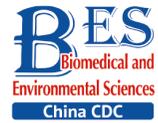


## Original Article

**Tra2 $\beta$  Enhances Cell Proliferation by Inducing the Expression of Transcription Factor SP1 in Cervical Cancer\***LI Mo Juan<sup>1,&</sup>, XIONG Dan<sup>2,&</sup>, WANG Shuai<sup>1,#</sup>, and HUANG Hao<sup>1,#</sup>

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**Abstract**

**Objective** In this study, the role and potential mechanism of transformer 2 $\beta$  (Tra2 $\beta$ ) in cervical cancer were explored.

**Methods** The transcriptional data of Tra2 $\beta$  in patients with cervical cancer from Gene Expression Profiling Interactive Analysis (GEPIA) and cBioPortal databases were investigated. The functions of Tra2 $\beta$  were evaluated by using Western blot, MTT, colony formation, Transwell assays, and nude mouse tumor formation experiments. Target genes regulated by Tra2 $\beta$  were studied by RNA-seq. Subsequently, representative genes were selected for RT-qPCR, confocal immunofluorescence, Western blot, and rescue experiments to verify their regulatory relationship.

**Results** The dysregulation of Tra2 $\beta$  in cervical cancer samples was observed. Tra2 $\beta$  overexpression in Siha and Hela cells enhanced cell viability and proliferation, whereas Tra2 $\beta$  knockdown showed the opposite effect. Alteration of Tra2 $\beta$  expression did not affect cell migration and invasion. Furthermore, tumor xenograft models verified that Tra2 $\beta$  promoted cervical cancer growth. Mechanically, Tra2 $\beta$  positively regulated the mRNA and protein level of SP1, which was critical for the proliferative capability of Tra2 $\beta$ .

**Conclusion** This study demonstrated the important role of the Tra2 $\beta$ /SP1 axis in the progression of cervical cancer *in vitro* and *in vivo*, which provides a comprehensive understanding of the pathogenesis of cervical cancer.

**Key words:** Tra2 $\beta$ ; Cervical cancer; Proliferation; SP1; Molecular targeted therapy

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**INTRODUCTION**

**M**ost cervical cancers could be prevented with human papillomavirus (HPV) vaccines, routine cervical

screenings, and prompt treatment for precancerous lesions. However, screening programs worldwide are inadequate, and cervical cancer remains the fourth most common malignancy diagnosed among women globally<sup>[1,2]</sup>. Over the past 20 years, the incidence and

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mortality rates of cervical cancer in women in China have increased every year<sup>[3]</sup>. Although traditional treatments such as surgery and chemoradiation are widely utilized in patients with cervical cancer, effective treatment options remain limited. Molecular targeted therapy is considered a potential treatment for cervical cancer because of its effectiveness and fewer side effects than traditional therapies. At present, targeted drugs for cervical cancer, such as the EGFR-targeting drug gefitinib, have greatly reformed the current treatment strategy, and targeted VEGF drugs, including Bevacizumab and Pazopanib, are in preclinical studies<sup>[4]</sup>. Searching for new oncogenic drivers in cervical cancer has become important for the development of targeted therapy.

Splicing regulator protein transformer 2 $\beta$  (Tra2 $\beta$ ), which contains two ribonucleoprotein domains and serine/arginine-rich domains, was originally identified as a new putative RNA-binding protein (RA301) in rat reoxygenated astrocytes, which is currently recognized as a sequence-specific pre-mRNA splicing enhancer<sup>[5]</sup>. It binds to the exonic splicing enhancer sequence, which is often found in an A/G-rich context, thereby modulating constitutive and alternative splicing of pre-mRNAs<sup>[6]</sup>. Accumulating body of evidence suggests that the RNA-binding protein Tra2 $\beta$  is deregulated in various malignant tumors, including colon cancer<sup>[7]</sup>, breast cancer<sup>[8]</sup>, endometrial carcinoma<sup>[9]</sup>, lung squamous cell carcinoma<sup>[10]</sup>, and prostate cancer<sup>[11]</sup>. Boris et al. showed that among 105 patients with cervical cancer, patients with high nuclear expression levels of Tra2 $\beta$  had poor prognoses compared with patients with weak amounts of nuclear Tra2 $\beta$ . However, the critical role of Tra2 $\beta$  in carcinogenesis remains elusive<sup>[12]</sup>. In this study, we primarily focused on the role of the splicing regulator Tra2 $\beta$  as a gene modulator in cervical cancer cells and provided a theoretical basis for potential targeted therapy for cervical cancer.

## MATERIALS AND METHODS

### Online Database Analysis

Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>) was utilized to analyze the Tra2 $\beta$  expression level and correlation between Tra2 $\beta$  expression and tumor stage in patients with cervical cancer from a TCGA cohort. cBioPortal databases (<http://www.cbioportal.org>) were used to investigate the Tra2 $\beta$  gene expression,

mutation, and correlation among *EGR1*, *SP1*, *NOLC1*, and *Tra2 $\beta$*  in CESC. Cervical cancer microarray data obtained from GSE7803 (Platform: GPL96) and GSE9750 (Platform: GPL96) were used to analyze the *SP1* expression.

### Cell Lines and Culture

Human normal cervical epithelial cells (HcerEpic) and human cervical cancer cell lines (Hela and Siha cells) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 (Sigma-Aldrich, St Louis, USA) medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C in a humidified 5% CO<sub>2</sub> incubator. All the cell lines used in this study were free of mycoplasma contamination.

### Transfection and Lentiviral Packaging

Full-length Tra2 $\beta$ 1 cDNA (human) was granted by Han Jiahui Lab (Xiamen University, Xiamen, China) and then cloned into the pLVX-EGFP-IRES-puro lentiviral vector. 293T cells were transfected with the recombinant lentiviral plasmids, psPAX2 packaging plasmids, and pMD2.G envelope plasmids. After incubating for 48 h, cell media were collected and used to infect Hela and Siha cells at 30% confluence. After incubating for 24 h, the virus-containing media were replaced by fresh media. After an additional 48 h, fresh media containing puromycin (2  $\mu$ g/mL, Sigma) were added to select infected cells for stable Tra2 $\beta$  expression. The mRNA or protein level of the selected cells was subsequently assessed by q-PCR or Western blot.

### RNA Interference (siRNA)

The sequences of the small interfering RNAs (siRNAs) used in this study are as follows: *Tra2 $\beta$* -siRNA-1: 5'-AGCUAAAGAACGUGCCAAUTT-3', *Tra2 $\beta$* -siRNA-2: 5'-CCGAUGUGUCUAUUGUAUATT-3'; *SP1*-siRNA: 5'-GCCAATAGCTACTCAACTA'; si-NC: 5'-AGUCUCCACGUGUACGUTT-3' (Genepharma, Suzhou, China). Hela or Siha cells were cultured in six-well plates (6  $\times$  10<sup>5</sup> cells/well), reaching 30% to 50% confluence before transfection. Then, the cells were transfected with 100 nmol/L of *Tra2 $\beta$*  siRNAs, *SP1* siRNAs, or non-target siRNAs using Lipofectamine™ 2000 (Invitrogen, USA) in a serum-free medium. After incubation for 6 h, the medium was replaced by fresh medium containing 10% fetal bovine serum, and the cells were incubated for another 24 h, followed by Western blot, MTT, colony formation, and Transwell assay.

### MTT Assays

A total of  $3 \times 10^4$  HeLa or SiHa cells stably overexpressing Tra2 $\beta$  or transfected with siRNAs targeting Tra2 $\beta$ 1 were seeded in RPMI-1640 medium in 96-well plates at 0.1 mL/well. MTT staining was used to determine the absorbance at 570 nm and assess cell viability.

### Colony Formation Assay

A total of 1,000 HeLa or SiHa cells stably overexpressing Tra2 $\beta$  or transfected with siRNAs targeting Tra2 $\beta$ 1 were seeded in six-well plates for 2 weeks. After 2 weeks, the cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% methanol for 10 min, and stained with crystal violet solution for 5 min. Then, the crystal violet was removed, and the wells were rinsed, dried, and scanned.

### Western Blot Assay

Western blot analysis was performed as previously described<sup>[13]</sup>. Total protein was extracted from cells or tumor xenograft tissues using a total protein extraction kit (Abcam, USA) according to the manufacturer's instructions. The BCA protein assay kit (Thermo Fisher Scientific, USA) was used to determine protein concentration. The following antibodies were used for staining: rabbit-Tra2 $\beta$  (Proteintech, USA), rabbit-GAPDH (Proteintech, USA), and rabbit-SP1 (Abcam, USA).

### Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from HeLa or SiHa cells using a total RNA extraction kit (Synthgene Biotech., China) according to the manufacturer's instructions and then transcribed into complementary DNA by using TransScript® one-step gDNA removal and cDNA synthesis supermix (TransGen, China). Quantitative real-time PCR reactions were performed using the PerfectStart® Green qPCR SuperMix (TransGen, China) on the StepOne™ Real-Time PCR System (Applied Biosystems, USA). The primers used were as follows: Tra2 $\beta$ 1 (F 5'-ATGACGACGATAAGAGCCCG-3', R 5'-TCGTGAGCACTTCCACTTC-3'), SP1 (F 5'-GTGGAGGCAACATCATTGCTG-3', R 5'-GCCACTGGTACATTGGTCACAT-3'), NOLC1 (F 5'-AGCTGGCCTGACGGTATG-3', R 5'-TTGGTCTGGCTGAGTACCG-3'), EGR1 (5'-CCAGTGGCTACCTCCTACC-3', R 5'-CCTGGGAGAAAAGGTCGCTG-3'), and GAPDH (F 5'-GTCTCTCTGACTTCAACAGCG-3', R 5'-ACCACCCTGTTGCTGTAGCAA-3'). GAPDH was used as a loading

control. The Tra2 $\beta$ -relative mRNA expression was calculated using the  $2^{-\Delta\Delta Cq}$  method.

### Cell Migration Assay

A total of  $4 \times 10^4$  transfected HeLa or SiHa cells stably overexpressing Tra2 $\beta$  or transfected with siRNAs targeting Tra2 $\beta$ 1 as required in 200- $\mu$ L serum-free RPMI-1640 medium were seeded in the upper compartment of the Boyden chambers (Corning, NY, USA), and 700  $\mu$ L of completed RPMI-1640 containing 10% FBS was added to the lower chamber. After incubation for 12 h at 37 °C, the cells migrating across the 8- $\mu$ m polycarbonate were fixed, stained with 0.1% crystal violet, and counted.

### Cell Invasion Assay

HeLa or SiHa cells ( $4 \times 10^4$ ) transfected with Tra2 $\beta$ 1 siRNAs or stably overexpressing Tra2 $\beta$  as required were re-suspended in 150  $\mu$ L of RPMI-1640 medium and then seeded into the upper chamber with 100  $\mu$ L of precoated Matrigel. Subsequently, 600  $\mu$ L of completed RPMI-1640 medium containing 10% FBS was set in the lower chamber as a chemoattractant. After incubation for 12 h, the invading cells were fixed, stained with 0.1% crystal violet, and counted.

### Xenograft Tumor Model

Female BALB/c nude mice aged 4–6 weeks and weighing 18–20 g were purchased from Jiangsu Jicui Yaokang Biotechnology Co., Ltd. (Nanjing, China), with five mice in each group, and all procedures were approved by the committee on the use of live animals in Jinan University (Ethical number: 20200807-01). Equal volumes of PBS and Matrigel were first used to suspend HeLa cells at  $5 \times 10^7$  cells/mL, and a 100- $\mu$ L cell-containing mixture was subcutaneously injected into the abdomen of mice to establish tumor xenografts. Tumor size was measured with calipers every two days, and tumor volume was calculated using the following formula:  $V = (\text{length} \times \text{width}^2) / 2$ . At 28 days after injection, the mice were sacrificed, and the tumors were collected for Western blot and immunohistochemical analyses.

### Immunohistochemistry (IHC)

For the detection of Tra2 $\beta$  and Ki67 antigens, tissue sections were deparaffinized and microwaved in 10-mmol/L sodium citrate for 10 min and then incubated in H<sub>2</sub>O<sub>2</sub> (0.3%) with methanol for 30 min. Nonspecific binding of antibodies was inhibited by incubating with normal goat serum (5%) for 30 min at room temperature. Then, tissue slides were

incubated with rabbit anti-Tra2 $\beta$  polyclonal antibody (40  $\mu$ g/mL) or mouse anti-Ki67 monoclonal antibody (5  $\mu$ g/mL) for 18 h at 4 °C. After washing, slides were incubated in horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 30 min, followed by chromogenic detection using DAB as the substrate.

#### **RNA Extraction, Library Preparation, and RNA-Seq**

Total RNA was extracted from HeLa cells by using TRIzol<sup>®</sup> Reagent (Invitrogen) according to the manufacturer's protocol. Then, the quality and integrity of total RNA were confirmed by agarose gel electrophoresis, and high-quality samples were sent to the Shanghai Biotechnology Co., Ltd. (Shanghai, China) for library construction and Illumina Genome Analyzer Ix sequencing. Libraries were prepared using the Illumina TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA) according to the manufacturer's protocol<sup>[14]</sup>. In brief, purified mRNAs were fragmented and converted to cDNA using reverse transcriptase. The resulting cDNAs were converted into double-stranded cDNAs and subjected to end-repair, A-tailing, and adapter ligation. The library was amplified by 8 PCR cycles. The library was quantified using a Qubit<sup>®</sup> 2.0 Fluorometer (Invitrogen) and validated using an Agilent 2100 bioanalyzer (Agilent, China). Clusters were constructed from the library using Illumina cBot diluted at a concentration of 10 pM and sequenced for 75 cycles on Illumina Genomic Analyzer Ix or 100 cycles on HiSeq-2000 (Illumina). High-quality reads that passed Illumina quality filters were retained for sequence analysis.

#### **Identification of Differentially Expressed Genes (DEGs)**

The input data of gene differential expression included ReadCount data obtained from gene expression level analysis. EdgeR was used for gene differential expression analysis of paired samples<sup>[15]</sup>. The absolute value of log<sub>2</sub> (fold change) > 1 and the false discovery rate < 0.01 were used to identify the significance of different gene expressions. Subsequently, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the DEGs were performed using DAVID Tools (<https://david.ncicrf.gov/>).

#### **Confocal Immunofluorescence**

Confocal immunofluorescence was performed as previously described<sup>[13]</sup>. HeLa cells transfected with pCMV-EGFP-Tra2 $\beta$ 1 plasmids or empty vectors

based on the experimental requirements were grown on polylysine-precoated slides. Cells were initially incubated with the rabbit-SP1 primary antibody at 4 °C overnight and then with Alexa Fluor 592-conjugated secondary antibody. Finally, DAPI nuclear counterstaining was performed. Slides were imaged using a 40 × NA 0.75 Oil Dic objective on an inverted laser-scanning confocal microscope (LSM700; Zeiss GmbH, Jena, Germany), and images were captured using Zeiss.

#### **RNA Immunoprecipitation (RNA IP)**

RNA IP was utilized to determine the interaction between SP1 mRNA and Tra2 $\beta$ . In brief, HeLa cell lysates were incubated with anti-Tra2 $\beta$  for 4 h at 4 °C. Then, beads were added and incubated for another 2 h. The precipitated RNAs were eluted and analyzed by RT-qPCR.

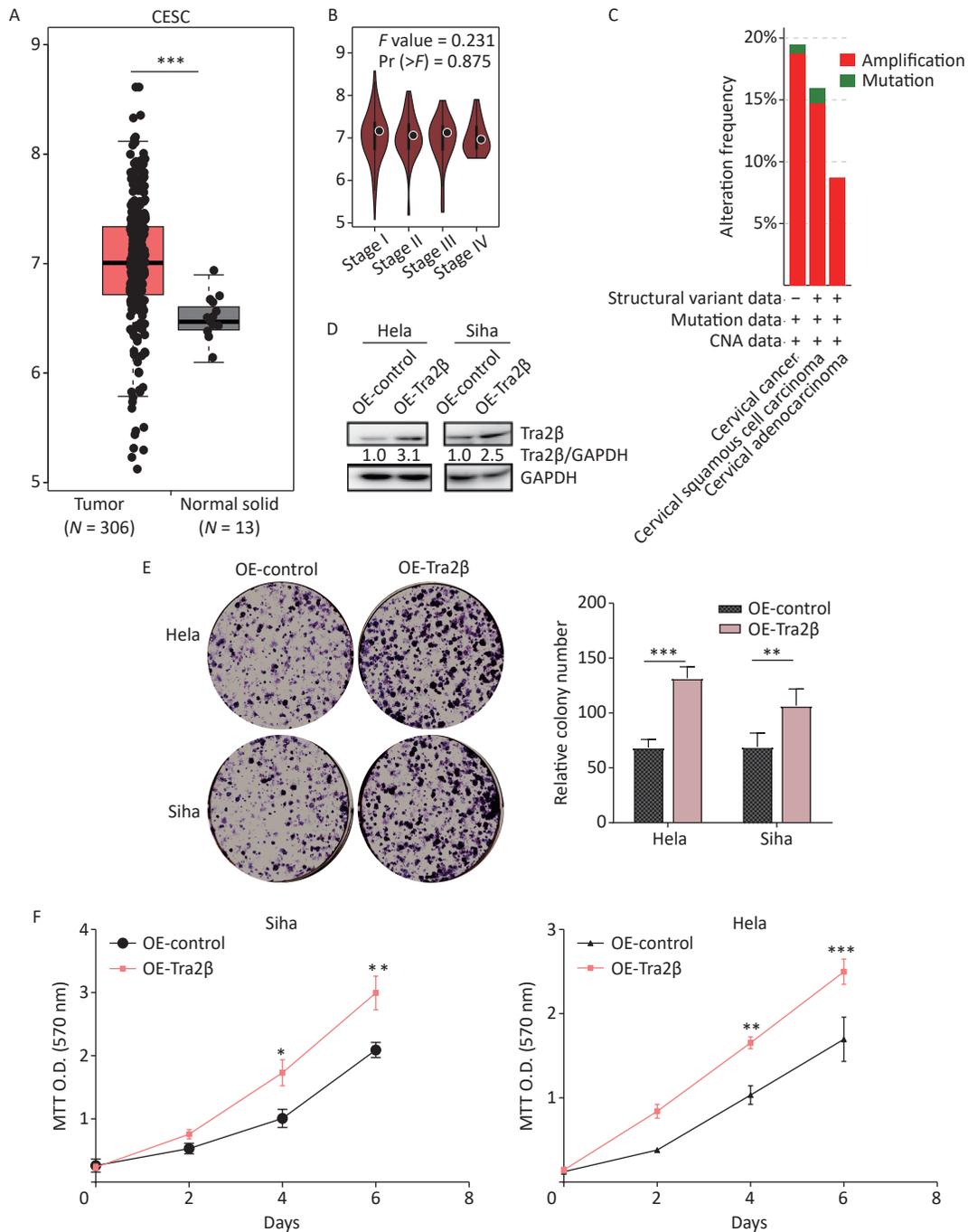
#### **Statistics Analysis**

The data were initially analyzed to assess for normality using the Shapiro-Wilk test and then presented as means  $\pm$  SD. When data were only compared with the control, two-way ANOVA followed by Dunnett's T3 *post hoc* test was used. Two-way ANOVA followed by Sidak's *post hoc* comparison was used when data were compared among all groups. A two-sided *P* value of less than 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism (La Jolla, USA). All experiments were repeated at least three times.

## **RESULTS**

#### **Tra2 $\beta$ Stimulated the Proliferation of HeLa and SiHa cells in vitro**

First, we investigated the transcriptional data of Tra2 $\beta$  in patients with cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) from GEPIA and cBioPortal databases. Data from GEPIA showed that the expression level of Tra2 $\beta$  was higher in cervical cancer tissues than in normal cervix tissues (Figure 1A). We also analyzed the expression of Tra2 $\beta$  with tumor stage for CESC. As shown in Figure 1B, no significant difference in Tra2 $\beta$  was observed among different tumor stages. The frequency of copy number amplification, deletion, and genetic mutation of Tra2 $\beta$  in patients with cervical cancer is shown in Figure 1C. About 17% of the clinical samples (102/605) had genetic amplification of Tra2 $\beta$ , and nearly 0.8% of the samples (5/605) had genomic mutations. However,

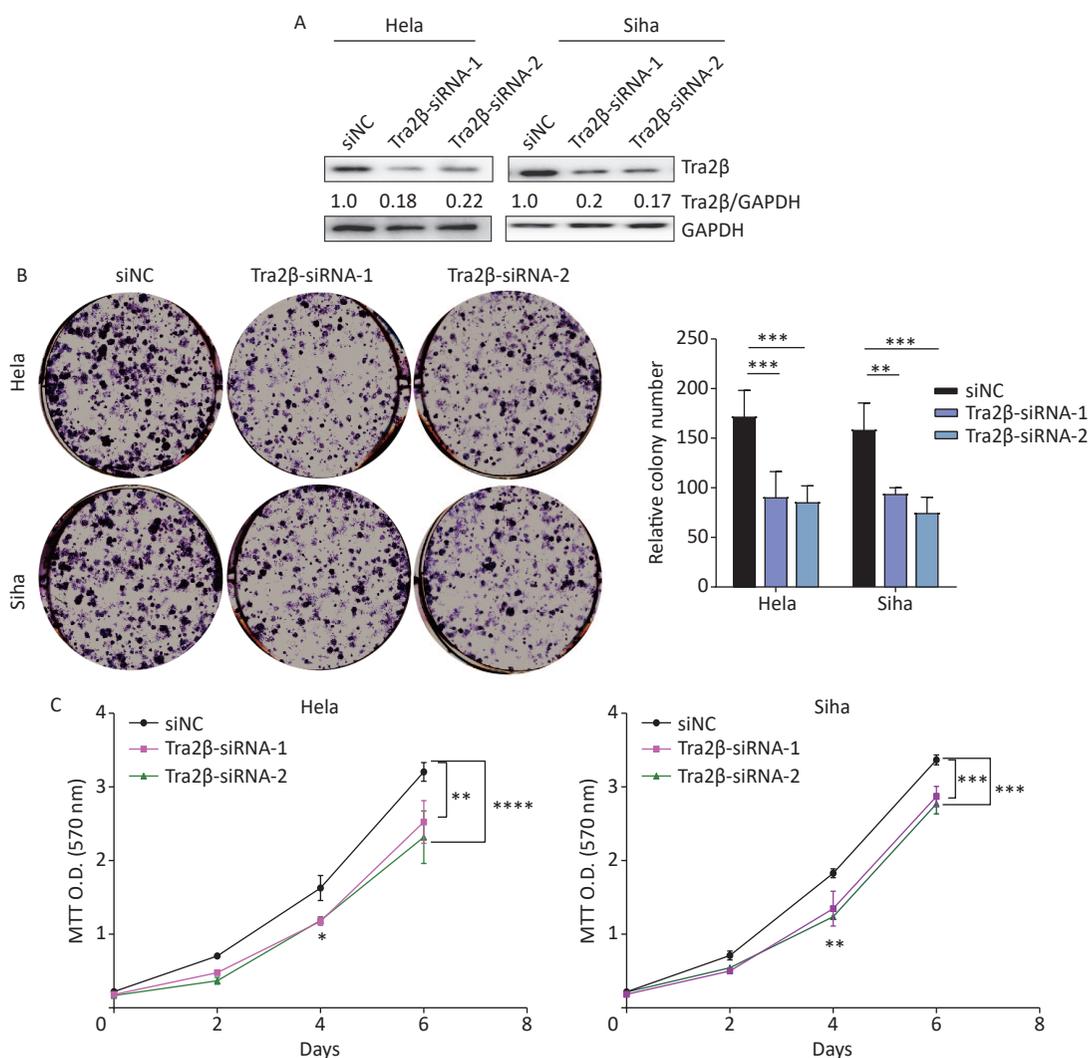


**Figure 1.** Overexpression of Tra2β promoted the proliferation of HeLa and SiHa cells *in vitro*. (A) The expression of Tra2β in patients with CESC (GEPiA, <http://gepia.cancer-pku.cn/>). (B) Correlation between Tra2β expression and tumor stage in patients with CESC (GEPiA, <http://gepia.cancer-pku.cn/>). (C) Tra2β gene expression and mutation analysis in CESC (cBioPortal, <http://www.cbioportal.org/>). (D) The confirmation of stable Tra2β overexpression in HeLa and SiHa cells using Western blot assay. (E) Cell proliferation ability of Tra2β overexpression in HeLa and SiHa cells was assessed by colony formation assays. (F) Cell viability of cells of Tra2β overexpression in HeLa and SiHa cells was measured by MTT assays. Two-way ANOVA with Dunnett comparison (E and F) was used. The data shown are the mean ± SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Kaplan-Meier plotter showed that patients with high *Tra2β* mRNA expression had better overall survival (OS,  $P = 0.014$ ), and no significant association was found between *Tra2β* expression levels and relapse-free survival (RFS) in patients with cervical squamous cell carcinoma ( $P = 0.064$ , Supplementary Figure S1 available in [www.besjournal.com](http://www.besjournal.com)). In testing whether the molecular properties of Tra2β are relevant in an oncologic context, we performed colony formation assays on HeLa and SiHa cells with Tra2β overexpression or knockdown. We generated stable Tra2β overexpression in HeLa and SiHa cell lines using the recombinant lentiviral expression system. The exogenous Tra2β expression level in

these cells was verified using Western blot (Figure 1D). Overexpressed Tra2β significantly promoted the cell clone formation *in vitro* (Figure 1E). In providing further evidence of Tra2β facilitating the cell growth of cervical cancer, MTT assays were performed on HeLa and SiHa cell lines with Tra2β overexpression. Consistent with the abovementioned results, Tra2β overexpression significantly increased the growth of HeLa and SiHa cells compared with control cells (Figure 1F).

Two siRNAs were designed and tested, and their respective cell lines were proven effective, reducing protein levels by more than 70% (Figure 2A). The downregulation of Tra2β significantly reduced clone



**Figure 2.** Knockdown of Tra2β suppressed the proliferation of HeLa and SiHa cells *in vitro*. siRNAs targeting Tra2β were transfected into HeLa and SiHa cells, followed by Western blot assay (A), colony formation assays (B), and MTT assays (C). Two-way ANOVA with Dunnett comparison (B and C) was used. The data shown are the mean ± SD of three independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .

formation in HeLa and SiHa cells (Figure 2B). Consistently, MTT colorimetric assay also showed that Tra2 $\beta$  silencing suppressed the proliferation of HeLa and SiHa cells compared with control cells (Figure 2C). Collectively, these results indicated that Tra2 $\beta$  promotes HeLa and SiHa cell growth *in vitro*.

#### **Knockdown or Overexpression of Tra2 $\beta$ had no Effect on the Migration and Invasion of HeLa and SiHa Cells *in vitro***

The migration and invasion of cancer cells are identified as key factors in cancer metastasis; thus, we further investigated the effects of Tra2 $\beta$  on the migration and invasion of HeLa and SiHa cells through *in vitro* Transwell migration and Matrigel invasion. Compared with control cells, the migratory and invasive abilities of HeLa cells have not changed significantly either with Tra2 $\beta$  overexpression (Figure 3A and B) or knockdown (Figure 3C and D). Consistently, no significant changes in migration and invasion of SiHa cells were found between the OE-Tra2 $\beta$  group and the control group (Figures 3E and F) or between the Tra2 $\beta$  knockdown group and the control group (Figure 3G and H). These results indicated that the alteration of Tra2 $\beta$  expression does not affect the migration and invasion of cervical cancer *in vitro*.

#### **Tra2 $\beta$ Promoted Cervical Cancer Growth *in vivo***

Tumorigenicity experiments were performed by subcutaneous injection of  $5 \times 10^6$  HeLa cells into the abdomen of female nude mice to confirm the role of Tra2 $\beta$  in driving cervical cancer growth. Twenty-eight days after injection, tumor size in the Tra2 $\beta$ -overexpressed group was generally larger than that in the control group (Figure 4A), and the average weight of the tumor in the Tra2 $\beta$ -overexpressed group was evidently heavier than that in the control group (Figure 4B). In addition, tumor growth was significantly accelerated in the Tra2 $\beta$ -overexpressed group (Figure 4C). Moreover, Tra2 $\beta$  overexpression increased the percentage of Ki-67-positive cells in the tumor xenografts of nude mice compared with the control group (Figure 4D). Moreover, the IHC assay showed that Tra2 $\beta$  was preferentially expressed in the nucleus, which was consistent with previous studies<sup>[12,16]</sup>. Collectively, these observations indicated that Tra2 $\beta$  plays a role in regulating cervical cancer formation and growth.

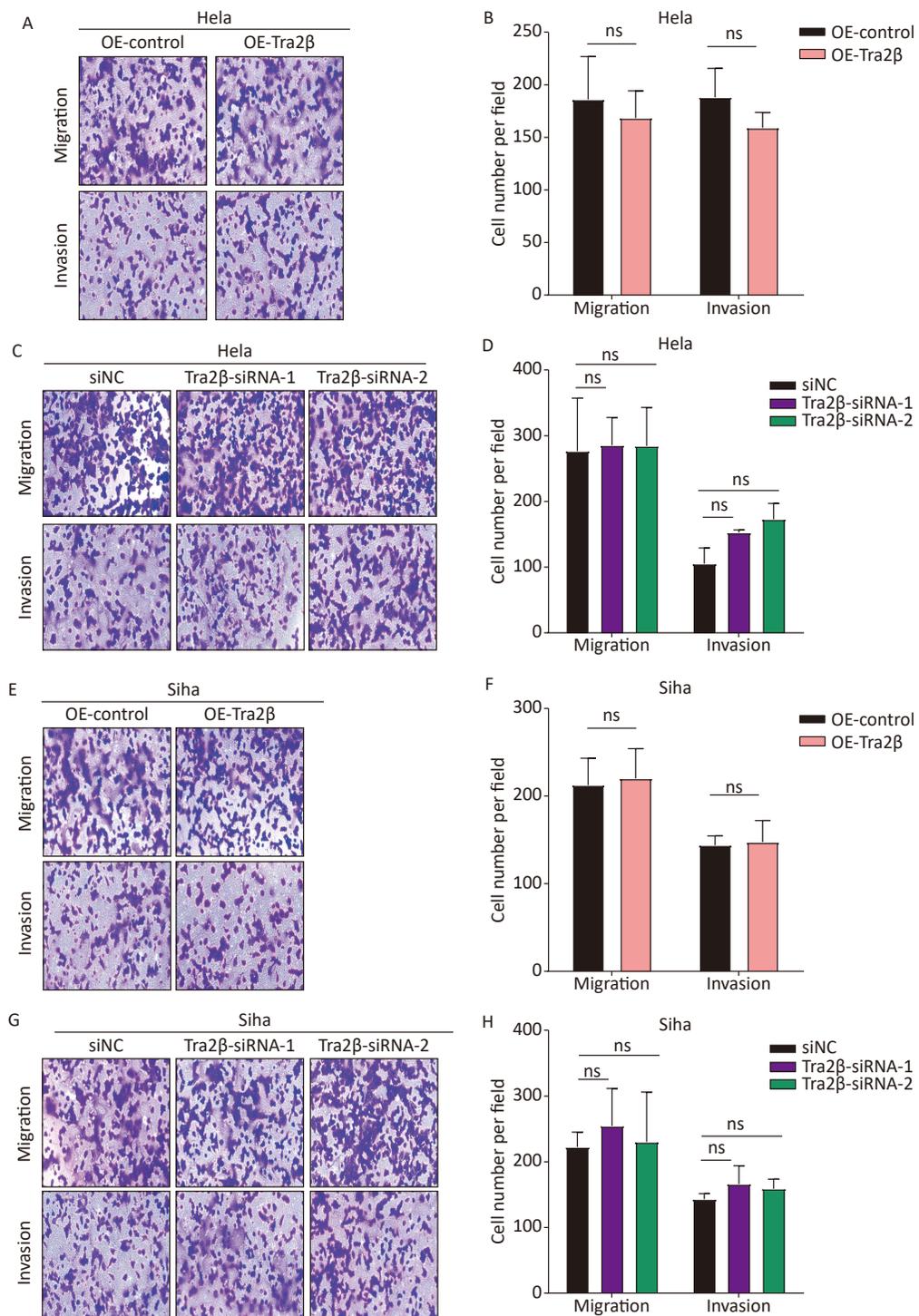
#### **Tra2 $\beta$ Could Regulate *EGR1*, *SP1*, and *NOLC1* Expression**

The major RNA-binding site for Tra2 $\beta$  is GAA,

particularly the AGAA-rich sequence, which lies within multiple mRNAs<sup>[17]</sup>. The transcriptomic profiles of HeLa cells with stable Tra2 $\beta$  overexpression and control cells were compared using the next-generation RNA-Seq to uncover Tra2 $\beta$ -regulated potential targets controlling cell growth. A total of 125 DEGs were identified, among which 56 genes were upregulated, and 69 genes were downregulated (Figure 5A and Supplementary Table S1 available in [www.besjournal.com](http://www.besjournal.com)). GO (Figure 5B) and KEGG pathway (Figure 5C) analyses identified significantly enriched pathways in DEGs, including the regulation of gene expression, protein-DNA complex assembly, chromatin assembly, protein metabolic process, transcriptional misregulation in cancer, VEGF signaling pathway, adherence junction, and p53 signaling pathway. RT-qPCR was used to confirm the RNA-Seq data. Of the top 10 DEGs, three (*EGR1*, *SP1*, and *NOLC1*) were randomly selected. As presented in Figure 5D, all these genes were highly expressed in Tra2 $\beta$ -overexpressed HeLa cells, as compared with control cells, which is with the RNA-Seq data. Furthermore, the correlation of *EGR1*, *SP1*, or *NOLC1* with Tra2 $\beta$  in CESC tissues was analyzed by using the ENCORI website. As shown in Figure 5E, *EGR1*, *SP1*, and *NOLC1* were positively correlated with Tra2 $\beta$  expression in 306 CESC tissue samples. Overall, Tra2 $\beta$  plays an important biological role in cervical cancer-like transcriptional dysregulation.

#### ***SP1* was Critical for Tra2 $\beta$ to Promote the Proliferation of HeLa and SiHa Cells *in vitro***

Based on previous reports, SP1 is identified as a transcription factor by binding to promoters of target genes and is believed to play important roles in the regulation of various key biological functions, such as cell proliferation, clonogenicity, cycle progression, and apoptosis<sup>[18]</sup>. First, the RNA-IP assay confirmed that Tra2 $\beta$  proteins could bind with *SP1* mRNA (Figure 6A). In exploring the significance of SP1 in the development of cervical cancer, we first validated the *SP1* expression of cervical cancer in the Gene Expression Omnibus database, including GSE7803 and GSE9750. As shown in Figure 6B, *SP1* mRNA expression was higher in cervical cancer tissues than in normal cervical tissues. Then, the protein level of SP1 was examined in normal cervical epithelial cells (HcerEpic) and cervical cancer cells (HeLa and SiHa). Western blot showed that the level of SP1 in the two cervical cancer cell lines was evidently higher than the normal cervical epithelial cells (Figure 6C). *SP1*-targeted siRNAs downregulated



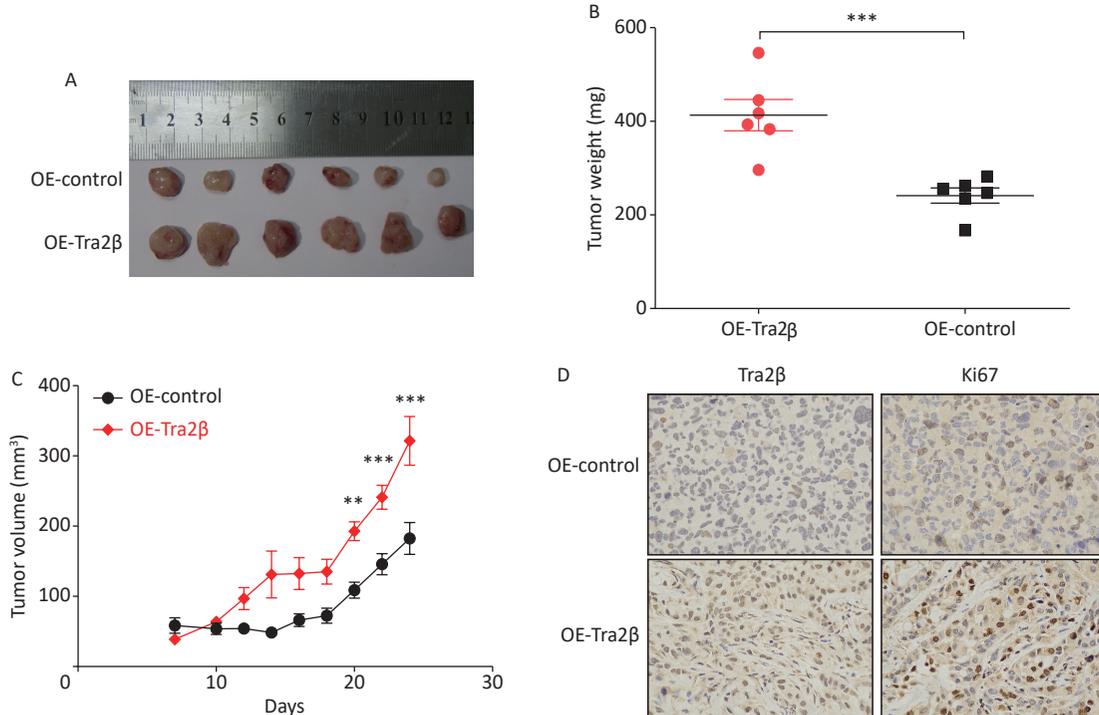
**Figure 3.** Tra2 $\beta$  had no effect on the migration and invasion of HeLa and SiHa cells *in vitro*. (A–D) Transwell assays were conducted to test the migratory (top) and invasive (bottom) ability of HeLa cells with stable Tra2 $\beta$  overexpression (A and B) or Tra2 $\beta$  knockdown (C and D). (E–H) Transwell assays were conducted to test the migratory (top) and invasive (bottom) ability of SiHa cells with stable Tra2 $\beta$  overexpression (E and F) or Tra2 $\beta$  knockdown (G and H). Five random fields of each well were counted under a microscope with a magnification of  $\times 100$  and followed by statistical analysis (B, D, F, and H). Two-way ANOVA with Dunnett comparison (B, D, F, and H) was used. Data shown are the mean  $\pm$  SD of three independent experiments; ns indicates no significant difference.

the expression of SP1 and its major downstream oncogenes (c-Myc and CDK1)<sup>[19]</sup>, which was consistent with previous findings, showing that high SP1 expression is correlated with the malignant progression of cervical cancer (Figure 6D). The expression of Tra2 $\beta$  and SP1 in tumor xenografts of nude mice formed by Hela cells was significantly higher than those in adjacent normal tissues (Supplementary Figure S2A available in [www.besjournal.com](http://www.besjournal.com)). In obtaining evidence regarding the Tra2 $\beta$ /SP1 axis in cultured Hela cells, we performed indirect immunofluorescence staining of Tra2 $\beta$  and SP1 followed by confocal scanning. The cells were transfected with pCMV-EGFP-Tra2 $\beta$ 1 or pCMV-EGFP empty vector for 24 h and then subjected to fluorescence staining as described in “Materials and Methods”. Figure 6E shows that in cells transfected with pCMV-EGFP empty vector, the EGFP protein was evenly distributed throughout the cell, with weak SP1 staining intensity. On the contrary, in cells transfected with pCMV-EGFP-Tra2 $\beta$ 1-expressing plasmids, the EGFP-Tra2 $\beta$  protein was primarily concentrated in the nucleus, accompanied by high-intensity immunoreactive SP1 in the nucleus, which

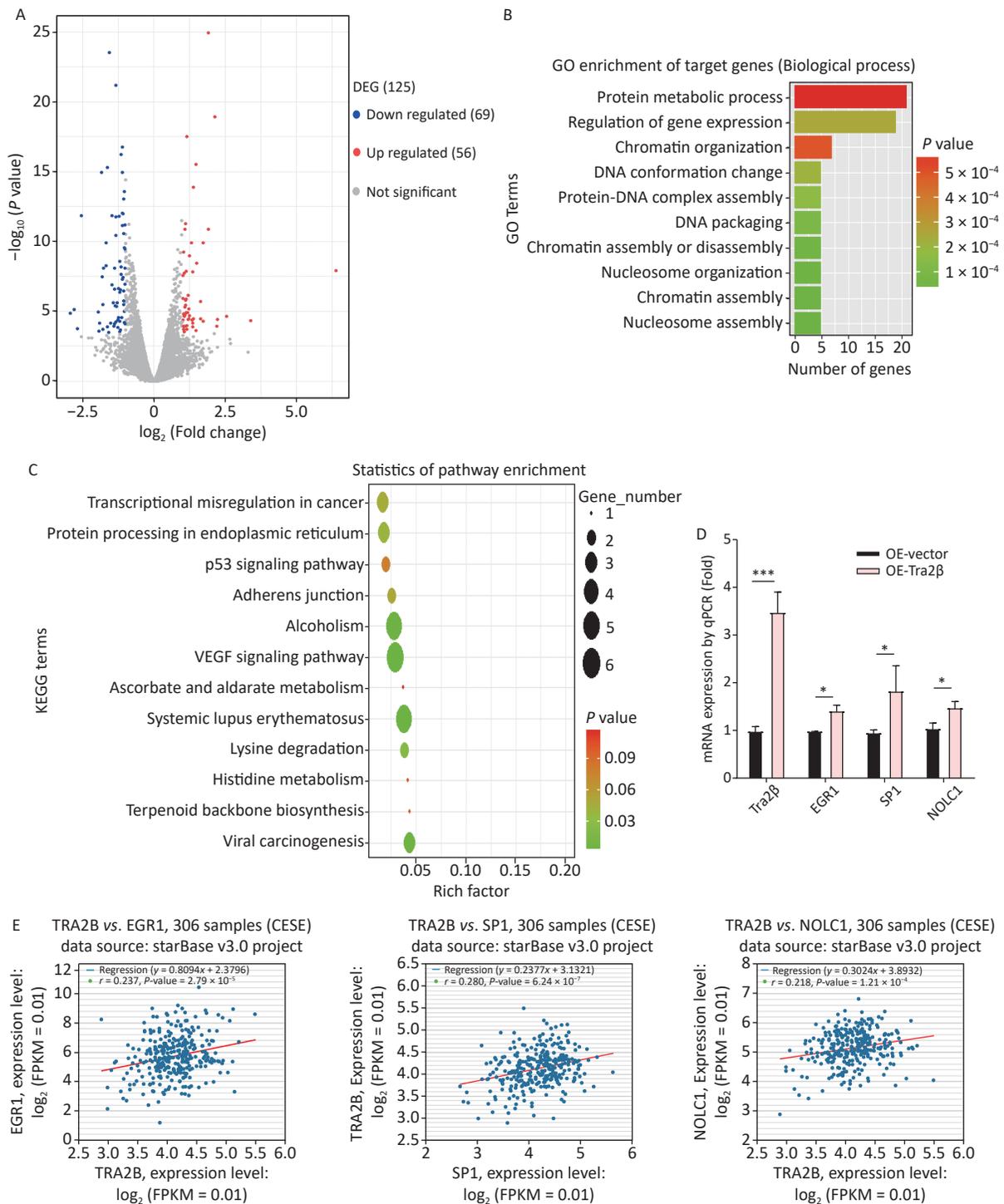
was conducive to the biological activity of transcription factor SP1. Functional rescue assays were performed by using Western blot, colony formation, and MTT to clarify whether SP1 contributes to the promotion effects of cervical cancer cell growth by Tra2 $\beta$ . As shown in Figure 6F, SP1 siRNAs effectively diminished SP1 expression in Siha cells and, to a lesser extent, in Hela cells. Tra2 $\beta$  overexpression (OE-Tra2 $\beta$  + si-NC group) dramatically upregulated SP1 expression when compared with the OE-control + si-NC group. On the contrary, the overexpression of SP1 (si-Tra2 $\beta$  + OE-SP1) alleviated SP1 deficiency compared with the si-Tra2 $\beta$  + OE-control group. Therefore, SP1 knockdown hindered the proliferation of Hela and Siha cells by Tra2 $\beta$  overexpression (Figure 6G and H). Collectively, these findings indicated that the Tra2 $\beta$ /SP1 axis promotes cervical cancer cell growth.

## DISCUSSION

The occurrence and development of cervical cancer is a broad pathological process, which involves not only the changes of proto-oncogenes E6



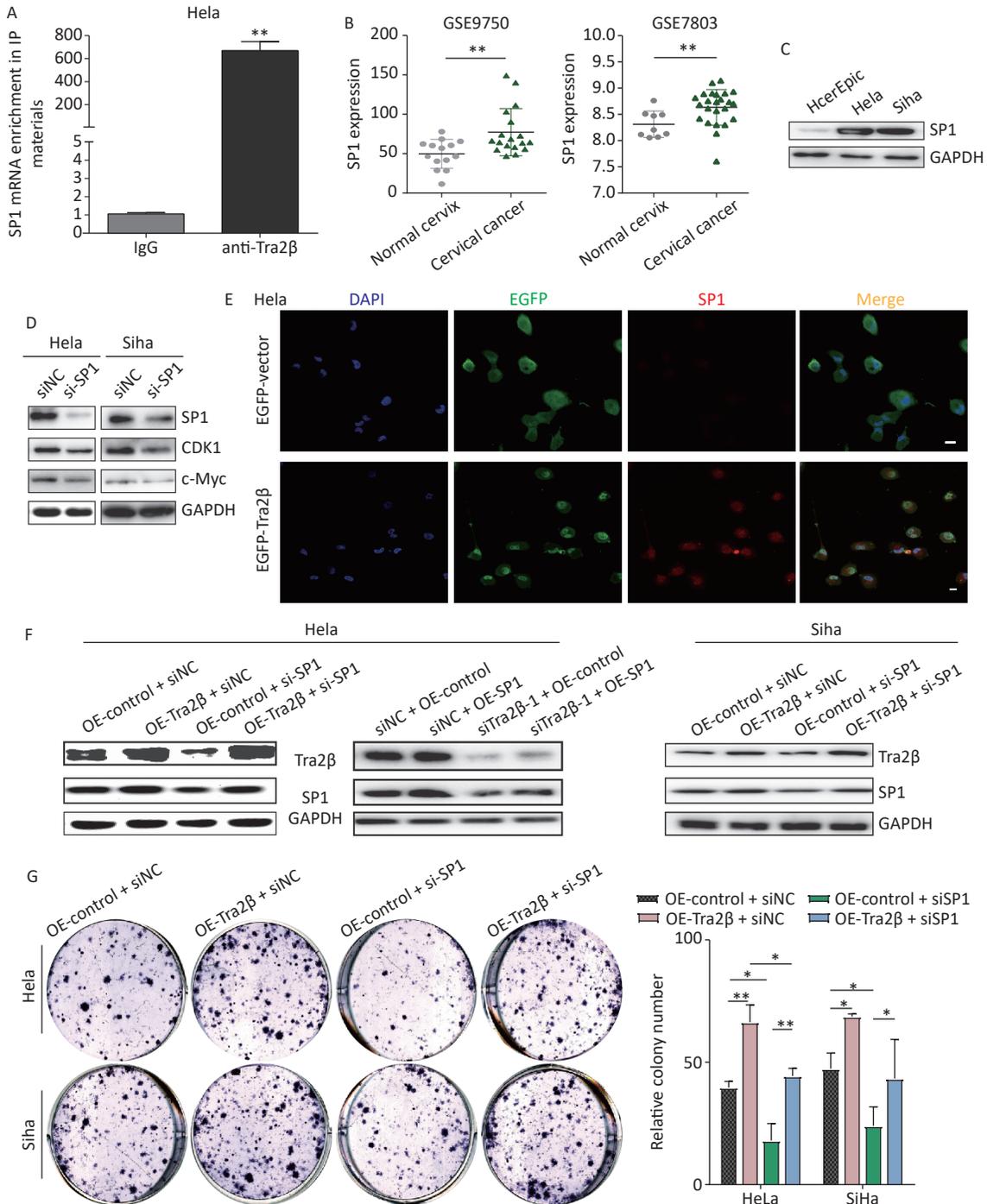
**Figure 4.** Tra2 $\beta$  promoted the tumor growth of cervical cancer *in vivo*. (A) Formation of tumors in nude mice injected with Tra2 $\beta$ -overexpressed or control Hela cells. (B) Measurement of subcutaneous tumor weight. (C) Tumor growth curves of nude mice during the experiment. (D) Tra2 $\beta$  and Ki67 proliferation index analysis in tumors by immunohistochemistry. Magnification of (D):  $\times 400$ . Two-way ANOVA with Dunnett comparison (C) and unpaired *t*-test (B) were used, \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

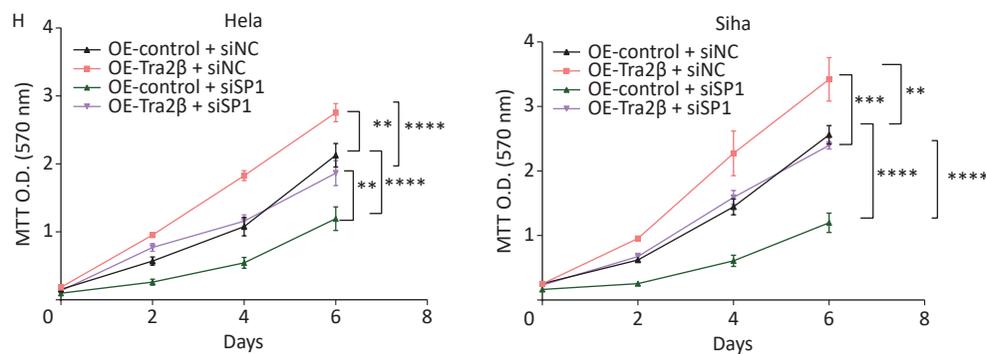


**Figure 5.** Tra2β could regulate *EGR1*, *SP1*, and *NOLC1* expression. (A) Volcano plot of global DEGs between Hela cells with stable Tra2β-overexpressed and control cells. (B–C) The function of DEGs was predicted by analysis using GO (B) and KEGG (C) by DAVID Tools (<https://david.ncifcrf.gov/>). (D) Relative mRNA levels of *Tra2β*, *EGR1*, *SP1*, and *NOLC1* in Hela cells with stable Tra2β-overexpressed or control cells were used to perform RT-qPCR experiments. The mRNA level of *GAPDH* was set as the internal control. (E) The correlation among *EGR1*, *SP1*, *NOLC1*, and *Tra2β* in 306 patients with CESC was analyzed by using the ENCORI database (<http://starbase.sysu.edu.cn/index.php>). Two-way ANOVA with Dunnett comparison (D) was used, \* $P < 0.05$ , \*\*\* $P < 0.001$ .

and E7 that promote the degradation of tumor suppressors p53 and pRb<sup>[20]</sup>, but also the intricate changes in the expression of various regulatory proteins that play a key role in cell proliferation, differentiation, drug resistance, apoptosis, migration, and invasion. Despite the development of treatments for cervical cancer, such as surgical resection, chemotherapy, and radiotherapy,

treatment failure and mortality in patients with cervical cancer remain high. Some recent reports have revealed that the abnormal expression of splicing factors in cancer cells might have important therapeutic significance<sup>[21]</sup>. Tra2 $\beta$  protein expression is important for cancer cell biology, and whether it exerts pro-tumor or anti-tumor effects depends on the important proteins and tumor type that it





**Figure 6.** SP1 was critical for Tra2β to promote the proliferation of HeLa and SiHa cells *in vitro*. (A) RIP enrichment was determined as RNA associated with Tra2β IP relative to the IgG control. The data represent the average and standard deviation of three independent experiments. (B) SP1 expression in normal cervical and cervical cancer tissues from GSE7803 and GSE9750. (C) SP1 expression level in the indicated cells was determined using Western blot analysis. (D) HeLa and SiHa cells were transfected with SP1 interfering RNA or scrambled small interfering RNA, and the level of downstream genes was determined using Western blot analysis. (E) HeLa cells were transfected with either pCMV-EGFP-Tra2β1 (EGFP-Tra2β) or pCMV-EGFP empty vector (EGFP-vector) and then subjected to immunofluorescence confocal microscopy for the subcellular localization of EGFP (Green) and endogenous SP1 (Red) proteins. DAPI was used for nuclear staining. Bar: 10 μm. (F–H) Functional rescue assays were performed as indicated by Western blot assay (F), colony formation assays (G), and MTT assays (H). Mann–Whitney test was used (A). Two-way ANOVA with Sidak comparison (G and H) was used. The data shown are the mean ± SD of three independent experiments. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .

regulates<sup>[16,22]</sup>. Watermann et al. reported that the significant induction of Tra2β at mRNA and protein levels is associated with invasive breast cancer<sup>[23]</sup>. Kuwano et al. reported that Tra2β inhibits the apoptosis of human colon cancer cells by upregulating the expression of Bcl-2 through its competition with miR-204 and the sensitivity of human lung adenocarcinoma cells (A549) to anticancer drugs<sup>[24]</sup>. The downregulation of Tra2β modified alternative splicing of *CD44* pre-mRNAs and inhibited cell growth from regulating cellular oxidative response in a gastric cancer cell line<sup>[25]</sup>. Data from GEPIA showed that *Tra2β* expression was higher in cervical carcinoma tissues than that in normal cervical tissues, and the expression of *Tra2β* was not associated with tumor stages (Figure 1A–B). However, patients with high *Tra2β* mRNA expression had better OS ( $P = 0.014$ , Supplementary Figure S1). The abovementioned findings and bioinformatic analysis of the clinical significance of Tra2β in cervical cancer led us to wonder whether Tra2β played oncogenic or tumor-suppressive roles in cervical cancer progression. Combining MTT, colony formation, Transwell assays, and nude mouse tumor formation experiments, we clearly demonstrated that Tra2β plays an important role in the growth of cervical cancer cells (Figures 1, 2, and 4) and that

Tra2β had no effect on cell migration, invasion, and apoptosis (Figure 3, Supplementary Figure S2B). The promotive effects of Tra2β on cell viability and proliferation might be mediated by the upregulation of transcription factor SP1 (Figures 5 and 6).

Previous studies have demonstrated the function of SP1 as a transcription factor, contributing to cell proliferation and colony-forming capacity<sup>[26]</sup>. Deng et al. showed that high SP1 expression was positively correlated with advanced International Federation of Gynecology and Obstetrics stage, lymph node metastasis, and lymphovascular space invasion of cervical cancer<sup>[19]</sup>. Nearly all cases of cervical cancer were due to HPV infection, particularly HPV16 and HPV18. The oncogenic potential of high-risk HPV types 16 and 18 depends on the expression of two viral oncogenes, E6 and E7. The HPV-18 upstream regulatory region contains SP1-binding sites, which have been shown to control the expression of E6 and E7 genes<sup>[27]</sup>. The downstream target genes of SP1 include well-known cyclin-dependent kinase inhibitors p27 and p21; CDK1, which is essential for G1/S and G2/M phase transitions of the eukaryotic cell cycle<sup>[19]</sup>; and oncogenes Bmi1, c-Myc, KLF4, and ABCG2, which were related to the acquisition of cancer stem cell phenotypes. Hence, we first hypothesized and focused on the possibility that

Tra2 $\beta$  might regulate the proliferation of cervical cancer cells by regulating the expression of SP1 and strived to verify this hypothesis. As shown in Figure 6, we have established a functional link between Tra2 $\beta$  and SP1 and demonstrated that the function of Tra2 $\beta$  in the regulation of the proliferative ability of HeLa and SiHa cells is dependent on SP1 expression. The knockdown of SP1 accelerated the overexpression of SP1 and inhibited G2/M phase arrest in cervical cancer cells<sup>[19]</sup>, indicating that Tra2 $\beta$  might play an important regulatory role in radioresistance and drug resistance in patients with cervical cancer who receive radiotherapy or chemotherapy. Metformin, an anti-diabetes drug, has been shown to induce the downregulation of SP1 and significantly reduce the mortality rate in elderly diabetic women diagnosed with cervical cancer<sup>[28]</sup>. If we can disrupt the stimulation of SP1 by Tra2 $\beta$  in cervical cancer, then the therapeutic effect of metformin is improved in elderly patients with cervical cancer and diabetes. Furthermore, the mechanism through which Tra2 $\beta$  regulates SP1 transcriptional levels must be investigated, whether by regulating alternative splicing, stabilizing mRNA, or enhancing transcription.

Considering that the G/A-rich enhancer elements are often found biologically important in alternatively spliced tumor genes, a total of 125 DEGs induced by Tra2 $\beta$  overexpression were screened in our study, and GO and KEGG analyses of DEGs revealed changes in biological functions and metabolic pathways, including the regulation of gene expression, transcriptional misregulation in cancer, VEGF signaling pathway, and p53 signaling pathway, which were related to the occurrence and development of malignant tumors<sup>[29,30]</sup>. Therefore, the changes in biological behavior in cervical cancer progression promoted by Tra2 $\beta$  may be broader phenotypic changes rather than a single regulatory effect.

Among the prognostic subgroups of cervical squamous cell carcinoma, patients with high Tra2 $\beta$  expression had better OS, and no significant association was observed between Tra2 $\beta$  expression levels and RFS (Supplementary Figure S1). First, Tra2 $\beta$  has no effect on the migration and invasion ability of cervical cancer cells (Figure 3), and tumor metastasis was the main cause of death in patients with cervical cancer, which may explain the small effect of Tra2 $\beta$  on prognosis. Second, our phenotypic results were obtained from immortalized cervical cancer cell lines and nude mice xenograft tumor models, excluding the role of tumor immunity. Third, the prognosis of

cervical cancer is related to perioperative factors, including anesthesia and anesthesia technology. Zhang et al. reported that sevoflurane enhances the proliferation and metastatic potential of cervical cancer cells by upregulating histone deacetylase 6, thereby increasing recurrence risk<sup>[31]</sup>. Another possible factor is the distinct cellular sublocalization of Tra2 $\beta$  in patients with cervical cancer. Li et al. demonstrated that the nuclear and cytoplasmic localizations of the Tra2 $\beta$  protein were correlated with its phosphorylation status. When Tra2 $\beta$  proteins are localized in the nucleus, active transcription and splicing occur. However, when Tra2 $\beta$  proteins shuttle to the cytoplasm, translational regulation, mRNA stability, and mRNA localization are their primary functions<sup>[32]</sup>. Liu et al. pointed out the potential role of Tra2 $\beta$  in mRNA trafficking, leading to the localized expression of the protein, which could influence cancer development<sup>[33]</sup>. Boris et al. reported that only high nuclear expression levels of splicing factor Tra2 $\beta$  have a direct regulatory influence on alternative mRNA processing and pronounced effects on the cellular function and tumor biology of the affected tumors, leading to poor patient outcomes<sup>[12]</sup>. These factors were cross-talked, which led to phenotypic changes. Our study has some limitations. For example, only two immortalized cervical cancer cell lines were used in the current study. Consequently, the biology of these cells is not as newly isolated as that of primary tumor samples from patients. The present study was focused on the molecular mechanism of Tra2 $\beta$ -induced cervical cancer cell malignancy in cell culture experiments and nude mouse models, whereas whether potential phenotype changes may occur during clinical trials and the mechanism through which Tra2 $\beta$  affects disease progression in patients with cervical cancer remain unknown and deserve further investigations.

Our study demonstrated the function of the Tra2 $\beta$ /SP1 axis in cervical cancer *in vitro* and *in vivo*, which provides a comprehensive understanding of the pathogenesis and improves the treatment strategy of cervical cancer.

#### CONFLICT OF INTEREST

None.

#### CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

LI Mo Juan: Study design and execution, data analysis, writing-original draft, writing-review, and editing. XIONG Dan: Formal analysis, writing-original

draft, Writing-review and editing. WANG Shuai: Data curation, software, formal analysis. HUANG Hao: Funding acquisition, conceptualization, supervision, writing-review, and editing.

### CONSENT FOR PUBLICATION

The manuscript is approved by all authors for publication.

### AVAILABILITY OF DATA AND MATERIAL

Datasets used and/or analyzed in this study can be obtained from the corresponding author upon reasonable request.

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