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Wwox suppresses breast cancer cell growth through modulation of the hedgehog–GLI1 signaling pathway

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ABSTRACT

Wwox is a tumor suppressor that is frequently deleted or altered in several cancer types, including breast cancer. Previous studies have shown that ectopic expression of Wwox inhibits proliferation of breast cancer cells. However, the underlying mechanism remains unclear. To better understand the molecular mechanisms of Wwox function, we investigated novel partners of this protein. Utilizing the coimmunoprecipitation assay, we observed a physical association between Wwox and the Gli1 zinc-finger transcription factor involved in the hedgehog pathway. Our results further demonstrated that Wwox expression triggered redistribution of nuclear Gli1 to the cytoplasm. Additionally, ectopic expression of Wwox reduced Gli1 expression in vitro. Furthermore, Gli1 Blocks Wwox-induced breast cancer cell growth inhibition. These findings suggest a functional crosstalk between Wwox and hedgehog–GLI1 signaling pathway in tumorigenesis.

1. Introduction

Breast cancers are complex diseases that are caused by alterations of both genetic and epigenetic factors [1,2]. Despite advances in chemotherapy, radiotherapy, and adjuvant hormonal therapy, one third of patients with breast cancers relapse and die of disease. New therapeutic strategies are needed to improve this outcome.

The tumor-suppressor gene, Wwox (WW domain containing oxidoreductase), spans one of the most active fragile sites, FRA16D, at chromosome 16q23.3-24.1, a region exhibiting loss of heterozygosity in breast, prostate, and other cancers [3,4]. Wwox encodes a 414 amino acid, 46 kDa protein. The full-length Wwox possesses two N-terminal WW domains (containing conserved tryptophan residues), a nuclear localization sequence (NLS), and a C-terminal short-chain alcohol dehydrogenase/reductase (SDR) domain [5]. The WW domains are believed to be involved in protein–protein interactions, including a number of transcription factors [6]. The SDR region of Wwox may be involved in sex-steroid metabolism due to its high amino-acid sequence homology to specific oxidoreductases [7].

Numerous studies showed Expression of Wwox is either altered or lost from epigenetic modification in multiple malignant cancers, such as breast, esophageal, lung, ovarian, colon, prostate, and gastric carcinomas [4,8–10]. Restoration of Wwox gene prevents the growth of lung cancer [11], prostate cancer [12], and breast cancer [13]. loss of one allele increased the incidence of mammary tumor formation [14], thus confirming that Wwox is a tumor suppressor. Previous works showed that the Wwox physically and functionally interacts with PPxY-containing proteins such as p73, AP-2gamma, c-Jun, RUNX2, ErbB4, TMEM207 and DeltaNp63alpha via its WW1 domain [6,15–20].

The Hedgehog (Hh) family of proteins regulates a wide variety of developmental processes, and malfunction of this pathway has been linked to numerous human disorders including cancer [21,22]. In vertebrates the Hh pathway begins with the binding of Hh ligands to the Patched receptors on the membrane, which negatively regulates the 7-transmembrane protein Smoothened (Smo), which regulate the transcriptional activity of Gli1 zinc-finger transcription factor [23]. A recent study demonstrated that Gli1 composed of a combination of two PPxYs and a phospho-serine/proline(pSP) motifs in C-terminal region. Numb recruits Gli1 into the complex with Itch to target Gli1 protein for ubiquitination and degradation [24].

In this work, we describe a physical and functional interaction between the Wwox tumor suppressor and the Gli1 zinc-finger transcription factor. We show that Wwox binds Gli1, changes its cellular localization, inhibits its expression in vitro, and counteracts Gli1-mediated effects to breast cancer cell growth.

2. Materials and methods

2.1. Cell culture

Breast cancer-derived cell lines were a gift from Dr. Shao Zhi-min (Fudan University, Shanghai, China). The human cancer

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cell lines MCF-7, T47D, SK-BR-3and ZR-75-30 were maintained in DMEM medium plus 10% fetal bovine serum (Gibco) and cultured in 5% CO_2 humidified atmosphere. MDA-MB-231 were maintained in L15 medium plus 10% FBS and cultured in 100% air humidified atmosphere.

2.2. Plasmids

Human Gli1 expression vector pRK5-Gli1 and pRK5-Myc-Sufu was kindly provided by Dr. Steven Y. Cheng (Nanjing Medical University, China). pRK5-HA-Gli1, pRK5-Myc-Wwox, pRK5-Wwox were constructed by ourself.

2.3. Oligonucleotides

Inhibitor and nonspecific control oligonucleotides for Wwox were purchased from: GenePharma. The template oligonucleotide sequences used were 5′-GATCCCCAAGTCCATGCAACAGGGCTTC CTGTCAGACCCTGTTGCATGGACTTGGTTTTTG-3′ and 5′-AATTCAAA AACCAAGTCCATGCAACAGGGTCTGACAGGAAGCCCTGTTGCATGGA CTTGGG-3′.

2.4. Transfections

Cells were plated in 6 well plates 18 h before transfection and cotransfected with the indicated amounts of expression constructs using FuGENE®HD Transfection Reagent (promega) per manufacturer's instructions.

2.5. RNA isolation and RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. A 0.5 μg aliquot of total RNAs was reverse transcribed into cDNA using random primers following the manufacturer's protocol (TransGen Biotech). Then, mature mRNAs were amplified by PCR using the appropriate primers. The PCR was carried out in a total volume of 20 μL containing 20 mmol/L Tris–HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.6 mmol/L of forward and reverse primers, and 2.5 U of Taq DNA polymerase. The housekeeping gene GAPDH was used as an internal control. The amplification cycles of Gli1, Wwox, ER and GAPDH were 30, 30, 30, 26, respectively. Denaturing, annealing, and extension reactions were performed at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, respectively.

2.6. Design of RT-PCR primers

The PCR primers were designed using the Primer 5.0 software. The primer sequences were: Wwox-200 bp: 5′-ATGTACTC CAACATTCATCGCAG-3′ (sense) and 5′-GTCTCTTCGCTCTGAGCTTCT -3′ (antisense); Gli1-473 bp: 5′-TGTTCAACTCGATGACCC-3′ (sense) and 5′-GTCATGGGGACCACAAGG-3′ (antisense); ER-180 bp: 5′-CCCACTCAACAGCGTGTCTC-3′ (sense) and 5′-CGTCGATTATCT-GAATTTGGCCT-3′ (antisense); GAPDH-159 bp: 5′-TTGATTTTG-GAGGGATCTCG-3′ (sense) and 5′-GTCATGGGGACCACAAGG-3′ (antisense). All primers were synthesized by BGI tech.

2.7. Coimmunoprecipitation

Cells were lysed by using Nonidet P-40 lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors. Lysates were precleared with mouse IgG, immunoprecipitations were carried out in the same buffer, and lysates were washed four times with the same buffer containing 0.1% Nonidet P-40 and 0.1% SDS. Antibodies used were mouse monoclonal anti-Myc (Santa Cruz Biotechnology). Western blotting was performed under standard conditions. Antibodies used for immunoblot were anti-HA-HRP (Roche Applied Science).

2.8. Immunofluorescence

T47D cells were seeded on fibronectin-covered cell culture slides (Becton Dickinson), fixed for 10 min in 3.7% PBS-buffered formaldehyde, permeabilized with 0.05% Triton X-100 in PBS for 5 min, blocked with goat serum (GIBCO BRL), and incubated with mouse monoclonal anti-Myc and rabbit monoclonal anti-HA antibodies. After washing thrice in PBS, sections were incubated for 1 h with secondary antiserum (goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 568) and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were examined by confocal microscopy (Bio-Rad, Hercules, CA) under ×63 magnification.

2.9. Cell growth assays

Cell growth was determined by the methyl thiazolyl tetrazolium (MTT) assay (Sigma). Briefly, T47D cells (2000 cells per well) plated in 96-well plates following transfected with indicated plasmids. MTT reagent was added to each well at 5 mg/mL in a 20 μL volume, and the reaction was incubated for another 2 h. The formazan crystals formed by viable cells were subsequently solubilized in dimethyl sulfoxide, and the absorbance at 490 nm was measured.

2.10. Cell cycle analysis

A total of 2×10^5 cells were plated in 6-well plates and were transfected with indicated plasmids. 72 h after treatment, logarithmically growing cells were collected and washed with PBS three times and fixed with 75% ethanol at −20 °C for at least 1 h. After extensive washing with PBS, the cells were suspended in Hank's balanced salt solution containing 50 mg/mL RNase A (Boehringer Mannheim) and 50 mg/mL propidium iodide (PI) (Sigma), incubated for 1 h at room temperature, and were analyzed by FACScan (Becton Dickinson).

3. Results

3.1. Expression of Wwox negatively correlates with Gli1 expression in breast cancer cells

To study the effects of restoration of Wwox on Gli1 expression in breast cancer cells, we first examined the endogenous mRNA expression of Wwox, Gli1 and ER in several human breast cancer cell lines by RT-PCR (Fig. 1A). In accordance with previous reports [32–35], our result showed that ER mRNA was detectable in estrogen-dependent cell lines, including MCF-7 and T47D cells, but not detectable in estrogen-independent cell lines, such as MDA-MB-231, SK-BR-3 and ZR-75-30 cells. The RT-PCR result showed that MCF7 and ZR-75-30 cells express high endogenous Wwox mRNA, whereas T47D and SK-BR-3 cells express low endogenous Wwox, and MDA-MB-231 cells are Wwox negative. Interestingly, we observed that expression level of Wwox correlates negatively with expression of Gli1 mRNA (Fig. 1A).

Because Gli1 is a target of the Hh pathway that acts in a positive feedback to reinforce the Gli1 activity [25], we assumed that increased Wwox expression may affect the expression of Gli1. To test this possibility, MDA-MB-231 and T47D cells with low levels of Wwox were transfected with RK5-Wwox expression construct. We found Gli1 mRNA levels in the RK5-Wwox transfected cells dramatically dropped compared with RK5-GFP transfected cells. About

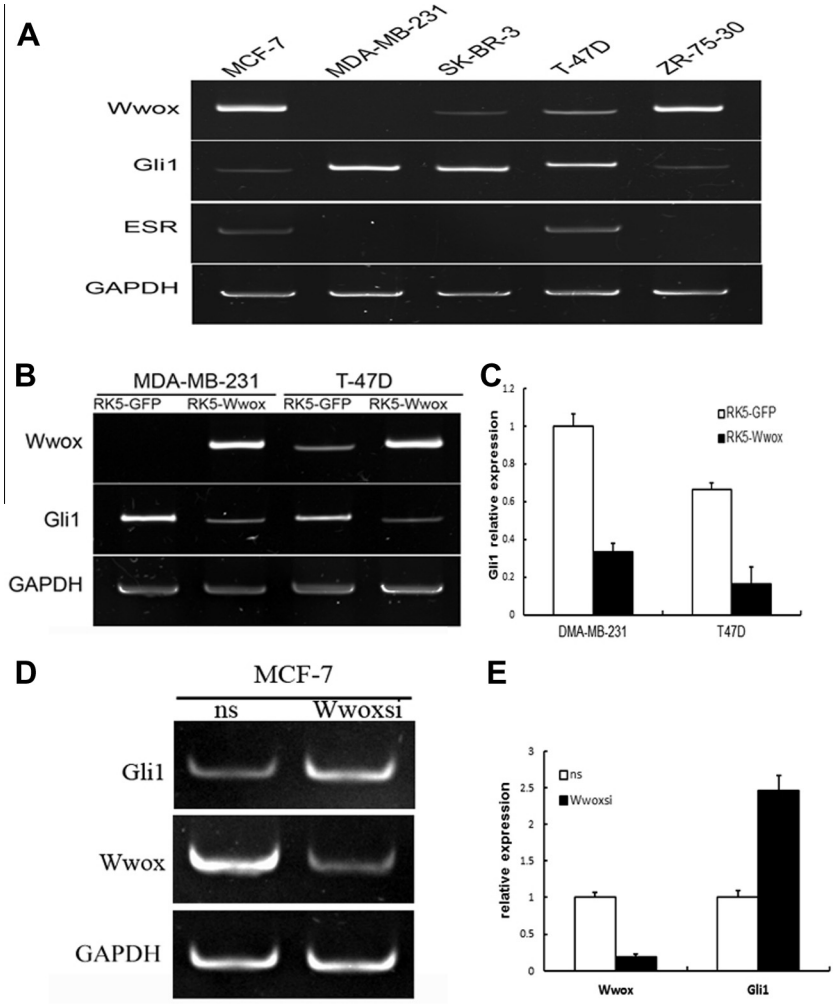

Fig. 1. Wwox down-regulates Gli1 in breast cancer cells. (A) Endogenous expression of Wwox and Gli1 in human breast cancer cells lines, ESR and GAPDH served as control. (B) Effects of Wwox on the Gli1 mRNA levels in MDA-MB-231 and T47D cells determined by RT-PCR, MDA-MB-231 and T47D cells were transfected with RK5-GFP or RK5-Wwox in and kept in growth medium for 48 h before RNA isolation. (C) The densitometry analysis performed and data presented as Mean ± SD of three independent experiments ($n = 3$). The RT-PCR result was quantified by ImageJ. (D) Effects of Wwox on the Gli1 mRNA levels in MCF-7 cells determined by RT-PCR, MCF-7 cells were transfected with Wwoxsi or nonspecific control oligonucleotides and kept in growth medium for 48 h before RNA isolation. (E) The densitometry analysis performed and data presented as Mean ± SD of three independent experiments ($n = 3$). The RT-PCR result was quantified by ImageJ.

66% and 75% decrease of Gli1 mRNA were observed in MDA-MB-231 and T47D respectively (Fig. 1B and C). MCF-7 cells with high levels of Wwox were transfected with Wwoxsi. We found Gli1 mRNA levels in the Wwoxsi transfected cells dramatically increase compared with RK5-GFP transfected cells (Fig. 1D and E). Taken together, our data indicate that Expression of Wwox negatively correlates with Gli1 expression in breast cancer cells lines.

3.2. Directly physical interaction between Wwox and Gli1

It has been reported that the Wwox physically and functionally interacts via its WW1 domain with the DeltaNp63alpha through its PPxY motif [18]. Because Gli1 also contains two distinct PPxY and phospho-serine/proline motifs in its sequence and has been shown to interact with WW domain-containing proteins [24,26], we hypothesized that Wwox might physically associate with Gli1 and regulate its transactivation activity. To examine whether Wwox binds to Gli1 directly, HEK293 cells were transiently cotransfected with Myc-Wwox and HA-Gli1 expression constructs. Cell lysates were immunoprecipitated (IP) with anti-Myc antibodies followed by immunoblotting (IB) with HRP-conjugated antibody to HA. Our results showed that Wwox interacts with Gli1 as determined by immunoprecipitation with anti-Myc and immunoblotting with anti-HA antibody (Fig. 2A), Gli1 interacts with Wwox as determined by immunoprecipitation with anti-HA and immunoblotting with anti-Myc antibody (Fig. 2B). Taken together, these results suggest that Wwox specifically binds Gli1.

3.3. Wwox–Gli1 interaction affects Gli1 intracellular localization

When the hedgehog signaling pathway is activated, Gli1 protein translocates to the nucleus, where it binds to the promoters of target genes and transactivates its target genes such as Ptc and Cyclin D2 [27,28]. In contrast, Wwox is a cytoplasmic protein. The question thus arises: in which subcellular compartment do Wwox and Gli1 interact? To answer this question, we studied the localization of both proteins with the aid of confocal microscopy. MYC-Wwox alone or transfected with HA-Gli1 was transiently expressed in T47D cells. Localization of the HA- or Myc-tagged proteins was then determined by immunofluorescent staining using the appropriate antibodies, as described in Section 2. Although the majority of Gli1 localizes to the nucleus, some cells also showed mild cytoplasmic staining of transfected T47D cells (Fig. 3A). In contrast, exogenous Wwox is mainly detected in the cytoplasm (Fig. 3B). Interestingly, in cells cotransfected with Myc-Wwox and HA-Gli1, 65–75% of cells showed cytoplasmic

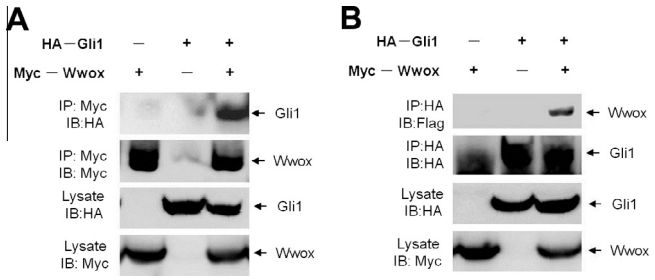

Fig. 2. Wwox physically interacts with Gli1 in vivo. 293 cells were transiently transfected with the expression plasmids encoding expressing various vectors (above lanes). (A) Whole-cell lysates were immunoprecipitated (IP) with anti-Myc antibodies 36 h after transfection. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-HA antibodies. (B) Whole-cell lysates were immunoprecipitated (IP) with anti-HA antibodies 36 h after transfection. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-Myc antibodies.

staining of Gli1 where it colocalizes with Wwox (Fig. 3C). Similar results were obtained with MCF-7 cells, which express high levels of endogenous Wwox protein, upon transfection with HA-Gli1 (Fig. 3D). These data suggest that binding of Wwox to Gli1 prevented Gli1 from entering the nuclei to activate or repress transcription of target genes such as Ptc.

3.4. Gli1 blocks Wwox-induced growth inhibition and apoptosis in T47D cells

We next investigated the biological effect of the binding of Wwox and Gli1. Previous work has shown that Wwox restoration

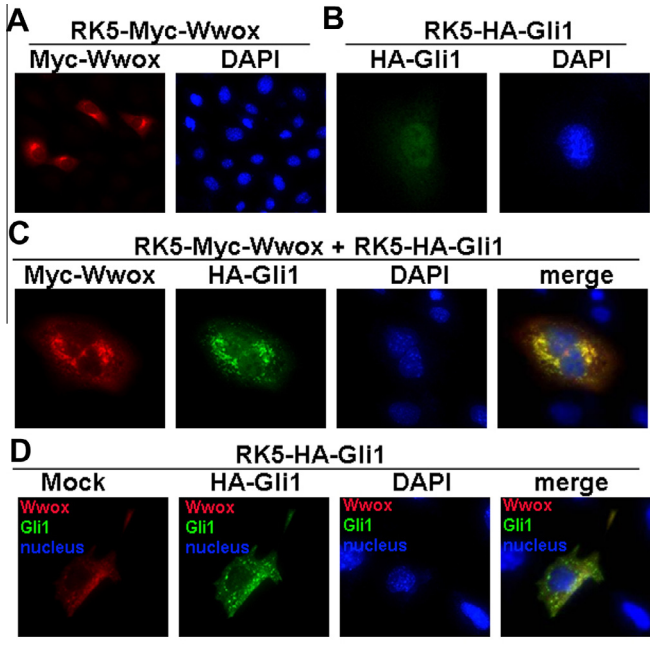

Fig. 3. Subcellular localization of Wwox and Gli1. (A) T47D cells were transfected with RK5-HA-Gli1. 36 h later, cells were fixed, permeabilized, and immunostained with monoclonal mouse anti-HA, followed by secondary goat anti-mouse Alexa Fluor 488, and nuclei were counterstained with DAPI and visualized by confocal microscopy. (B) T47D cells were transfected with RK5-Myc-Wwox, cells were immunostained with monoclonal rabbit anti-Myc antibodies, secondary goat anti-rabbit Alexa Fluor 568. (C) T47D cells were transfected with RK5-HA-Gli1 and RK5-Myc-Wwox, 36 h later, cells were fixed, permeabilized, and immunostained with monoclonal mouse anti-HA and polyclonal rabbit anti-Myc antibodies, followed by secondary goat anti-mouse Alexa Fluor 488 and secondary goat anti-rabbit Alexa Fluor 568. (D) MCF-7 cells were transfected with RK5-HA-Gli1. Cells were prepared as B. Antibodies used were polyclonal anti-Wwox (red) and monoclonal anti-HA (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

suppressed breast cancer cell growth [13]. The Gli1 has been found to be elevated in breast cancer tissues [29,30], and overexpression of Gli1 led to estrogen-responsive breast cancer cells proliferation through sustaining activity of the cyclin-Rb axi [31]. We hypothesized that Wwox might suppress Breast cancer cell growth by altering the nuclear localization of Gli1. Growth assays were done to determine if binding of Gli1 by Wwox has an effect on cell growth. T47D cells were transiently-transfected with Wwox in the presence or absence of wild type Gli1. The cells were cultured 72 h and subjected to cell cycle analysis by flow cytometry. The results showed that Wwox-mediated reduction of cellular distribution in the G0/G1, S and G2/M phases was reversed by Gli1 (Fig. 4A and B). Furthermore, MTT showed significant inhibition of cell growth in RK5-Wwox transfected cells compared with RK5-GFP transfected cells, and Wwox-induced growth inhibition was reversed by Gli1. Thus, our data strongly support the protective role of Gli1 in blocking Wwox–Wwox-induced growth inhibition.

4. Discussion

In the current study, we first demonstrate that Wwox down-regulates Gli1 in breast cancer cells. Several groups analyzed Wwox and Gli1 expression in breast cancer in cell lines, but their results are not entirely consistent. For example, Mukherjee showed high Gli1 mRNA levels in estrogen-dependent cells (MCF-7, T47D) and estrogen-independent cells (MDA-MB-231 and SK-BR-3) [29]. and Zhao J. and Zhang X. found lacking of Gli1 mRNA expression in MCF-7 cells and high levels in T47D, MDA-MB-231, SK-BR-3 cells [31,32]. Different primers or cell culture conditions may result in the differences between the studies. In this study we clearly showed that expression of Gli1 mRNA was very low in MCF-7 and ZR-75-30 but high in MDA-MB-231, T47D and SK-BR-3. In contrast, expression of Wwox mRNA was high in MCF-7 and ZR-75-30 but low in MDA-MB-231, T47D and SK-BR-3. Because Gli1 is a target of the Hh pathway that acts in a positive feedback to reinforce the Gli activity [25], we next set to determine whether Wwox might down-regulated Gli1 in breast cancer cell lines. To test this hypothesis, we used RK5-Wwox to transiently transfected breast cancer cell lines MDA-MB-231 and T47D. Gli1 mRNAs in the RK5-Wwox transfectants was tested to compare with Gli1 mRNAs in the RK5-GFP transfectants cells. Our data indicate that Gli1 mRNA was down-regulated by overexpression of Wwox.

Previous characterization of Wwox partners revealed its WW domains interaction with PPxY-containing proteins [6,15–20]. Nevertheless, other non-PPxY members were also reported including Jnk1, Tau and Mdm2 [33–35]. Z. Salah' results demonstrate that WWOX interaction with DeltaNp63alpha is not mediated by the PPxY motif of DeltaNp63alpha. These data might suggest that WW1 domain of WWOX might interact with other proline-rich motifs rather than the canonical PPxY motif. In fact, it was shown recently that classical WW domains, known to interact with canonical PY motifs, could also bind non-canonical pSP or pTP motifs highlighting the plasticity of WW domains interactions [24,36]. L. Di Marcotullio' data demonstrate that Itch physically interacts with either PPxY and pSP motifs of Gli1. Our further research would be necessary to decipher the mechanisms of physical interaction between Wwox and Gli1.

Hedgehog–GLI1 signaling pathway plays an essential role in vertebrate organogenesis as well as the development of some cancers, including breast cancer [37]. Numerous studies show that the transcriptional activity of Gli1 is regulated by other proteins such as Itch and AKT [24,27]. In this work, we identify a novel molecular mechanism of Wwox-induced regulation of the hedgehog–GLI1 signaling pathway (Fig. S1).

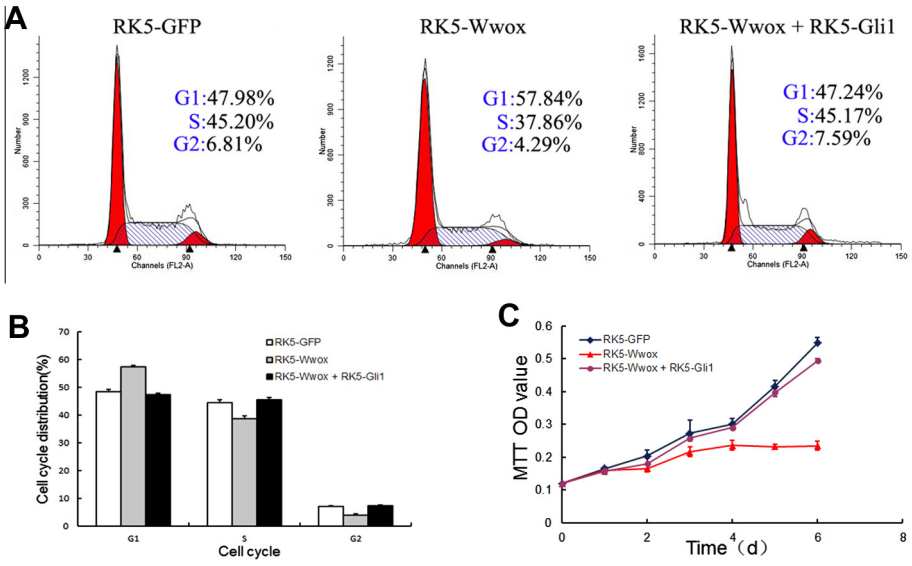

Fig. 4. Wwox suppresses Gli1 – induced T47D cell growth. (A) T47D cells were transfected with the indicated plasmids, and cells harvested at 72 h and subjected to flow cytometry analysis and cell cycle distribution was presented as the percentage of cells at each phase. (B) Above data presented as Mean ± SD of two independent experiments (n = 3). (C) Cell proliferation was determined by MTT assay, T47D cells (2000 cells per well) plated in 96-well plates following transfected with indicated plasmids, Medium was changed 6 h later. Cells were incubated with MTT (1 mg/mL) for 2 h at 37 °C every 24 h up to 6 days.

In conclusion, our results strongly suggest that overexpression of Wwox suppresses Breast Cancer cell growth, and molecular mechanisms of Wwox in breast Cancer cell growth are Physical interacting with Gli1 and sequestering Gli1 in the cytoplasm. The detailed molecular mechanisms of physical interaction between Wwox and Gli1 are not yet fully elucidated but will be a future area of research.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.133.

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