Analysis of chromatin accessibility in $p53$ deficient spermatogonial stem cells for high frequency transformation into pluripotent state

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Abstract

Objectives: Spermatogonial stem cells (SSCs), the germline stem cells (GSCs) committed to spermatogenesis in niche, can transform into pluripotent state in long-term culture without introduction of exogenous factors, typically in $p53$ deficiency condition. As the guardian for genomic stability, $p53$ is associated with epigenetic alterations during SSCs transformation. However, the mechanism is still unknown, since complicated roles of $p53$ baffle our understanding of the regulating process.

Materials and Methods: The chromatin accessibility and differentially expressed genes (DEGs) were analysed in $p53^+/+$ and $p53^{-/-}$ SSCs using the Assay for Transposase-Accessible Chromatin with high-throughput Sequencing (ATAC-seq) and RNA-sequencing (RNA-seq), to explore the connection of $p53$ and cell fate at chromosomal level.

Results: Several transcription factors (TFs), such as CTCF, SMAD3 and SOX2, were predicted as important factors mediating the transformation. Molecular evidence suggested that SMAD3 efficiently promoted pluripotency-associated gene expression both in fresh and long-term cultured SSCs. However, $p53$ knockout (KO) is insufficient to induce SMAD3 expression in SSCs.

Conclusions: These observations indicate that SMAD3 is a key factor for SSCs transformation, and an unknown event is required to activate SMAD3 as the prerequisite for SSCs reprogramming, which may occur in the long-term culture of SSCs. This study demonstrates the connection of $p53$ and pluripotency-associated factors, providing new insight for understanding the mechanisms of SSCs reprogramming and germline tumorigenesis.
INTRODUCTION

Testicular germ cell tumours (TGCTs) are rare among pediatric ages, making up 0.5% of pediatric malignancies, but rise to 14% in adolescent malignancies, and become the most common solid tumour in young adults, representing 0.4% of new cases from all sites. The incidence rate of testicular germ cell tumours starts to increase in the late teens (10 years old) and reaches its peak in the young adult age group. The underlying mechanism of germ cell transformation into tumour cell is not clear, yet. Notably, p53 deficient mice have a high frequency of testicular teratoma, and clinical observations showed that p53 dysfunction is usually concomitant with enhanced expression of NANOG in TGCTs. Therefore, TGCTs are possibly associated with p53 dysfunction and the dedifferentiation of SSCs from male puberty to adult.

SCCs are germline stem cells with capacities of self-renewal and production of functional sperm through multi-steps of differentiation. SSCs are believed to be unipotent when they reside in their microenvironment (also called niche), since their fates are under the control of signals from the niche. However, transformation of SSCs into embryonic stem cells-like (ES-like) state is occasionally observed during long-term culture in vitro. The morphology of the transformed cells is distinct from a typical SSCs cluster, but very similar to embryonic stem cells (ESCs) colony. Moreover, the expression of germine markers is hardly detected. Instead, the pluripotent markers, such as Nanog, Sox2, are highly expressed in the transformed ES-like cells. Subcutaneous injection of ES-like cells into nude mice could form teratoma with a comparable efficiency with ESCs, which confirms their pluripotency identity.

This special phenomenon is interesting and important, since the dedifferentiation process does not rely on any transgenic operation or stimulation by chemicals. In contrast to reprogramming using Yamanaka factors, the underlying mechanism of SSCs transformation is still ambiguous. Oct4, a Yamanaka factor essential for pluripotency, is ubiquitously expressed in germine, including primordial germ cells (PGCs), SSCs, female germline stem cells (FGSs) and oocytes. However, the endogenous expression level of Oct4 is relatively low in wild type of SSCs compared to ESCs or transformed SSCs, according to published studies and observation in our laboratory. Enhanced Oct4 expression is essential for the transformation of primed ESCs to higher hierarchy, naive state, which indicates that the alteration of Oct4 expression level may play a key role in SSCs transformation. Moreover, Shinohara and his colleagues noticed that the loss of p53 improved the transformation efficiency of SSCs. Further studies revealed that epigenetic modification played an important role in SSCs transformation, and explained that p53 deficiency rescued SSCs from extensive cell apoptosis during transformation induced by the rewriting of DNA methylation profiles in SSCs. However, they also commented that the underlying mechanism was more complicated than that, since knockdown of Bax failed to promote SSCs transformation into pluripotent state. Notably, the activity of p53 has been identified as an effective factor for cell reprogramming, since activated p53 could suppress the expression of Nanog, a key pluripotent gene, and p53 is pivotal in maintenance of the genomic stability. Therefore, p53 is believed as a key bottleneck for reprogramming, since overexpression of the reprogramming factor (OCT4, SOX2, KLF4 and c-MYC, which are oncoproteins) always activates p53 to cause cell cycle arrest, apoptosis and senescence, and simultaneously suppresses the expression of Nanog in somatic cells.

Based on these observations, we proposed that the impact of p53 deficiency on chromatin accessibility is pivotal to elucidate the mechanism of p53 in the suppression of pluripotency transformation. However, it is complicated to reveal the exact roles of p53 in reprogramming, since p53 targets on multiple regions of chromosomes. In recent years, Assay for Transposase-Accessible Chromatin with high-throughput Sequencing (ATAC-seq) has been developed to explore the link between chromatin accessibility and biological phenomenon. By analysing the open regions of chromatin, the genomic regions with altered chromatin accessibility could be profiled and allow the identification of potential transcriptional regulators involved in cellular reprogramming.

Here, we employed ATAC-seq to compare the difference of transcription active regions in the chromatin of p53+/− and p53−/− SSCs, to explore the underlying connection between the p53 deficiency and transformation into pluripotent state at chromosomal level. RNA-seq and molecular assays were subsequently exerted to verify the predicted genes and related pathways associated with SSCs transformation. This result enhances our further understanding of the connection of chromatin accessibility mediated by p53 and SSCs fates, which provides a new insight into the prevention and curing of testicular tumours.

MATERIALS AND METHODS

2.1 Mice

The p53−/− transgenic allele-carrying mice were purchased from the Shanghai Model Organisms Center, and C57BL/6 mice were supplied by Yangzhou University. For the genotyping of p53−/− mice, genomic DNA samples extracted from mouse tail tips were used for polymerase chain reaction (PCR) as follows: The touchdown-PCR was carried out according to the following cycling programme: 94°C for 2 min, followed by 10 cycles at decreasing annealing temperatures in decrements of 0.5°C per cycle, 94°C for 20 s, 65°C for 15 s and 68°C for 10 s, then 25 cycles of 15 s at 94°C, 15 s at 60°C, 10 s at 72°C, and final extension at 72°C for 2 min (primer 1: TGGATGGTGGTATACTCAGAGC, primer 2: CAGCCCTCTGGTCCACATACACT, primer 3: AGGCCCTAGAGGTCACACTG).
2.2 | SSCS purification using Fluorescence-Activated Cell Sorting (FACS)

Testes from 5-day-old p53+/+ or p53−/− mice were harvested for SSCS sorting using the protocol of the previous study.21 Briefly, tunica albuginea removed testes were sliced into small pieces and digested with collagenase IV at 37°C in a water incubator for 20 min. After washing with D-Hanks, the seminiferous tubule fragments were incubated with 0.05% trypsin at 37°C for 5 min in a water incubator. After removal of the enzyme solution via centrifugation, the cell pellet was resuspended and filtered with a 70-μm filter. The cell sample was washed with phosphate-buffered saline (PBS) and was resuspended with FACS buffer at a concentration of 1 × 10^7 cells/ml, followed by incubation with anti-THY1 and anti-c-kit antibodies at 4°C for 0.5–1 h. After centrifugation and removal of the antibody-containing supernatant, the cells were re-suspended in FACS buffer for FACS sorting. The THY1+c-kit+ fraction was collected for centrifugation and resuspended to a desired concentration before plated on mouse embryonic fibroblast (MEF) feeder layers. The protocol for preparing MEF was as previously described.21

2.3 | Cell culture and transformation

Both p53+/+ and p53−/− SSCSs were able to be maintained in Shinohara's Iscove's Modified Dulbecco's Medium (IMDM)/foetal bovine serum (FBS) culture medium22 for 30 passages with a minor modification. Briefly, insulin, putrescine and transferrin were replaced with N2. Isolated SSCSs were placed on MEF feeder layers and were subcultured every 5–6 days for 6–8 passages, and every 3 days later.

A few ES-like colonies formed around 25 passages, and these colonies were picked under microscope and transferred to ESC culture medium. It took around 5–7 days for the subculture of ES-like cells in the first several passages, and the average subculture time reduced to 3 days after 10 passages. The components of modified Shinohara's GSC medium and ESC medium are summarized in Table S1.

2.4 | SSCSs labelling and transplantation

SSCSs cultured on MEF for more than 12 passages were infected with green fluorescent protein (GFP) expressing lentivirus. The lentivirus package was identical to that used in a previous study.23 The uninfected SSCSs were eliminated with puromycin, and the injection procedure followed the reported protocol24 with minor modifications: the GFP-labelled SSCSs were digested into single-cell suspension and filtered with a 70-μm filter, and trypan blue was added to monitor the cell injection efficiency.

Immunofluorescence (IF) staining, alkaline phosphatase (AP) staining and Western blotting.

The protocol for IF assay was identical to that given in a previous study.25 Briefly, cells were fixed with Carnoy for 20 min at −20°C and were rinsed with neutral PBS for three times before blocking with 10% goat serum for 30 min at room temperature. Cells were incubated with primary antibodies at 4°C overnight and were incubated with appropriate secondary antibodies for 1 h after rinse. Finally, DAPI (4′,6-diamidino-2-phenylindole) was used for counterstaining.

The BCIP/NBT (5-bromo-4-chloro-3-indoly-phosphate/nitro blue tetrazolium)/alkaline phosphatase staining kit (Beyotime, C3206) was used to detect alkaline phosphatase activity. Briefly, ESCSs, ES-like cells from p53+/+ or p53−/− SSCSs and primary SSCSs were rinsed with PBS and incubated with BCIP/NBT solution for 30 min in dark. After the removal of BCIP/NBT solution, the cell samples were rinsed with Millipore H2O to terminate staining, and finally were analysed under the microscope.

The protocol for Western blotting identical to previously described21 was briefly listed: protein lysates were separated with sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels, and the gels were transferred to nitrocellulose membranes for blotting. Nitrocellulose membranes were blocked in 5% milk for 1 h prior to the addition of primary antibody at 4°C overnight and then were rinsed twice with TBST (Tris-buffered saline with Tween 20). Peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG was used to detect the primary antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) and exposed to the film. The intensity of the bands was quantified using the ImageJ software.

The information of antibodies used for IF and Western blot is listed in Table S2.

2.5 | Reverse transcription-polymerase chain reaction (RT-PCR)

For reverse transcription, total RNA extracted from SSCSs with TRNZol (Tiangen, DP424) was converted into complementary DNA (cDNA) using GoScript™ Reverse Transcription System (Promega, A5001). Subsequently, PCR was performed using Premix Ex Taq (Takara). The information of primers is listed in Table S3.

2.6 | Transfection

The pcDNA3-FLAG-Smad3 expression vector was constructed, as described previously.26 The protocols for transfection assays were identical to those used in a previous study.25 Briefly, for the overexpression of SMAD3, SSCSs after 20 passages were transfected with pcDNA3-FLAG-Smad3 or empty vector using Lipofectamine 3000 (Thermo Fisher, L3000015), and the samples were harvested 48 h posttransfection for Western blot analysis. Similarly, the scrambled or Smad3 siRNA (5′-GAGAUUCGAUUGCGUAATT-3′27) was transfected into newly isolated or long-term cultured SSCSs using
Lipofectamine 3000, and the cells were harvested 48 h posttransfection for gene expression analysis.

2.7 ATAC-seq and data analysis

THY1+ and c-Kit+ cells were collected from p53+/− and p53−/− mice using flow cytometry and amplified on MEF for 5–6 passages. Around 1x10⁶ cells were collected for the ATAC-seq assay. ATAC-seq library preparation and sequencing were performed according to previously described. All paired-end reads were first subjected to adaptor trimming using cutadapt (v2.10). Then, the clipped reads were aligned to the mouse genome (10 mm) using bowtie2 (v2.3.3.1) with the parameters: -t -q -N 1 -L 25 -X 2000 no-mixed discordant. PCR amplicon duplicates were then removed using PicardTools (v2.2.4). For downstream analysis, nonuniquely mapped reads or reads mapped to the mitochondrial genome, Y chromosome and unmapped contigs were removed. To visualize the ATAC-seq signal in Integrative Genomics Viewer (IGV, v2.5.3), bam files were converted to bigwig (BW) files using deetools (v3.3.0). Peaks were called for each sample using MACS2 (v2.2.6) in narrowPeak mode. Peaks overlapping with encoded backlist regions (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeMapability/wgEncodeDacMapabilityConsensusExcludable.bed.gz) were removed using bedtools (v2.26.0–148-gd1953b6). We performed differential chromatin accessibility (DA) analysis using the DiffBind package with the DESeq2 algorithm. DA was defined with the criteria of a false discovery rate (FDR) <0.05 and absolute log2 (fold change) ≥1. To identify TF motifs in p53 KO-enriched peaks enriched in p53 KO SPCs, a homer (v4.10.4) de novo motif analyser was used by setting parental enriched peaks as the background. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID).

The original data of ATAC-seq assay have been uploaded to https://www.ebi.ac.uk/fg/annotare/, and E-MTAB-10012 is the code to review the original data.

2.8 RNA-seq and data analysis

Around 10⁵ p53+/− and p53−/− SSCs were collected using the identical protocols for ATAC-seq assay. RNA-seq library preparation and sequencing were performed according to previously described. Total RNA was extracted using Trizol (Ambion Life Technologies) according to the Ambion standard RNA isolation procedure, and messenger RNA (mRNA) was purified using the NEBNext Poly (A) mRNA Magnetic Isolation Beads (NEB, USA). Then, the mRNA library was constructed with a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7420S/L, NEB) and sequenced with Illumina HiSeq 2000. DEGs analysis was performed to compare p53+/− and p53−/− SSCs using the DESeq2 R package. DEGs were defined with the criteria of q-value <0.05 and absolute log (fold change) ≥1.5 (p < 0.03). Gene ontology (GO) and KEGG analysis were performed with DAVID.

For analysis, the connection of chromatin change and DEGs in Venn diagram, individual peaks separated by <100 bp were joined together using bedtools. Peak annotation was performed using HOMER. The duplicate genes in the RNA-seq results have been removed for Venn analysis.

The original data of RNA-seq assay have been uploaded to https://www.ebi.ac.uk/fg/annotare/, and E-MTAB-10608 is the code to review the original data.

2.9 Quantification and statistical analysis

Data were analysed by Excel and were presented as mean ±SD (standard deviation), and statistical significance was determined by the t-test.

3 RESULTS

3.1 Collection and verification of SSCs from p53 deficient mouse

Based on several protocols that can efficiently enrich SSCs and achieve SSCs long-term culture, we sorted SSCs (Figure 1 A-B). They express undifferentiated spermatogonia markers (Figure 1C) and SSCs marker ID4 (Figure 1D–C), could be maintained on MEF feeder layer for more than 30 passages in IMDM/FBS condition and restore the capacity to reconstitute the fertility of busulfan-treated mice (Figure 1D–F). Interestingly, we frequently observed that a few SSCs always transformed into ES-like state around 25 passages under this culture condition, and these colonies could be stably maintained on MEF feeder layers (Figure 1D) with a higher proliferation ratio than untransformed SSCs (Figure 1E). SSCs spontaneous reprogramming was reported in 2004, despite the fact that their culture medium was slightly different. Shinohara and his colleagues also reported that the p53 deficiency remarkably increased the transformation efficiency, and indicated that this event was associated with epigenetic change caused by p53 loss. Therefore, we focused on the role of p53 in regulating SSCs fate, to further demonstrate the molecular mechanism of SSCs reprogramming, and to reveal the connection of p53 expression with age and SSCs fate. First, SSCs isolated from 5-day-, 30-day- and 42-day-old mice were examined using RT-PCR, to track the expression change of p53 in SSCs of neonatal, juvenile and young adult testes. The expression level of p53 in SSCs of 30-day testes decreased by 35% compared to that in SSCs of 5-day testes, and decreased by about 60% in SSCs harvested from 42-day testes (Figure 1F,G). On the contrary, expression of Nanog remarkably increased with age at mRNA level (Figure 1F,G). Expression of NANOG was not detected in SSCs at protein level using Western blot (data not shown). These observations suggested...
FIGURE 1 Identification of spermatogonial stem cells (SSCs) from $p53^{+/+}$ or $p53^{-/-}$ mice. The schematic illustration of SSCs sorting is exhibited (A), and a representative of SSCs sorting using fluorescence-activated cell sorting (FACS) (PE: c-kit, PerCP: THY1) (B). Sorted cells were identified for SSCs markers using reverse transcription-polymerase chain reaction (RT-PCR), M. marker, T. testis, S. SSCs, N. negative control (C). The morphologies of SSCs of the first passage, transformed embryonic stem cells-like (ES-like) cells (29 passages) and embryonic stem cells (ESCs) colonies is exhibited (D). The growth curves of SSCs and ES-like are exhibited (E). SSCs sorted from 5-day-, 30-day- and 42-day-old mice were determined for $p53$ and Nanog expression using RT-PCR (F), and the results were statistically exhibited ($n = 3$, *$p < 0.05$, **$p < 0.01$) (G). A representative result of genotyping for the litters at 3 days postpartum using PCR (H). The SSCs sorted from 5-day-old $p53^{+/+}$ (I) or $p53^{-/-}$ mice (J) were cultured on mouse embryonic fibroblast (MEF), and the morphology was exhibited. Immunostaining of promyelocytic leukaemia zinc finger (PLZF) was used to verify SSCs from $p53^{+/+}$ (K) or $p53^{-/-}$ mice (L), and the percentage of PLZF+ cells was statistically analysed (M). RT-PCR was used to detect the expression of $p53$, Id4, Plzf, Mvh and Gapdh in SSCs from $p53^{+/+}$ and $p53^{-/-}$ mice ($n=3$) (N). The data represent the means ± SD (*$p < 0.05$; **, $p < 0.01$). Scale bar = 20 µm.
that the expression of p53 and the potential of pluripotency transformation may increase in SSCs during ageing. Considering that p53 loss promotes the spontaneous reprogramming of SSCs into pluripotent state, we hypothesize that the increased rate of tumorigenesis in adolescent and adult testes is possibly associated with p53 loss or dysfunction in SSCs.

To understand the potential mechanism of increased transformation efficiency caused by p53 deficiency, we harvested SSCs from testes of 5-day-old p53<sup>−/−</sup> and p53<sup>−/+</sup> mice for investigation (Figure 1H). Testes were digested to single-cell suspension for SSCs sorting using FACS, and THY1<sup>c-kit</sup> populations were collected for identification, and both p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs formed typical SSCs clusters (Figure 1I,J). The purity of sorted cells was determined using promyelocytic leukaemia zinc finger (PLZF) (the marker of undifferentiated spermatogonia) IF staining (Figure 1K,L), and statistical analysis revealed that the PLZF<sup>+</sup> ratio is approximate to 93% (93.6 ± 2.9% in p53<sup>+</sup>/+ SSCs and 94.6 ± 1.4% in p53<sup>−/−</sup> SSCs) (Figure 1M). The expression levels of p53, SSCs markers Id4 and Plzf, germline marker Mvh were determined using RT-PCR, which revealed identical expression levels of SSCs markers Id4 and Plzf in p53<sup>+</sup>/+ and p53<sup>−/−</sup> SSCs, but Mvh expression was remarkably decreased (Figure 1N). Therefore, we confirmed that SSCs-enriched populations were collected, and p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs were indistinguishable for their morphological characteristics, but the expression of molecular markers was probably not identical.

3.2 Verification of the transformation capacity of p53 deficient SSCs

Before exploring the transformation mechanism, we tested the transformation capacity of p53 deficient SSCs under in vitro condition. According to the protocol (Method/Cell culture and transformation, and Table S1), both p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs were able to form ES-like state before 30 passages, and they formed typical ES-like colonies when transferred into ESC medium (Figure 2A–C), which was consistent with reported observations. Moreover, the expression levels of germline and pluripotent markers were also altered: high expression levels of pluripotent marker genes, including OCT4, NANOG and SOX2, were detected in both ESCs and ES-like cells derived from p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs, confirming the transformation into pluripotent state. The undifferentiated spermatogonia marker PLZF was only detected in untransformed SSCs, and a very low level of germline marker mouse vasa homologue (MVH) was detected in ES-like cells derived from p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs (Figure 2D), indicating that the expression profile of transformed ES-like cells was similar to that of pluripotent cells, despite the fact that a few of the germ cells still remained in the cell mixture. Meanwhile, NANOG IF staining was employed to further test the purity of transformed cells, and the NANOG<sup>+</sup> ratio was approximate to 100% in ES-like cells derived from p53<sup>−/−</sup> or p53<sup>−/+</sup> SSCs (Figure 2E,F). Finally, ESC, ES-like cells derived from p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs and untransformed SSCs were subcutaneously injected into nude mice for teratoma assay. Teratomas were observed in recipients of ESCs, p53<sup>−/−</sup> and p53<sup>−/+</sup> ES-like cells, but not in SSCs recipients (Figure 2G–J), and the histology of teratoma was analysed (Figure 2K). Moreover, strong alkaline phosphatase signals were detected in ESCs, p53<sup>−/−</sup> and p53<sup>−/+</sup> ES-like cells (Figure 2L–N), but not in SSCs after 10 passages (Figure 2O). These observations suggested that p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs have transformed into pluripotent state, which confirmed the spontaneous reprogramming capacity of SSCs.

3.3 The landscape of chromatin accessibility

Previous studies revealed the connection of epigenetic events and SSCs transformation into pluripotent state, but the exact mechanism, especially the role of p53, is still largely unknown. Here, we confirmed SSCs’ identity and their transformation potential during long-term culture, and subsequently harvested SSCs from p53<sup>−/−</sup> and p53<sup>−/+</sup> neonatal mice for ATAC-seq analysis, to detect the change of genomic chromatin accessibility caused by p53 deficiency. The ATAC-seq data from p53<sup>−/−</sup> and p53<sup>−/+</sup> SSCs were generated, and differential chromatin accessibility patterns were exhibited by the volcano map. Totally, 4,898 differentially accessible peaks were identified in p53<sup>−/−</sup>: SSCs when comparing its wild-type (WT) counterpart (FDR <0.05 and fold change ≥2) (Table S4, to obtain the details via this link: https://figshare.com/s/2663b87fd48c819c3ab9). Among them, 2798 regions with increased openness and 2100 areas with decreased openness were detected in p53<sup>−/−</sup>: SSCs (Figure 3A).

First, we noticed that accessibility of several pluripotency-associated genes was increased, including Nanog, Sox2, Mycn and Tgfb1 (Figure S2), confirming the link of p53 and expression of pluripotent genes. Subsequently, the genes with increased chromatin accessibility in p53<sup>−/−</sup> SSCs were analysed using the gene ontology (GO) analysis (p value <0.05). The Biological Process items of the canonical Wnt signalling pathway, Notch signalling pathway, embryo development, cellular response to the epidermal growth factor stimulus, cell fate commitment, cell-cell adhesion, and regulating type II RNA polymerase binding, were significantly enriched (Figure 3B). According to the Molecular Function, the genes associated with poly(A)RNA binding, GTPase activator activity, transcription activator and RNA polymerase II core promoter proximal region sequencing-specific binding were significantly enriched (Figure 3C). From the perspective of Cellular Components, genes in the nucleus, cytoplasm and cell membrane were most significantly changed (Figure 3D). In addition, KEGG results showed that the genes with increased accessibility were mainly involved in the regulation of stem cell pluripotency, cell cycle and Wnt signalling pathway (Figure 3E). These results were consistent with the phenotypes we observed: transformed cells gained pluripotency, increased in proliferation rate and colonies became more compacted (Figure 1 and Figure 2), compared to SSCs.

In addition, we made the computational prediction of TF potentially bound in the altered chromatin regions in p53<sup>−/−</sup> SSCs using the de novo TF motifs discovery software HOMER, yielding 29 transcription factors (Figure 3F). Among them, we noticed...
several transcription factors related to pluripotency, including CTCF, POU5F1: SOX2, SOX1, SMAD3, SMAD4, LHX2, NFATC2, E2F and E2F4, five embryonic development–related transcription factors, ARID3A, HNF1B, ZFP57, PRDM15 and DMRT3, two cell cycle–related transcription factors, MYBL and ELK4. The binding domain recognized by p53 became more open, indicating that the
transcriptional activity of its potential target sites might be increased after p53 deletion, which was in line with our expectation, and confirmed the reliability of ATAC-seq results. We also noticed that the most significant change in openness is CTCF, a key structural protein for the high-order chromatin folding of pluripotent stem cells. A recent study indicated that CTCF is an insulator-binding protein, which
The increased accessibility of CTCF-binding regions preliminarily indicated that SSCs tended to transform into pluripotency. Increased accessibility of domains recognized by TF POU5F1: SOX2 after p53 deficiency also implied the potential of transformation. POU5F1, also known as OCT4, is a key transcription factor for cell reprogramming. It has been reported that OCT4 was up-regulated by SOX2 during SSCs transformation, which coincided with our experimental results. Meanwhile, we analysed the motifs with increased accessibility in ESCs or induced pluripotent stem cells (iPSCs), and noticed that domains recognized by CTCF and OCT4 had a lower nucleosome occupancy, indicating the up-regulated transcriptional activity of these regions. This is consistent with our ATAC-seq data. Several members
of the Pit-Oct-Unc (POU) family, POU2F2, POU2F3, were also detected in ESCs, while the binding domain of POU3F4 was increased in p53 deleted SSCs (Figure 3F), implying a different mechanism of reprogramming in SSCs.

Importantly, the binding domains of SMAD3 and SMAD4, two key members of the transforming growth factor-β (TGF-β) signalling pathway, became more accessible, indicating that the transcriptional regulation of SMAD3 and SMAD4 on their targets may altered after formation.

In addition, the binding domain of E2F4 also became open. Under the stimulation of TGF-β, E2F4 can form a complex with SMAD3 to enter the nucleus, and combine with SMAD4 to regulate the expression of c-Myc. These results facilitated us to preliminarily understand the characteristics of gene expression changes in SSCs caused by p53 deletion. Therefore, we proposed that the increase of chromosomal accessibility of the regions recognized by SMAD3 and SMAD4 was an important event that led to pluripotency transformation of SSCs after p53 deletion.

### 3.4 The differential expression profiles of p53+/+ and p53−/− SSCs in transcriptome analysis

To examine the differential gene expression of p53+/+ and p53−/− SSCs, we performed RNA-seq analysis. Totally, 4726 differentially expressed genes (DEGs) after p53 deletion (q < 0.05, and absolute log (fold change) ≥1.5) were detected, including 1942 up-regulated genes and 2784 down-regulated genes (Table S5, to see full data, please use this link: https://figshare.com/s/a02ad4af1650888742f). The results are displayed and analysed by the volcano map (Figure 4A).

Furthermore, the gene ontology (GO) analysis was performed with the up-regulated or down-regulated genes. According to the results of the Molecular Function analysis, in p53 deleted SSCs, the expression of genes related to Notch signalling pathway, histone H3-K36 demethylation, extracellular matrix (ECM) organization and activation of the mitogen-activated protein kinase (MAPK) signalling pathway significantly increased, while the gene expression levels related to TGF-β signalling pathway inhibition, canonical Wnt signal activation, cell proliferation and stem cell maintenance were significantly down-regulated (Figure 4B, C). Subsequently, we analysed the differential expression genes in p53+/+ and p53−/− SSCs using KEGG. The results showed that the up-regulated genes in p53 knockout SSCs included the ECM-receptor interaction, focal adhesion, phosphatidylinositol signal system, Notch and gonadotropin-releasing hormone (GnRH) signalling pathways, etc., while the down-regulated genes mainly included AMP-activated protein kinase (AMPK), PI3K-AKT, Hippo and TGF-β signalling pathways (Figure 4D). These observations were consistent with ATAC-seq results, which revealed the increased accessibility in the genes of these signalling pathways, including Wnt, Notch, cell adhesion, etc. This indicated that p53 affected these genes via regulating chromosomal accessibility in SSCs. Meanwhile, we focused on the pluripotent genes with increased accessibility (Wnt10b, Zfhx3, Wnt10a, Wnt2b, Fzd2, Rip1, Dlx5, Esrb, Pik3r1, Isl1, Bmp2, Katsa, Akt3, Akt1, Smarcd1, Pcgf1, Nanog, Bmpr1b, Neurog1 and Nodal) (Figure 3E, Table S4), but most of them have not been activated, yet (Table S5). This was in accord with our expectation: p53-deficient SSCs have not been transformed (Figure 2). Moreover, the dysregulated genes associated with change of chromatin accessibility were analysed. Deletion of p53 led to 4898 regions with altered chromatin accessibility in SSCs, and the expression of 4726 genes was significantly affected (1.5-fold change). By analysis of the 2798 open peaks, 2284 genes were identified, and 1693 genes were up-regulated, after p53 deficiency. Similarly, 1662 genes with closed chromatin state were identified from 2100 peaks, and 2449 genes were down-regulated. About 9.76% and 11.9% altered ATAC-seq peaks were, respectively, mapped to differentially expressed genes (DEGs) (Tables S6 and S7), matching with the range of a previous study (only 5%–12% cell type-specific ATAC-seq peaks mapped to genes with differential expression).

In the 230 up-regulated genes whose chromosomal regions became more open (Figure 4F and Table S6), we found several genes of the TGF-β signalling pathway (Tgfbi, Tgb3, Inhbb and Acrv1b) and pluripotency-associated genes including Axin2, E2f2 and Gli2. In the 265 down-regulated genes with decreased accessibility after p53 knockout (Figure 4G and Table S7), we found several germ-line markers, such as Bmp8b, Dazl, Ddx4 and Rassf8, and genes of the Wnt signalling pathway, such as Apc, Prdm5, Tcf4 and Wnt2, and pluripotency-associated gene Klf4. To further dig into the molecular cues caused by p53 loss, we specifically compared the expression changes of several pivotal genes in Wnt (Figure 4H), TGF-β (Figure 4I) and Notch (Figure 4J) signalling pathways, since they are potentially associated with the regulation of cell fate. Expression levels of Tgfa, Tgb1, Notch3, Notch4 and Jag2 were remarkably increased, and expression levels of Apc, Gsk3b and Tgb1, Acrv2a and Smurf2 were remarkably decreased in p53 deficient SSCs (q-value < 0.05 and absolute log (fold change) ≥1.5 (p < 0.03)) (Table S5), implying the activation of Wnt, TGF-β and Notch signalling pathways in p53 deficient SSCs. Based on these observations, we concluded that p53 deletion activated Wnt, TGF-β and Notch signalling pathways through activating opened-up chromatin state.

Furthermore, several genes associated with stem cell fate were chosen for analysing the expression difference in p52+/+ and p53−/− SSCs, which was exhibited in the heatmap (Figure 4K). The expression levels of genes related to pluripotency, including Bmp4, Oct1, Oct4, Nanog, Nodal, Sal1, Stat3, Klf2 and Foxo1, were not significantly changed, and the expression of Sox2, Klf4 and Utf1 was remarkably decreased, suggesting pluripotency was not obtained in p53 deficient SSCs, yet. However, the chromosomal regions of Nanog, Nodal, Sal1, Sox2 and Foxo1 became open state (Table S4), indicating that these genes tended to be transcriptionally activated. Considering that these pluripotent-associated genes were not activated after p53 deletion, we speculated that these genes probably
did not participate in the initiation step of transformation, or the expression levels of several spermatogonia markers, including Cdh22, Itga6, Nanos2, Nanos3, Etv5, Lhx1, Bcl6b and Sohlh2, were not significantly changed, but the undifferentiated spermatogonia marker E-cadherin (Cdh1), Gfra1 and germline marker Ddx4 were remarkably decreased. Meanwhile, the expression levels of differentiation markers including c-kit, Sycp1 and Sycp2 were not remarkably changed, and sycp3 was down-regulated, which hinted that p53 deficient SSCs tended to lose germline characteristics, rather than differentiate.

Notably, the binding domain of SMAD3 became open (Figure 3F), and TGF-β signalling pathway tended to be activated (Figure 4F, I, Table S5), but neither the chromatin state nor the expression levels of Smad3 and Smad4 were significantly changed. On the other hand, genes of the Wnt signalling pathway, including Wnt3, Axin2 and Tcf3, were up-regulated, but the chromatin state and expression level of β-catenin were not changed, either. This demonstrated that these two pathways connecting p53 deficiency to SSCs reprogramming have not been fully activated, yet.

Tumour suppressor gene Pten was inhibited in p53 deficient SSCs, and the expression levels of some cell cycle or proliferation-associated genes Axin2 and Cyclin D1 were increased (Figure 4K), indicating that p53 loss accelerates cell proliferation. Notably, the Log2(fold change) describing the expression changes of Akt1 and mTOR were 0.46 and 0.45 (Table S5), representing that their expression levels were about 1.37-fold as control. They were defined as these differences could be observed in the heatmap (Figure 4K).

Moreover, the expression of methylation-regulated genes Dnmt1 and Dnmt3 were not remarkably affected (Table S5), indicating that the methylation modification mediated by these two genes has not occurred after p53 deletion.

These results suggested that several signalling pathways associated with stem cell fate, especially TGF-β and Wnt signalling pathways, and some pathways associated with cell cycle, epigenetic modification and DNA repair, were affected by p53 loss. Combined with the changes of chromatin accessibility revealed by ATAC-seq, we further speculated that p53 deletion affected chromatin openness and activity of signalling pathways related to cell fate.

### 3.5 | The relationship between chromatin accessibility and RNA-seq measured gene expression

Furthermore, we focused on six representative genes which were differentially expressed in p53+/− and p53−/− SSCs, including the pluripotency-related genes Sox2 and Utft1, germline marker gene Ddx4 and signalling pathway-related genes Axin2, Gli2 and Tgfb1 (Figure 5A). The expression levels of Sox2 and Utft1 were decreased in p53 deficient SSCs, indicating that after deletion of p53 gene, pluripotency was not immediately increased. However, both chromatin accessibility and expression levels of Axin2, Gli2 and Tgfb1, the key genes of TGF-β and Wnt signalling pathways, were activated, implying that p53 regulated their expression at chromosomal level. Interestingly, the transcription of germline marker gene Ddx4 became inactive, and its chromatin was less accessible after p53 deficiency (Table S6), suggesting the potential role of p53 in the maintenance of Ddx4 through regulating chromosomal state. Combined with the results of RNA-seq that showed no significant increase in the expression levels of most pluripotent genes in p53−/− SSCs (Table S5), we further confirmed that p53 deficient SSCs were still adopting germline identity, even if they already tended to transform into pluripotent state accompanied with decreased expression of some germline markers, which was probably related to TGF-β and Wnt signalling pathways.

To verify the RNA-seq results, we selected several genes associated with pluripotency, germline and TGF-β signalling pathway to detect their expression at mRNA and protein levels. RT-PCR results revealed that the expression levels of Id4, Pitzf and Oct4 were not remarkably changed, but the expression levels of some germline markers and spermatogonia markers including Itgb1, E-cadherin and Mvh (Ddx4) were down-regulated in p53−/− SSCs (Figure 5B, C), indicating that part of spermatogonia characteristics was decreased at mRNA level. However, the expression of Smad3 was down-regulated in p53 deficient SSCs (Figure 5B, C), but RNA-seq results showed that the expression of Smad3 was not remarkably changed. To further verify these results, expression levels of these genes were determined at protein level. Both expression and phosphorylation levels of SMAD3 were down-regulated by p53 deficiency (Figure 5D, E), indicating decreased function of the antiproliferative effector in p53 deficient SSCs were decreased. And we noticed that the expression levels of Cdk2 and Cdk4, two cell cycle kinases that regulate SMAD3 phosphorylation to inhibit SMAD3’s transcriptional activity and antiproliferative function, were about 1.39-fold increased (Log2(fold value) = 0.477 and 0.471, respectively (Table S5)), and the expression of Cdc25a, the cell cycle gene inhibited by TGF-β, was remarkably up-regulated, confirming the down-regulated expression and phosphorylation of SMAD3 after p53 deficiency. Notably, the transformation of SSCs is a long-term process, which needs more than 25 passages in vitro.

Thus, we proposed that SMAD3 was not activated in the early stage of transformation. Similarly, we also noticed that the binding domain of ZFP57 (a key TF related to methylation regulation in pluripotency) was more open (Figure 3F), but the chromatin region of Zfp57 gene and transcriptional level of Zfp57 were not increased in p53 KO SSCs (Tables S4 and S5), indicating that p53 loss led to the increased accessibility of ZFP57’s target genes, rather than affect Zfp57 gene itself. The expression of Nanog was up-regulated in p53 deficient SSCs (Figure 5B, C), but in RNA-seq data the expression of Nanog was not remarkably changed (Table S5). However, the expression values of Nanog detected by RNA-seq were 0.087 and 0, respectively, indicating that the expression level was too low to be detected using RNA-seq. Moreover, NANOG signal was detected neither in p53+/− nor in p53−/− SSCs using Western blot (Figure 5D, E). This extremely low expression level of Nanog may explain the inconsistency between molecular assays and RNA-seq. Since the
sensitivity of RT-PCR and Western blot was higher than that of RNA-seq, we confirmed that the expression of Nanog was up-regulated at mRNA level after p53 loss, and its chromosomal accessibility was increased, as well.

Subsequently, the expression changes of genes in TGF-β signalling pathway detected in RNA-seq were analysed and exhibited in a heatmap (Figure 5F), and the expression levels of these genes were verified using RT-PCR (Figure 5G,H). Compared with p53+/+ SSCs, the expression levels of all selected genes: Lefty1, Smurf2, Thbs1 and Acvr2a were significantly down-regulated in p53−/− SSCs. As upstream repressive genes of the TGF-β family, Lefty1, Smurf2, Thbs1 and Acvr2a bind to various TGF-β receptors to recruit and activate SMAD family transcription factors, to regulate gene expression. These results further confirmed that TGF-β signalling pathway...
was activated in p53 deficient SSCs, and we therefore proposed that TGF-β signalling pathway was pivotal for SSCs spontaneous transformation.

3.6 | Pivotal genes associated with pluripotent signal network in differential accessible regions and potential mechanism

Next, we analysed the expression levels of TFs whose binding domains were more accessible after p53 deficiency (Table 1). Although the binding regions of these 29 TFs (Figure 3F) were more accessible after p53 deletion, the transcriptional activity of TFs was not up-regulated (Table S5), indicating that p53 is associated with the chromosomal structure of these TFs’ target genes, rather than regulating the expression levels of these TFs. Moreover, the expression levels of pluripotent genes, such as Sox2 and Nanog, were not up-regulated, but the accessibility of their binding domains was increased, indicating that their chromosomal states were affected by p53 deficiency. On the other hand, although the expression of these pluripotency-associated TFs, such as SMAD3, was not increased either, it should be noted that this did not mean that the expression levels of SMAD3’s target genes were not changed, since the increased chromatin openness of target genes normally enhances the binding efficiency of TF to regulate gene expression.49 Therefore, the target genes of SMAD3 were possibly affected by p53 deficiency, for example, Sox2 was predicted as a putative target of SMAD3, and the expression of Sox2 was positively related to SMAD3 expression (Table 2), indicating a direct regulatory effect of SMAD3 on Sox2 in SSCs.

Subsequently, we analysed whether SMAD3 could bind to some pluripotency-associated genes and found SMAD3’s potential binding sites in Foxo1, Axin2, Cdh1, Itgb1, Mycn, Nanog, Nodal, Sall4, Sox2, Tgfb1 and Tgfb3 genes. Consistently, expression levels of Axin2, Tgfb1 and Tgfb3 were up-regulated (Table 2), suggesting the direct binding and regulatory effect on these genes. Above conclusions showed that SMAD3 was not activated in SSCs. As a potential target of SMAD3, the expression of Nanog was extremely low, which was not detectable at protein level in wild-type and p53 knockout SSCs (Figures 2D and 5C.D). It was not sure whether the low expression of Nanog was associated with transcriptional level of Smad3 in SSCs. Moreover, we analysed the binding sites of SMAD4, which cooperates with SMAD3 for DNA binding. The potential binding sites were predicted in Cdh1, Itgb1, Mycn, Nanog, Nodal, Sall4, Fox1, Tgfb1 and Tgfb3 genes (Table 3), which were very close to those of SMAD3, and the only difference was that the binding sites were not found in Sox2 and Axin2. These results further suggest that SMAD3 may cooperate with SMAD4 to bind to and regulate the above-mentioned pluripotency-related genes in p53 deficient SSCs, and SMAD3 probably directly binds to and regulates Sox2 and Axin2 genes.

Table 1  The expression changes of transcription factors that recognize the accessibility increased domains after p53 deficiency in spermatogonial stem cells (SSCs)

| gene_ID | locus | sample_1 | sample_2 | value_1(Sample_1) | value_2 (Sample_2) | log2 (fold_change) | Relative expression (p53 KO vs WT) |
|---------|-------|----------|----------|------------------|------------------|-------------------|---------------------------------|
| Ctf1    | chr:105636346-105682924 p53 KO | WT | 17.851 | 23.3467 | −0.38722 | \ |
| E2f4    | chr:105297662-105305370 p53 KO | WT | 28.003 | 19.8986 | 0.492913 | \ |
| Smad3   | chr:9:63646764-63757994 p53 KO | WT | 27.0548 | 21.1877 | 0.352657 | \ |
| Smad4   | chr:17:3639012-73703741 p53 KO | WT | 36.8651 | 45.3384 | −0.29848 | \ |
| Pou5f1  | chr:17:35506031-35510777 p53 KO | WT | 27.1936 | 34.8058 | −0.35606 | \ |
| Dmrt3   | chr:19:25610536-25623921 p53 KO | WT | 9.87694 | 8.14039 | 0.278966 | \ |
| Stat1   | chr:1:52119437-52233232 p53 KO | WT | 10.9315 | 9.66132 | 0.178198 | \ |
| Sox2    | chr:3:34650390-34677993 p53 KO | WT | 0.687556 | 1.95592 | −1.5083 | Down-regulated |
| Elk4    | chr:13:2007604-132068062 p53 KO | WT | 4.46601 | 7.87563 | −0.81841 | Down-regulated |
| Mybl2   | chr:2:163054634-163084732 p53 KO | WT | 20.8014 | 10.9112 | 0.930868 | Up-regulated |

Note: According to the results of ATAC-seq, among the transcription factors corresponding to the open transcription factor binding domain, we selected the transcription factors that may be related to pluripotency transformation, including Ctf1, E2f4, Smad3, Smad4, Stat1, Sox2, Pou5f1, Dmrt3 and Mybl2. Sox2 and Elk4 expression was down-regulated, while Mybl2 expression was significantly increased (Log2FC>0.585 means expression level is remarkably increased after p53 KO, Log2FC<-0.585 means decreased).

3.7 | SMAD3/SMAD4 regulates the pluripotent signalling network in SSCs

Above observations demonstrated the potential connection of SMAD3 and many pluripotency-associated genes. However, it is unexpected that the expression level of SMAD3 in newly isolated p53−/− SSCs was lower than that in p53+/+ SSCs (Figure 5B-E), indicating that loss of p53 could not activate expression or phosphorylation of SMAD3. Thus, we wonder whether activation of SMAD3 could regulate these pluripotent genes in SSCs. First, newly isolated SSCs were treated with SMAD3 activator alantolactone to enhance the SMAD3 activity, leading to downregulated expression of PLZF and up-regulated expression of SOX2 in alantolactone-treated SSCs (Figure 6A,C). Combined with
Note: Log2FC > 0.585 means expression level is remarkably increased after p53 KO, Log2FC < −0.585 means decreased.

Log2FC > 0.585 means expression level is remarkably increased after p53 KO, Log2FC < −0.585 means decreased.

Note: Log2FC > 0.585 means expression level is remarkably increased after p53 KO, Log2FC < −0.585 means decreased.

Table 2: Prediction of the binding sites of SMAD3 in some pluripotency-associated genes

| gene_ID | locus | sample_1 | sample_2 | value_1(Sample_1) | value_2(Sample_2) | log2(fold_change) | Relative expression (p53 KO vs WT) |
|---------|-------|----------|----------|-------------------|-------------------|------------------|-----------------------------------|
| Foxa1   | chr3:52268336-52350109 | p53 KO     | WT        | 34.7167           | 49.0257           | −0.49791         | \                                 |
| Mync    | chr12:12936092-12941996 | p53 KO     | WT        | 25.9709           | 33.9265           | −0.38552         | \                                 |
| Nanog   | chr6:122707488-122714633 | p53 KO     | WT        | 0.087124          | 0                 | --               | \                                 |
| Sall4   | chr2:168748331-168767201 | p53 KO     | WT        | 37.6655           | 41.1992           | −0.12937         | \                                 |
| Nodal   | chr10:61417971-61425337 | p53 KO     | WT        | 0.148573          | 0.075261          | 0.981201         | \                                 |
| Axin2   | chr11:108920348-108950781 | p53 KO     | WT        | 7.96963           | 4.57413           | 0.800106         | Up-regulated                     |
| Tgfb1   | chr7:25687001-25704996 | p53 KO     | WT        | 17.7919           | 9.58317           | 0.892643         | Up-regulated                     |
| Tgfb3   | chr12:86056742-86079041 | p53 KO     | WT        | 17.8388           | 10.4755           | 0.767994         | Up-regulated                     |
| Cdh1    | chr8:106603209-106670248 | p53 KO     | WT        | 11.5823           | 21.3651           | −0.88334         | Down-regulated                   |
| Itgb1   | chr8:128685653-128733579 | p53 KO     | WT        | 61.5212           | 110.308           | −0.84239         | Down-regulated                   |
| Sox2    | chr3:234560380-34677993 | p53 KO     | WT        | 0.687556          | 1.95392           | −1.5083          | Down-regulated                   |

Table 3: Prediction of the binding sites of SMAD4 in some pluripotency-associated genes

| gene | locus | sample_1 | sample_2 | value_1 | value_2 | log2.fold_change | Relative expression (p53 KO vs WT) |
|------|-------|----------|----------|---------|---------|-----------------|-----------------------------------|
| Itgb1 | chr8:128685653-128733579 | treat | ctrl | OK | 61.5212 | 110.308 | \ |
| Mync | chr12:12936092-12941996 | treat | ctrl | 25.9709 | 33.9265 | −0.38552 | \ |
| Foxa1 | chr3:52268336-52350109 | treat | ctrl | 34.7167 | 49.0257 | −0.49791 | \ |
| Sall4 | chr2:168748331-168767201 | p53 KO | WT | 37.6655 | 41.1992 | −0.12937 | \ |
| Nanog | chr6:122707488-122714633 | p53 KO | WT | 0.087124 | 0 | -- | \ |
| Cdh1 | chr8:106603209-106670248 | treat | ctrl | 11.5823 | 21.3651 | −0.88334 | Down-regulated |
| Nodal | chr10:61417971-61425337 | p53 KO | WT | 0.148573 | 0.075261 | 0.981201 | \ |
| Tgfb1 | chr7:25687001-25704996 | p53 KO | WT | 17.7919 | 9.58317 | 0.892643 | Up-regulated |
| Tgfb3 | chr12:86056742-86079041 | treat | ctrl | 17.8388 | 10.4755 | 0.767994 | Up-regulated |

Note: Log2FC > 0.585 means expression level is remarkably increased after p53 KO, Log2FC < −0.585 means decreased.

FIGURE 6 SMAD3 plays an important role in regulating the pluripotent signalling network in spermatogonial stem cells (SSCs). The expression levels of mouse vasa homologue (MVH), NANOG, SOX2, SMAD3 and p-SMAD3 in primary SSCs treated with alantolactone (10 μM, 6 h) (A) or scrambled/Smad3 siRNA (B) were detected using Western blot, and the results of Alan-treated (C) or small interfering RNA (siRNA)-treated groups (D) were statistically analysed (n = 3), p53+/− and p53−/− SSCs cultured in vitro for 20 passages were harvested to detect the relative expression levels of germline markers Plzf, Mvh, and pluripotent markers Sox2, Nodal and Nanog (n = 3) (E). SSCs cultured in vitro for 20 passages were incubated with alantolactone (F), SMAD3 inhibitor SIS3 (G), scrambled/Smad3 siRNA (J) or pcDNA3-Smad3 (K) for 48 h, and expression levels of MVH, promyelocytic leukaemia zinc finger (PLZF), SOX2, NANOG, SMAD3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and phosphorylation level of SMAD3 were detected, and these results were statistically analysed (H). Alan-treated long-term SSCs, I. SIS3-treated long-term SSCs, L. Smad3 siRNA transfected long-term SSCs, M. pcdNA3-Smad3 transfected long-term SSCs. n = 3. The p53 KO and wild-type (WT) SSCs of passage 1 and passage 20 were harvested to determine the expression and phosphorylation of SMAD3 using Western blot (N), and the results were statistically analysed (n = 3) (O). Illustration of the potential regulatory network of p53 in mediating pluripotency transformation of SSCs (P). The data represent the means±SD (*, p < 0.05; **, p < 0.01)
Subsequently, we cultured $p53^{+/+}$ and $p53^{-/-}$ SSCs for around 20 passages in vitro, to detect the expression change of germline and pluripotent genes. Expression of Sox2 and Nanog was detected (Figure 6E), confirming that after long-term culture, both $p53^{+/+}$ and $p53^{-/-}$ SSCs gradually transformed, at least could be observed at mRNA level. Meanwhile, expression levels of germline marker were decreased and pluripotent markers were significantly increased in $p53^{-/-}$ SSCs (Figure 6E), confirming that transformation efficiency was higher in $p53$ deficient SSCs. Therefore, we proposed that these long-term cultured $p53^{+/+}$ and $p53^{-/-}$ SSCs partially possessed some of the characteristics of pluripotent stem cells at molecular levels, and selected SSCs around 20 passages to study the
regulatory role of SMAD3 in SSCs transformation. The dosage effect and treatment time of SMAD3 activator alantolactone or SMAD3 inhibitor SIS3 were tested in these long-term cultured SSCs (Figure S3). Alantolactone treatment increased expression levels of SOX2, SMAD3 and NANOG, and enhanced the phosphorylation level of SMAD3 (Figure 6F,H). Consistently, SIS3 remarkably inhibited the expression of SOX2, NANOG, SMAD3, and inhibited SMAD3 phosphorylation (Figure 6G,J). And the expression of SSCs marker PLZF was negatively regulated by SMAD3 (Figure 6F–I), implying that activation of SMAD3 promoted SSCs transformation and led to the loss of SSCs characteristics. Likewise, expression of Smad3 was disturbed in SSCs around 20 passages using siRNA to eliminate the off-target effects of small molecules, and identical effect on the expression of germline and pluripotent markers was observed (Figure 6J,L). As expected, transfection of SMAD3 expression plasmid in long-term SSCs enhanced the expression levels of SOX2 and NANOG (Figure 6K,M), confirming the regulatory role of SMAD3 in pluripotency in the later stage of SSCs transformation. Finally, we noticed that the expression level and phosphorylation level of SMAD3 were remarkably up-regulated after long-term culture in p53 KO and wild-type SSCs (Figure 6N,O). Combined with the results that the expression level of SMAD3 in newly isolated p53−/− SSCs was lower than that of wild-type control (Figure 5B–E), we proposed that SMAD3 activation occurred in the middle or late stage of transformation, to further promote NANOG expression.

Based on the reported conclusions and results of this study, we proposed a potential model of p53 in mediating pluripotency transformation of SSCs (Figure 6P). As a tumour suppressor, p53 inhibits many target genes and maintains the stability of chromosomes. Deletion of p53 increases accessibility of many domains in chromatin, and TFs detected in the ATAC-seq assay (such as CTCF, OCT4:SOX2 complex, E2F4, SMAD3 and SMAD4) can bind to these opened regions to regulate their expression more efficiently. Some of them are reprogramming-associated genes, including Yamanaka factors, c-Myc, Sox2, and they were identified as the putative targets of SMAD3, SMAD4 and E2F4 in this study. Additionally, SSCs endogenously express Oct4 (Figure 2D). Loss of p53 provides many, if not all, essential conditions for pluripotency transformation, and activation of Nanog, the core gene of pluripotency, is more efficient during in vitro culture. However, expression and phosphorylation of SMAD3 are not up-regulated after p53 deletion, and p53 deficient SSCs still need long-term culture to transform into pluripotent fate, indicating a key event may happen during this process, which is the prerequisite to activate SMAD3 and induce SSCs transformation. This is similar to "two-hit hypothesis" in tumorigenesis, that p53 deficient SSCs fail to maintain their fate when a stimulus appears. In the future research, we will screen this unknown event of SSCs transformation to pluripotent fate.

4 | DISCUSSION
The relationship between p53 and pluripotency is complex. Here, we observed that p53 loss led to open chromatin state of Nanog, and increased expression of Nanog mRNA. However, p53 deficiency was insufficient to drive SSCs transformation, since most of the pluripotent genes were not up-regulated (especially NANOG protein was not detectable), and germline characteristics still remained. Increased expression of NANOG at protein level was detected only in long-term culture SSCs, which was further enhanced by SMAD3 activator or overexpression. Considering that SSCs transformation composed of several steps, we proposed that SMAD3 was not activated in the early stage, and the underlying mechanism in the whole transformation process was more complicated than our expectation. Besides the role of inhibiting Nanog expression in pluripotent transformation, evidence showed that p53 deficiency promoted reprogramming by avoiding cell arrest or apoptosis. p53 also regulates pluripotent genes such as Leukaemia Inhibitory Factor (LIF) and some long non-coding RNAs (lncRNAs) to maintain the pluripotency of ESC. On the other hand, inhibition of p53 activity is the key to maintaining the versatility of ESCs under stress-free condition. The results of ATAC-seq showed that the binding domains of OCT4:SOX2, E2F4, SMAD3 and other transcription factors were more accessible in p53 deficient SSCs, and the key adhesion signal genes, such as Itgb1 and E-cadherin, were down-regulated. This observation implied that p53 played a role for SSCs maintenance by inhibiting the accessibility of chromosomal regions by recognizing pluripotent transcription factors. Based on these conclusions and our observations, we propose that p53 is more likely to be responsible for maintaining the original cell fate under the stimulation of inducing factors. If the change of cell fate is irreversible, it will trigger cell stagnation or death. In this case, loss of p53 is a key step for cell differentiation or reprogramming.

It is worth noting that the activity of Oct4 is essential during the embryo development of sperm-egg binding. Our observations are consistent with reported evidence regulation of Oct4 expression and stemness is closely associated. In germline, Oct4 expression is maintained at a low level, while after fertilization or reprogramming, the expression of Oct4 is activated and cells obtain pluripotency.

Although the accessibility of many domains is increased in p53 deficient SSCs, neither the expression levels of TFs we identified nor the expression levels of pluripotent genes recognized by these TFs were significantly up-regulated in p53 KO SSCs. This suggests that p53 deleted SSCs retain the characteristics of germ cells and have not obtained pluripotency, yet, and that is why SSCs need further culture in vitro to transform into pluripotent state. Typically, in primary SSCs, alantolactone is able to stimulate SOX2 expression, indicating that SMAD3 activation is able to drive pluripotency transformation at the early stage, even though NANOG was not detectable at protein level (Figure 6A). When SSCs around 20 passages already expressed NANOG, the expression level of NANOG was positively related to SMAD3 (Figure 6). These observations also suggest that SMAD3 promotes pluripotency transformation throughout SSCs culture, but in the beginning, there is no inducing factor to activate SMAD3 expression. Thus, SSCs transformation takes several steps, which may be divided by the expression of NANOG protein as a key event, and the expression of Nanog gene is regulated by
SMAD3 throughout the process. Moreover, SMAD3 probably plays different roles in each step.

A study revealed that phosphorylated SMAD2 or SMAD3 could bind to the Nanog proximal promoter region to regulate Nanog expression in human ESCs and in mouse epiblast stem cells (EpiSCs). We also predicted Nanog as a direct target of SMAD3 and SMAD4 according to the bioinformatics analysis (Tables 2 and 3). Thus, we proposed that SMAD3 positively regulates the expression of Nanog, to regulate the pluripotency transformation of SSCs. However, further molecular evidence is required to support this hypothesis. This also suggested that the regulation of stem cells’ fate may be related to multiple signal networks, and the regulatory effect of a single transcription factor is limited.

Although the accessibility of SMAD3’s binding domains was increased in the newly isolated p53 deficient SSCs (Figure 3F), it is not clear why the expression level and phosphorylation level of SMAD3 were declined in these cells (Figure 5D). Considering that alantolactone treatment enhanced the expression of pluripotent genes and attenuated the expression of germline genes both in newly isolated SSCs and in long-term culture SSCs, we propose that there are probably some endogenous mechanisms to prevent SSCs transformation, and an essential factor/step is required to activate SMAD3 expression in SSCs, which is the prerequisite of SSCs transformation. TGF-β signalling pathway is probably involved in this unknown event. However, further research is needed to verify this hypothesis.

In addition, the relationship between p53 and E2F4 is also very important. We found that in p53 deficient SSCs, the openness of E2F4’s binding domain was also increased. E2F4 can form a complex with SMAD3, which enters the nucleus under the stimulation of TGF-β and combines with SMAD4 to regulate the expression of c-Myc. Therefore, SMAD3 and SMAD4 may activate c-Myc and Nanog to promote SSCs transformation by interacting with E2F4. This hypothesis needs to be further investigated, as well.

Shinohara’s team revealed that SSCs transformation was associated with p53 and Dnmt1. Deficiency of p53 combining with epigenetic changes jointly affected the genomic stability and expression profile of SSCs, which were also involved in the cooperation with OCT4: SOX2. Here, we noticed that the expression levels of Dnmt1 and Dmr1 were not significantly changed, indicating that methylation modification has not occurred, yet. And we observed that the expression levels of SMAD3/4’s target genes Itgb1 and E-cadherin decreased in p53 deficient SSCs, indicating the reduced cell adhesion. Considering that this event can enhance the undifferentiated state of pluripotent stem cells, we believe that the altered cell surface interaction is also important for SSCs transformation.

A study compared the expression profiles of SSCs and reprogrammed pluripotent SSCs using RNA-seq, and predicted some potential transcription factors associated with three pluripotency-related processes including cell proliferation, stem cell maintenance and epigenetic regulation. Totally, 15 TFs were predicted as two groups, 4 of them (OCT4, CUX1, ZFP143, E2F4) were associated with the early stage of reprogramming and 11 regulated pluripotency-related processes at the late stage, based on bioinformatics analysis. Here, the chromatin accessibility changes demonstrated using ATAC-seq provided direct biological evidence for key TFs associated with SSCs transformation. Consistently, OCT4 and E2F4 were also identified in our system. The TFs identified in two studies were not identical, since Jeong et al. collected data from normal SSCs and transformed SSCs, while we selected newly isolated p53+/− and p53−/− SSCs for analysis.

5 | CONCLUSION

This study explored the impact of p53 deletion on the fate of SSCs and the underlying molecular mechanism. Due to the complexity of p53’s function and regulatory network, we screened regions with increased accessibility in the whole chromosome, to analyse the opening degree of key genes related to pluripotency induced by p53 deficiency, and revealed the molecular mechanism of SSCs transformation. This study is helpful to understand the role and molecular mechanism of p53 in maintaining the genome stability and cell fate, which is conducive to revealing the connection of stemness transition at chromatin level, and will provide theoretic reference for ageing, tumour biology or clinical research.

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CONFLICT OF INTEREST

The authors have no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Sitolong Liu: Collection and assembly of data, data analysis and interpretation, manuscript writing. Rui Wei: Collection and assembly of data, data analysis and interpretation, manuscript writing. Hongyang Liu: Collection and assembly of data. Ruqi Liu: Collection and assembly of data. Pengxiao Li: Data analysis and interpretation. Xiaoyu Zhang: Data analysis and interpretation. Xiaodong Zhao: Data analysis. Xiaomeng Li: Data analysis, financial support. Yang Yang: Data analysis. Xueqi Fu: Conception and design, financial support. Kang Zou: Conception and design, financial support, manuscript writing, final approval of manuscript. All of the authors in the authorship list agreed to publication of this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the method part and supplemental materials.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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