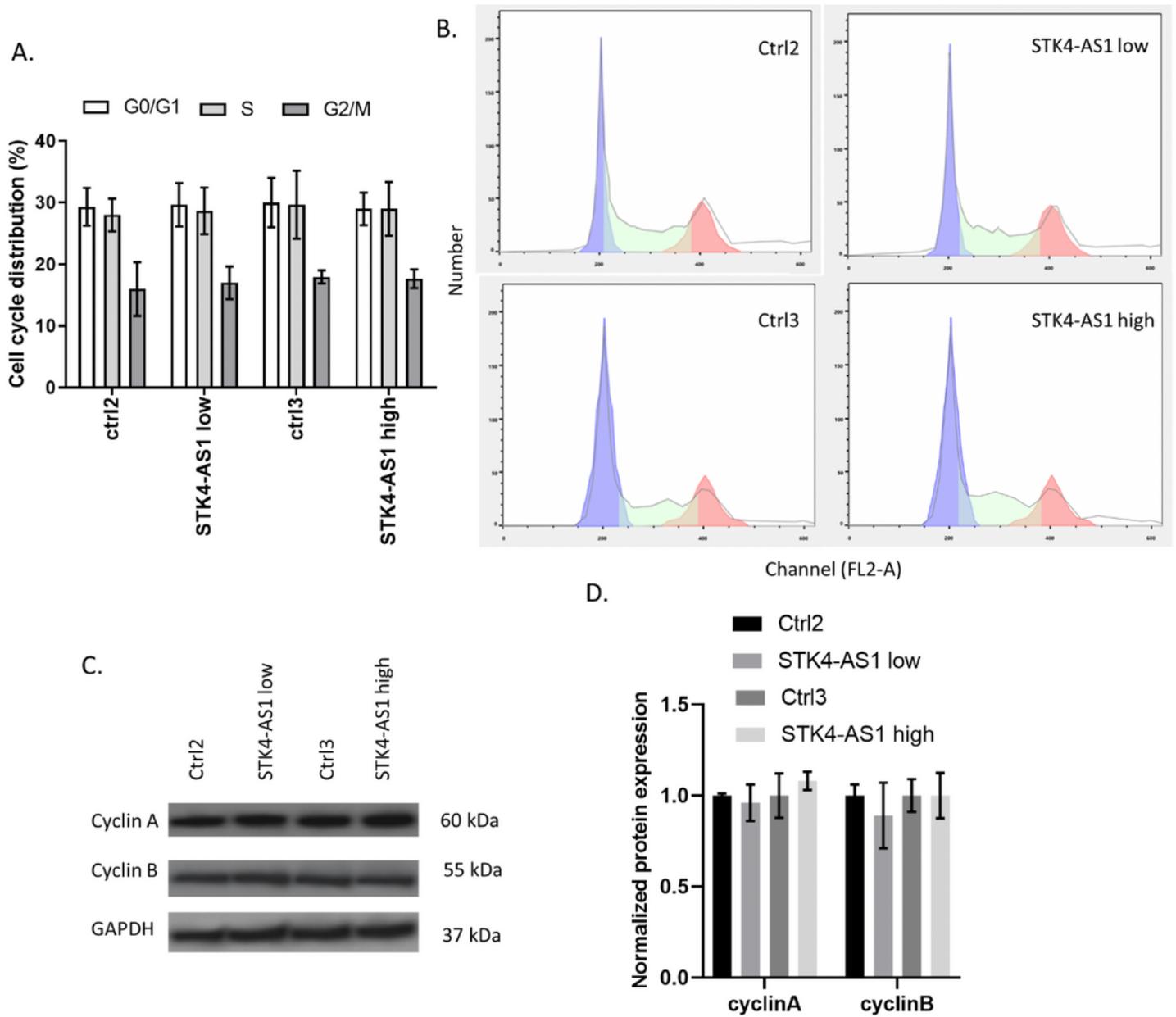


Figure 6

Effect of STK4-AS1 on the cell cycle of MG63 cells. (A) Effect of knockdown and overexpression of STK4-AS1 on MG63 cell cycle. (B) Representative images of cell cycle analysis. (C) Protein expression of STK4-AS1 overexpressed MG63 cells with p53 or p21 rescue. (D) Representative images of p53/p21 western blotting in MG63 rescue experiments. (E) The cell viability of STK4-AS1 overexpressed MG63 cells with p53 or p21 rescue. (* $p < 0.01$. Ctrl 1: cells without treatment; ctrl 2: cells with negative control siRNA; ctrl 3: cells with negative control empty vector.)

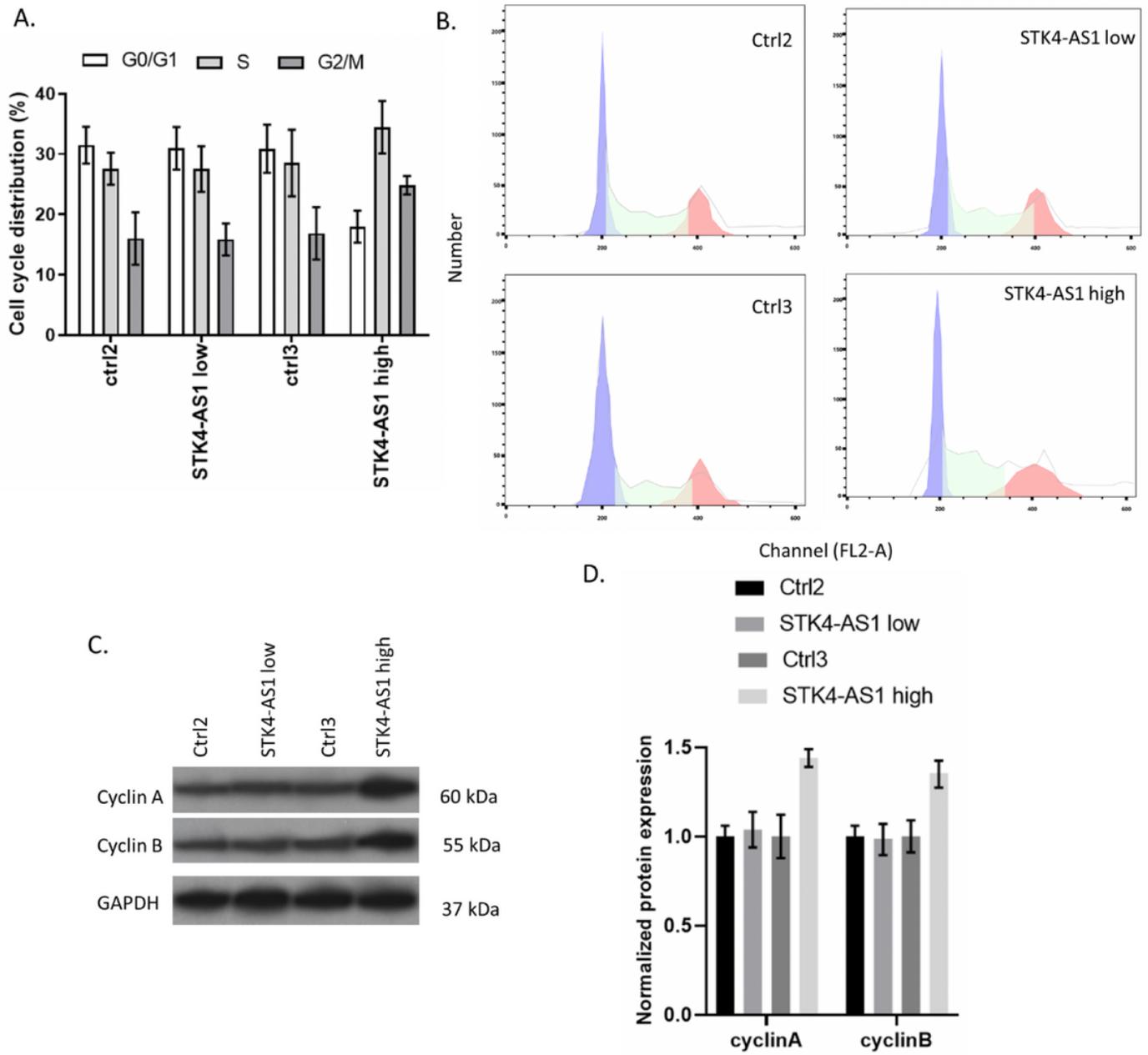


Figure 7

Effect of STK4-AS1 on the cell cycle of hFOB. (A) Effect of knockdown and overexpression of STK4-AS1 on hFOB cell cycle. (B) Representative images of cell cycle analysis. (C) Protein expression of STK4-AS1 overexpressed hFOB cells with p53 or p21 rescue. (D) Representative images of p53/p21 western blotting in hFOB rescue experiments. (E) The cell viability of STK4-AS1 overexpressed hFOB cells with p53 or p21 rescue. (* $p < 0.01$. Ctrl 1: cells without treatment; ctrl 2: cells with negative control siRNA; ctrl 3: cells with negative control empty vector.)

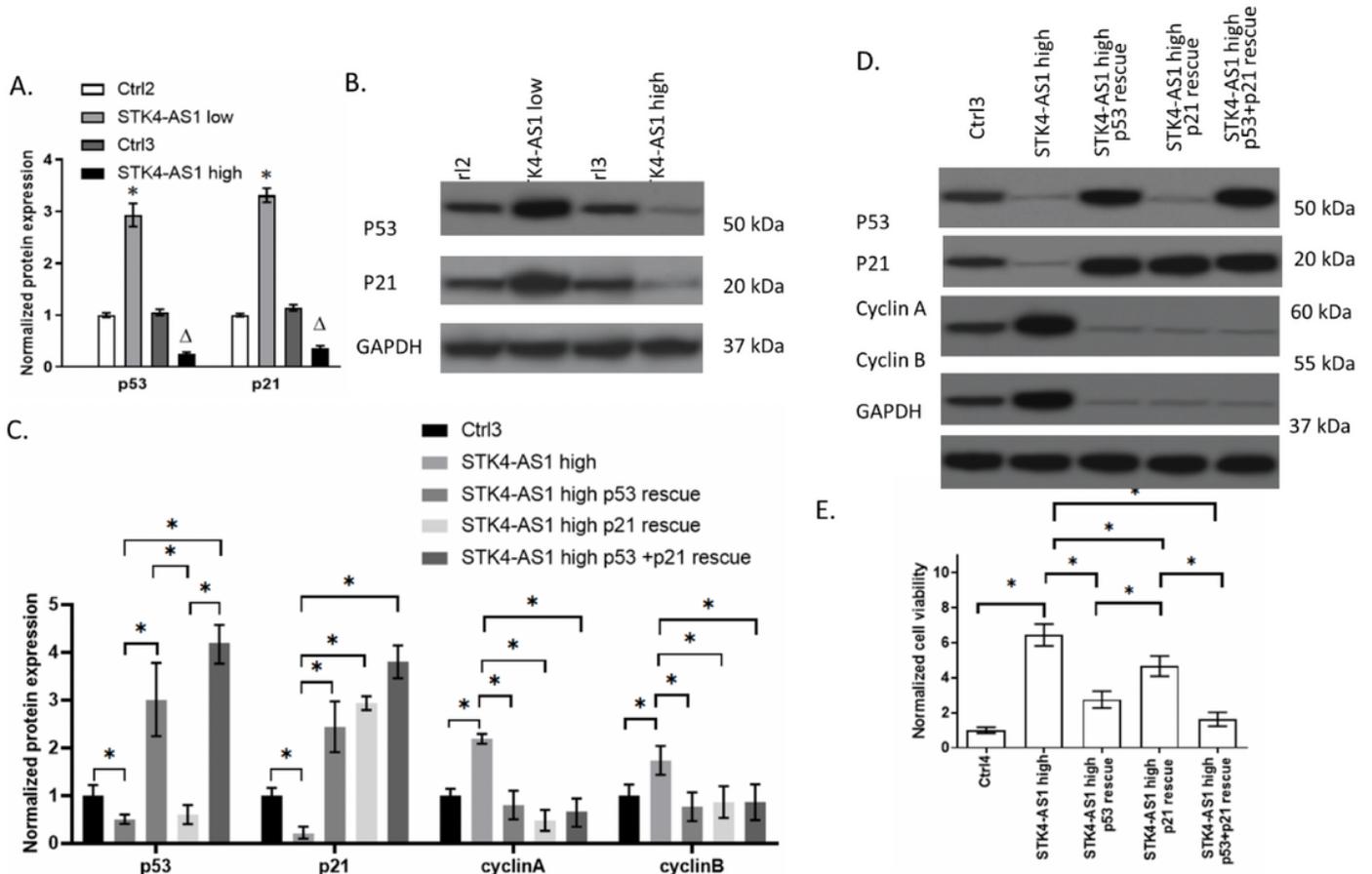


Figure 8

The effect of STK4-AS1 on p53/p21 regulation in hFOB cells. Protein expression was determined using western blotting and the cell viability was determined using the MTT assay. (A) Effect of STK4-AS1 on the expression of p53 and p21. (B) Representative images of p53/p21 western blotting in hFOB. (C) Protein expression of STK4-AS1 overexpressed hFOB cells with p53 or p21 rescue or p53/p21 co-rescue. (D) Representative images of p53/p21 western blotting in hFOB rescue experiments. (E) The cell viability of STK4-AS1 overexpressed hFOB cells with p53 or p21 rescue. (* $p < 0.01$. Ctrl 1: cells without treatment; ctrl 2: cells with negative control siRNA; ctrl 3: cells with negative control empty vector.)



Figure 9

Interaction of STK4-AS1 and TP53. The analysis was conducted using LncRRISearch. (A) Subcellular localization (subcytoplasm and subnucleus compartments) of STK4-AS1 and TP53 mRNA. (B) Global base-pairing interaction analysis of STK4-AS1 and TP53.

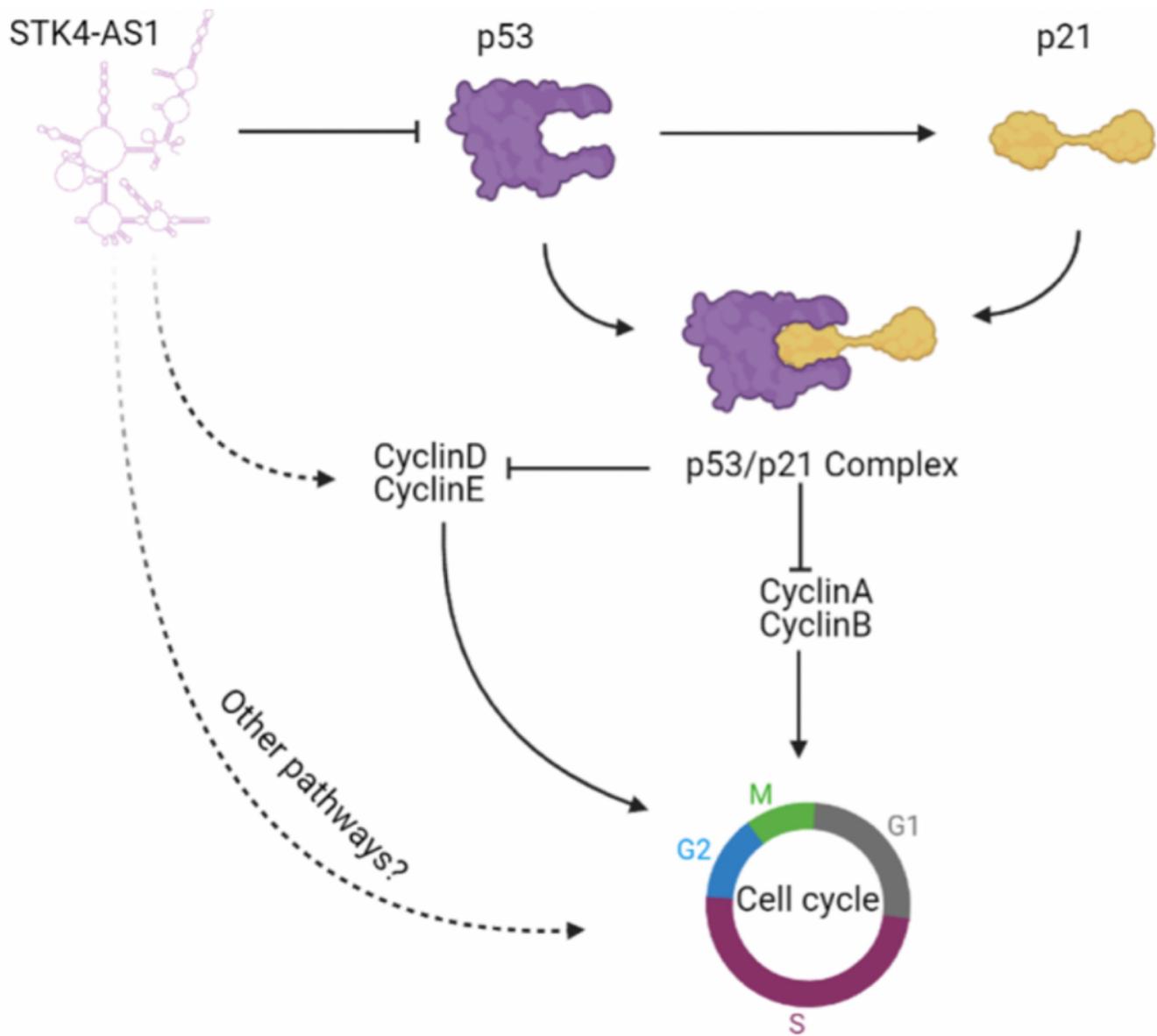


Figure 10

Proposed model for the role of STK4-AS1 in osteosarcoma cell cycle regulation.

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