



## Introduction

Glioblastoma (GBM) is one of the most deadliest cancers in humans.<sup>1</sup> Proliferation and invasion are the devastating characteristics of GBM and contribute to the high mortality rate.<sup>1</sup> A variety of clinical treatment strategies, including surgical treatment, radiation therapy combined with temozolomide chemotherapy, are currently used for the patients with GBM, and the prognosis of GBM is still very poor and the average survival time of patients is still 12-15 months.<sup>2</sup> Novel and specific drug targets for prevention of GBM cell proliferation and invasion are urgently needed due to the limited therapeutic effects of current strategies.<sup>3</sup>

Williams syndrome transcription factor (WSTF) is encoded by bromodomain adjacent to zinc finger domain 1B gene and has been identified as a hemizygous deletion gene in patients with Williams syndrome.<sup>4</sup> WSTF, the component of WSTF-Imitation Switch (ISWI) ATP-dependent chromatin-remodeling complex, contains the C-terminal bromine domain adjacent to plant homologue domain to participate in DNA damage through tyrosine phosphorylation of gamma-H2A.X.<sup>5</sup> Loss of WSTF was involved in heterochromatin formation and resolution and participated in the pathogenesis of Williams-Beuren syndrome.<sup>6</sup> WSTF was recruited to the DNA damage site induced by UV-C to specifically facilitate the transcription recovery.<sup>7</sup> Recent studies have found that WSTF also plays a very important role in the occurrence and development of tumors. For example, estrogen receptor signaling was activated by WSTF to promote breast cancer cell growth.<sup>8</sup> WSTF can form a complex with neuregulin-3 to activate the oncogenic pathways in colon tumor cells.<sup>9</sup> Downregulation of gamma-H2A.X phosphorylation by mouse double minute 2 homolog-WSTF-eyes absent transcriptional coactivator and phosphatase 3 complex promoted the progression of gastric cancer.<sup>10</sup> However, the effects of WSTF on GBM cell proliferation and invasion have not been reported until now.

In this study, the expression level of WSTF in GBM cells was firstly identified, and its effects on cell proliferation and invasion of GBM cells were then investigated. The investigation of downstream signaling pathways might provide theoretical basis for identification of WSTF as the potential therapeutic target for the prevention of GBM.

## Materials and Methods

### Validation of WSTF

GEPIA database (<http://gepia.cancer-pku.cn/>) was used to detect the expression of WSTF in TCGA RNA-seq raw data from 163 GBM tissues and 207 normal tissues.

### Cell culture, transfection, and treatment

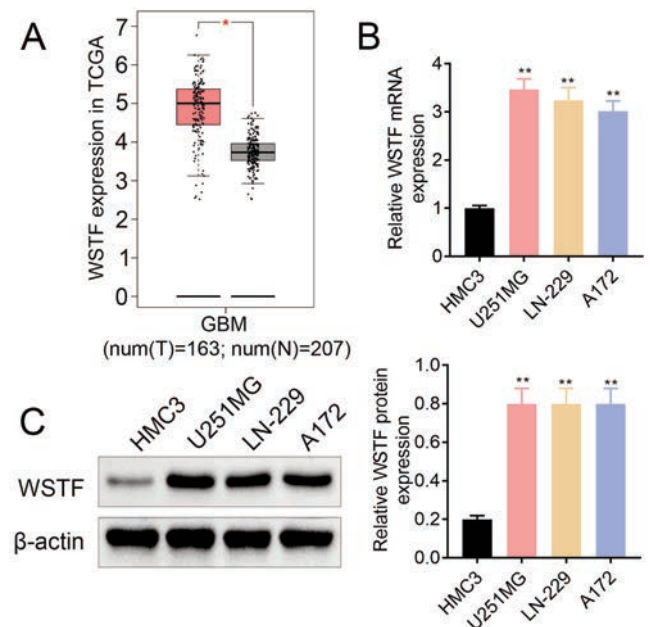
GBM cell lines (U251MG, LN-229, A172) and human microglial clone 3 cell line (HMC3) were received from ATCC (Manassas, VA, USA) and then cultured in DMEM medium with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) in a 37°C humidified chamber. pcDNA3.1-WSTF and shRNAs targeting WSTF (shWSTF-1# or 2#) were constructed by RiboBio (Guangzhou, China). U251MG and LN-229 cells were transfected with pcDNA vectors or shRNAs by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). U251MG and LN-229 under pcDNA vectors transfection were also incubated with 15  $\mu$ M LY294002 (Sigma-Aldrich, St. Louis, MO, USA) for 48 h, and the cells were used for functional assays.

### Cell viability and proliferation

U251MG and LN-229 cells with indicated transfection and treatment were plated in a 96-well plate for 24 h. A total of 10  $\mu$ L CCK8 solution (Sigma-Aldrich) was added to each well for 2 h before measurement of absorbance at 450 nm under spectrometer (Thermo Fisher Scientific). U251MG and LN-229 cells with indicated transfection and treatment were also plated in a 6-well plate for two weeks. Formaldehyde-fixed cells were stained with 0.4% crystal violet (Sigma-Aldrich) for the measurement of colonies under a light microscope (Olympus Corp., Tokyo, Japan).

### Cell apoptosis, migration and invasion

U251MG and LN-229 cells with indicated transfection and treatment were suspended in 100  $\mu$ L Annexin V-binding buffer (Thermo Fisher Scientific), and then incubated with 5  $\mu$ L Annexin V-FITC (Thermo Fisher Scientific). Following incubation with 400  $\mu$ L Annexin V-binding buffer containing 2  $\mu$ L PI solution (2 mg/mL), the cell apoptosis rate was analyzed by Attune™ Flow Cytometer (Thermo Fisher Scientific). For investigation of cell migration, U251MG and LN-229 cells with indicated transfection and treatment were suspended in 200  $\mu$ L serum-free DMEM medium and plated in the upper chamber of well (Corning, Tewksbury, MA, USA). The lower chamber was filled with 400  $\mu$ L DMEM containing 10% fetal bovine serum. Twenty-four h later, the invasive cells in lower chamber were stained with 1% crystal violet and measured under the microscope (Olympus). For detection cell invasion, the well was performed with matrigel coating (BD Bioscience, San Jose, CA, USA).



**Figure 1.** Upregulation of WSTF in GBM tissues and cells. A) mRNA expression of WSTF was up-regulated in GBM tissues (n=163) compared to the normal tissues (n=207). B) mRNA expression of WSTF was upregulation in GBM cells (U251MG, LN-229, A172) compared to human microglial clone 3 cell line (HMC3). C) Protein expression of WSTF was up-regulated in GBM cells (U251MG, LN-229, A172) compared to human microglial clone 3 cell line (HMC3). \*vs control  $p < 0.05$ ; \*\*vs control  $p < 0.01$ .

## RT-qPCR

RNAs were isolated from GBM cell by Trizol (Thermo Fisher Scientific), and reverse transcribed into cDNAs by MMLV reverse transcriptase (Promega, Madison, WI, USA). The mRNA expression of WSTF and GAPDH were determined via SYBR Green Master (TaKaRa, Dalian, China) with following primer sequences: GAPDH (Forward: 5'-AGGTCGGTGTGAACGGATTTG-3' and Reverse: 5'-TGTAGACCATGTAGTTGAGGTC-3') and WSTF (Forward: 5'-GGGCTCAGACACAGATGACA-3' and Reverse: 5'-TGGGGCTCAAACCTTCACAAT-3').

## Western blot

GBM cells were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific), and acid protein kit (Thermo Fisher Scientific) was applied to detect the protein concentrations in the cellular lysates. The lysates were separated by SDS-PAGE, electro-transferred onto PVDF membrane (Millipore, Bedford, MA, USA) and blocked in 5% BSA. Following overnight probe with the primary antibodies: anti-WSTF (1:2000, Cell Signaling, Beverly, MA, USA), anti-Bax (1:3000, Cell Signaling), anti-cleaved caspase-3 (1:3500, Cell Signaling), anti-AKT, anti-p-AKT and anti- $\beta$ -actin (1:4000, Cell Signaling), the membranes were incubated with corresponding horseradish peroxidase-labeled secondary antibody (1:5000; Cell Signaling). The immunoreactivities were detected by enhanced chemiluminescence (KeyGen, Nanjin, China).

## Statistical analysis

Data with at least triple replicates were expressed as mean  $\pm$  SEM, and performed with one-way analysis of variance or

Student's *t*-test under GraphPad Prism software. The *p* value <0.05 was considered as statistically significant.

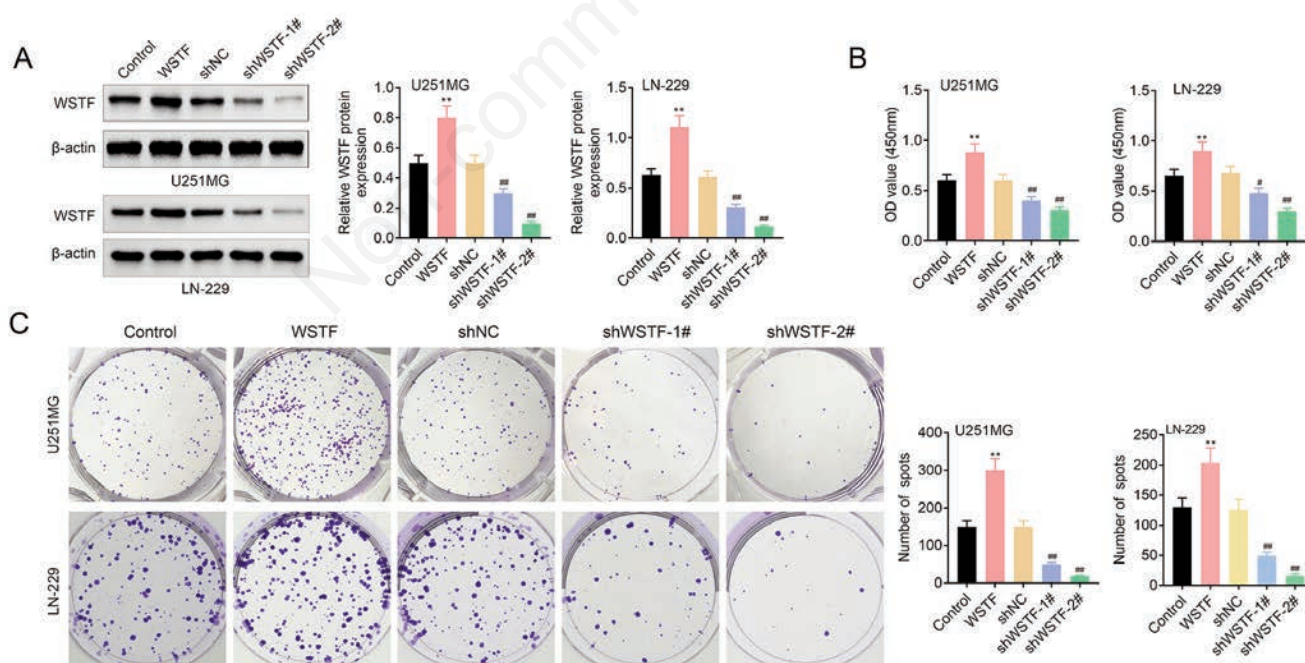
## Results

### Upregulation of WSTF in GBM tissues and cells

In order to evaluate WSTF expression in GBM tissues, GEPIA database (<http://gepia.cancer-pku.cn/>) was applied to detect TCGA RNA-seq raw data from 163 GBM tissues and 207 normal tissues. Result showed that WSTF was upregulated in GBM tissues compared with the normal tissues (Figure 1A). Both of mRNA (Figure 1B) and protein (Figure 1C) were also elevated in GBM cells (U251MG, LN-229, A172) compared with human microglial clone 3 cell line (HMC3). These results suggested that WSTF might be involved in GBM progression.

### WSTF contributed to GBM cell proliferation

U251MG and LN-229 were transfected with pcDNA-WSTF or shRNAs-WSTF for gain- of loss- of functional assays. Protein expression of WSTF was enhanced in U251MG and LN-229 transfected with pcDNA-WSTF or reduced by transfection with shRNAs-WSTF (Figure 2A). pcDNA-mediated over-expression of WSTF increased cell viability of U251MG and LN-229 (Figure 2B) and promoted the cell proliferation (Figure 2C). However, the cell viability (Figure 2B) and proliferation (Figure 2C) of U251MG and LN-229 were repressed by silencing of WSTF, indicating that WSTF contributed to GBM cell proliferation.

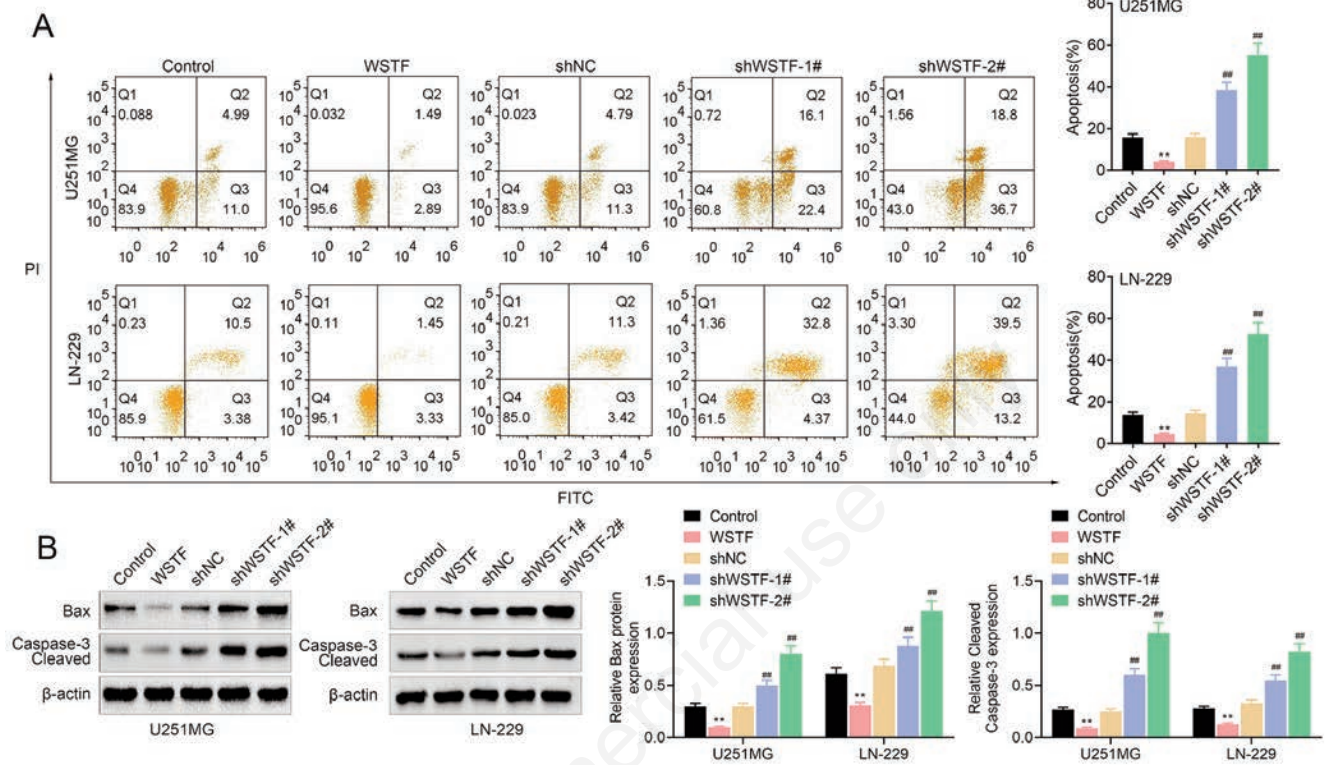


**Figure 2.** WSTF contributed to GBM cell proliferation. A) Protein expression of WSTF was enhanced in U251MG and LN-229 transfected with pcDNA-WSTF AND reduced by transfection with shRNAs-WSTF. B) pcDNA-mediated over-expression of WSTF increased cell viability of U251MG and LN-229, while shRNAs-mediated knockdown of WSTF suppressed the cell viability. C) pcDNA-mediated over-expression of WSTF increased cell proliferation of U251MG and LN-229, while shRNAs-mediated knockdown of WSTF suppressed the cell proliferation. \*\*vs control; ##vs shNC *p*<0.01.

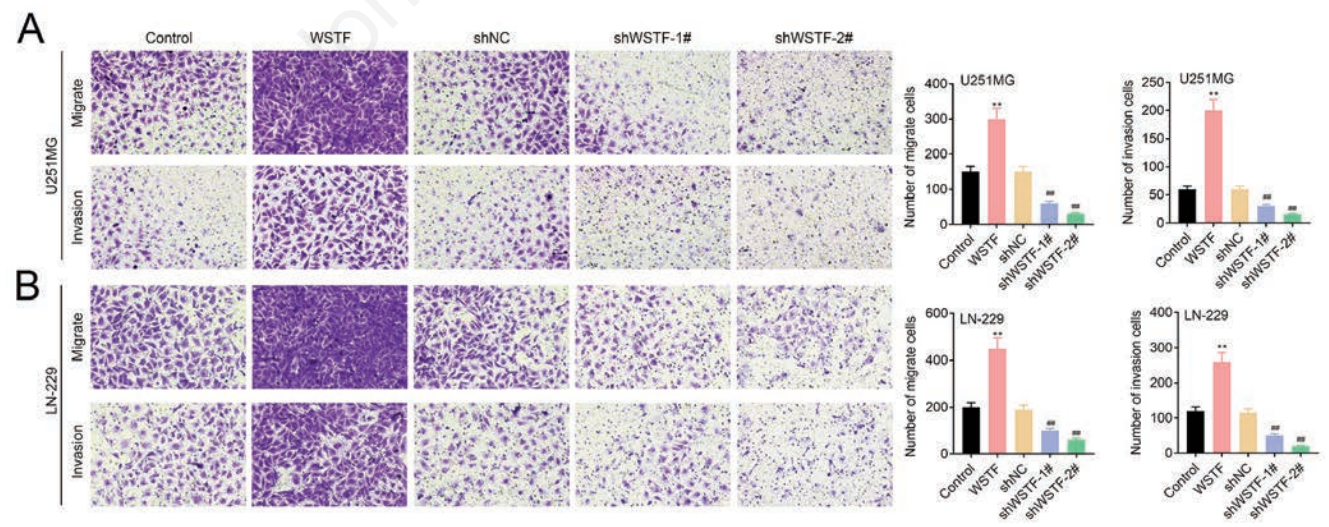
### Silencing of WSTF contributed to GBM cell apoptosis

Flow cytometry analysis revealed that over-expression of WSTF suppressed cell apoptosis of U251MG and LN-229 (Figure 3A), while silencing of WSTF promoted cell apoptosis (Figure

3A). Transfection with pcDNA-WSTF decreased protein expression of Bax and cleaved caspase-3 in U251MG and LN-229 (Figure 3B), while shWSTF transfection increased the expression of Bax and cleaved caspase-3 (Figure 3B), indicating the anti-apoptotic role of WSTF in GBM cell.



**Figure 3.** Silence of WSTF contributed to GBM cell apoptosis. A) pcDNA-mediated over-expression of WSTF repressed cell apoptosis of U251MG and LN-229, while shRNAs-mediated knockdown of WSTF promoted the cell apoptosis. B) pcDNA-mediated over-expression of WSTF decreased protein expression of Bax and cleaved caspase-3 in U251MG and LN-229, while shRNAs-mediated knockdown of WSTF increased the protein expression. \*\*vs control; ##vs shNC p<0.01.



**Figure 4.** WSTF contributed to GBM cell invasion. pcDNA-mediated over-expression of WSTF promoted cell migration and invasion of U251MG and LN-229, while shRNAs-mediated knockdown of WSTF suppressed the cell migration and invasion. \*\*vs control; ##vs shNC p<0.01.

### WSTF contributed to GBM cell invasion

In addition to the proliferative and anti-apoptotic roles of WSTF in GBM cell, transwell assay was applied to assess the effect of WSTF on migration and invasion. Cell migration and invasion of U251MG and LN-229 were promoted by pcDNA-mediated WSTF over-expression, whereas repressed by shRNAs-mediated WSTF silencing (Figure 4), indicating the anti-invasive role of WSTF silencing in GBM cell.

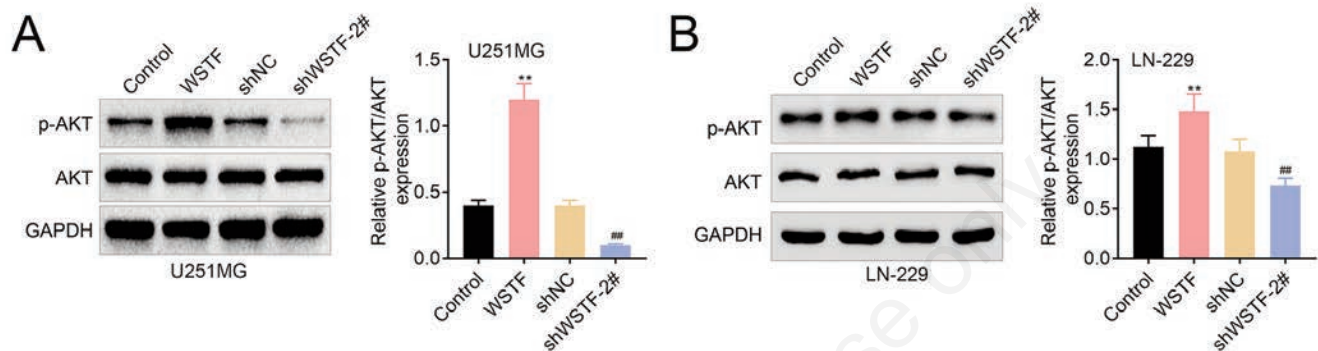
### WSTF contributed to AKT activation in GBM cell

Protein expression of AKT was not significantly affected by

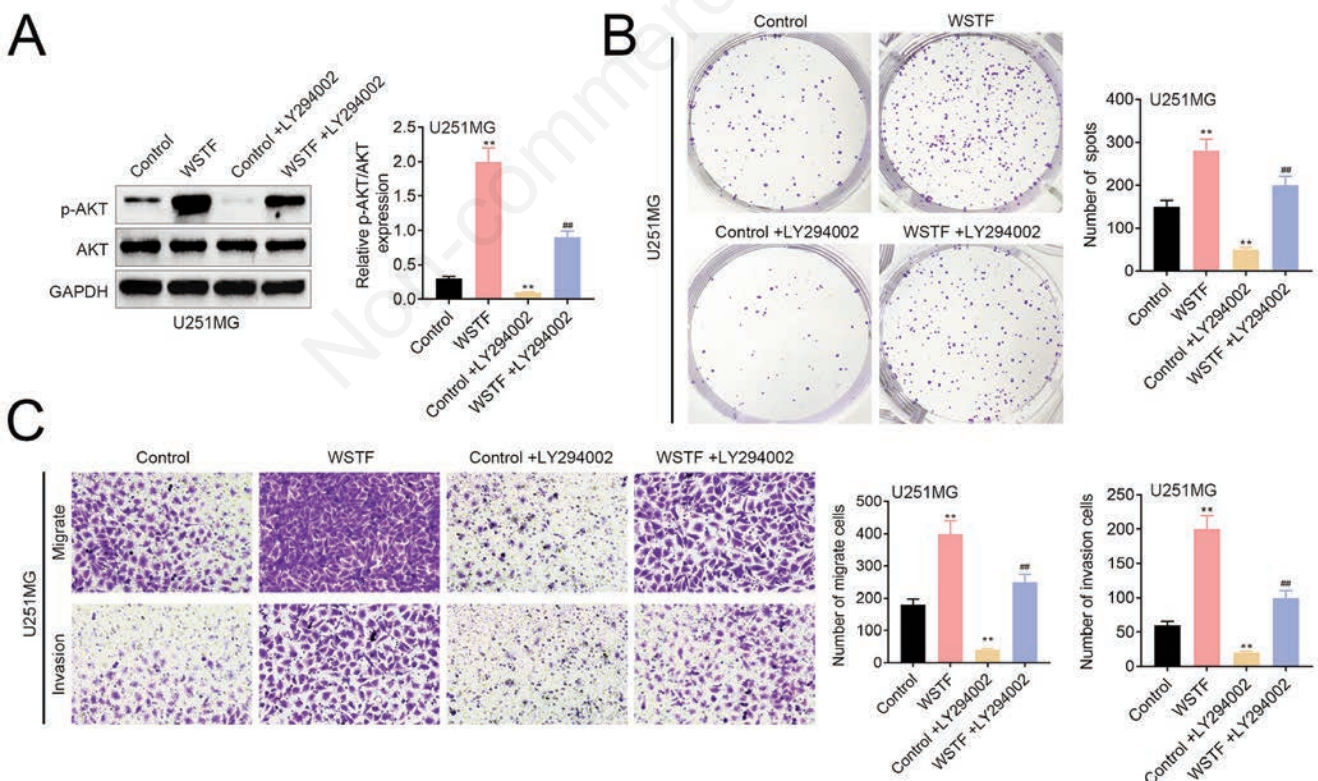
pcDNA-mediated WSTF over-expression or shRNA-mediated WSTF silencing (Figure 5). However, AKT phosphorylation was enhanced in U251MG and LN-229 transfected with pcDNA-WSTF and reduced by shWSTF transfection (Figure 5), demonstrating that WSTF contributed to AKT activation in GBM cell.

### Inhibition of PI3K/AKT attenuated oncogenic role of WSTF in GBM cell

To validate whether WSTF contributed to GBM cell progression through activation of PI3K/AKT pathway, U251MG cells transfected with pcDNA-WSTF were treated with LY294002,



**Figure 5.** WSTF contributed to AKT activation in GBM cell. pcDNA-mediated over-expression of WSTF increased protein expression of p-p-AKT in U251MG (A) and LN-229 (B), while shRNAs-mediated knockdown of WSTF decreased the protein expression. \*\*, #  $p < 0.01$ .



**Figure 6.** Inhibition of PI3K/AKT attenuated oncogenic role of WSTF in GBM cell. A) LY294002 treatment attenuated WSTF over-expression-induced increase of AKT phosphorylation in U251MG. B) LY294002 treatment attenuated WSTF over-expression-induced increase of cell proliferation in U251MG. C) LY294002 treatment attenuated WSTF over-expression-induced increase of cell migration and invasion in U251MG. \*\* vs control; ## vs control + LY294002  $p < 0.01$ .

inhibitor of PI3K. Western blot analysis showed that LY294002 treatment attenuated WSTF over-expression-induced increase in AKT phosphorylation (Figure 6A). Moreover, the cell proliferation (Figure 6B), migration and invasion (Figure 6C) induced by WSTF over-expression were also attenuated by LY294002 treatment. These results revealed that WSTF contributed to GBM cell progression through activation of PI3K/AKT pathway.

## Discussion

Chemo- or radio-therapeutic agents stimulate cytotoxicity in GBM cells through DNA damage, and the DNA repair pathways could prevent the DNA damage through activation of damage sensors, such as gamma-H2A.X.<sup>11</sup> The activation of DNA repair pathways contributes to the standard chemo- or radio-therapies.<sup>12</sup> Therefore, checkpoint/repair molecules were involved in regulation of DNA damage repair and tumor cell apoptosis, thus participating in the pathogenesis of GBM.<sup>12</sup> Since WSTF functions as gamma-H2A.X-binding protein to regulate DNA damage response in tumor cells,<sup>13</sup> and WSTF activates oncogenic pathways in tumor cells,<sup>9</sup> the regulatory role of WSTF in GBM cell growth and invasion were investigated in this study.

Bioinformatics analysis from TCGA database in this study firstly showed that mRNA expression of WSTF was enhanced in GBM tissues, and GBM cells also showed higher expression of WSTF than that of the normal control. Functional assays then indicated that over-expression of WSTF suppressed GBM cell apoptosis, and contributed to the cell proliferation, migration, and invasion. The GBM cell growth and invasion were repressed by silencing of WSTF. Considering that proliferation and invasion are the devastatingly characteristics of GBM, and suppression of GBM cell growth and invasion provided potential therapeutic targets for the treatment of GBM,<sup>14</sup> WSTF might also be regarded as a novel therapeutic strategy for GBM. However, the *in vivo* suppressive role of WSTF silencing on carcinogenesis of GBM should be investigated in the further research.

A variety of signaling pathways have been reported to be implicated in GBM cell proliferation and invasion.<sup>15</sup> PI3K/Akt/mTOR pathway that participates in GBM cell survival, growth, angiogenesis, invasion or drug resistance is the main therapeutic targets for drug design for GBM.<sup>16</sup> Aberrant activation of PI3K/AKT up-regulates anti-apoptotic factors and down-regulates pro-apoptotic factors to contribute to GBM cell growth.<sup>16</sup> The inhibitors of PI3K/AKT/mTOR pathway are widely used in clinical prevention for the brain tumors,<sup>17</sup> especially in GBM.<sup>18</sup> Transcriptional factors, eukaryotic initiation factors, have been regarded as promising targets for glioma patients through regulation of PI3K/AKT/mTOR pathway.<sup>19</sup> WSTF promoted epithelial to mesenchymal transition of lung cancer through activation of PI3K/AKT pathway.<sup>20</sup> Therefore, WSTF might be involved in gliomagenesis through PI3K/AKT pathway. Here, results in this study showed that protein expression of AKT phosphorylation was upregulated in GBM cells transfected with pcDNA-WSTF, while reduced by silencing of WSTF. Moreover, inhibition of PI3K attenuated WSTF over-expression-induced increase in GBM cell proliferation, migration and invasion. Therefore, WSTF might contribute to GBM cell growth and invasion through activation of PI3K/AKT pathway. Post-translational phosphorylation of WSTF at Ser158 residue regulated chromatin remodeling activity,<sup>21</sup> and acetylation of WSTF at Lys 426 promoted the cell proliferation and invasion of tumor cells.<sup>22</sup> The post-translational modification of WSTF involved in GBM cell progression should be investigated in the further research.

In conclusion, the present study for the first time indicated that WSTF over-expression promoted GBM cell proliferation and inva-

sion through activation of PI3K/AKT pathway, and silencing of WSTF contributed to suppression of GBM progression. Therefore, WSTF might be considered as a promising therapeutic target for GBM.

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