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BRCA1 regulates transforming growth factor-β (TGF-β1) signaling through Gadd45a by enhancing the protein stability of Smad4

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ABSTRACT

BRCA1 is a well established tumor suppressor gene, which is involved in many cellular processes, including DNA damage repair, cell cycle control, apoptosis, as well as transcriptional control. In this work, we have found that BRCA1 is involved in regulating TGF-β1/Smad pathway. The loss of endogenous BRCA1 greatly attenuated TGF-β1-induced growth inhibition and cell cycle G1 arrest. BRCA1 greatly maintains stability of Smad4 protein, and the loss of BRCA1 results in Smad4 down-regulation, which is likely related to its downstream gene Gadd45a. Gadd45a is able to interact with β-Trcp1, a-F-box protein of SCF E3 ligase, and consequently suppresses the ubiquitin-degradation of Smad4 by $SCF^{\beta\text{-}trcp1}$, as reflected by the observations that the induction of Gadd45a substantially stabilizes Smad4 protein. In addition, exogenous expression of Gadd45a can largely rescue the protein level of Smad4 in BRCA1 deficient cells. These results further demonstrate that BRCA1 may act as an important negative regulator in cell cycle progression and tumorigenesis through regulating the stability of Smad4, and define a novel link that connects BRCA1 to TGF-β1/Smad pathway.

1. Introduction

Transforming growth factor-beta (TGF-β1) is a multifunctional signaling protein which regulates cell proliferation, apoptosis, differentiation and migration. Following TGF-β1 signaling, regulatory Smads (R-Smads) containing Smad2 and Smad3 are phosphorylated by the activated receptor I, which is phosphorylated by the constitutively active receptor II, then oligomerized with a common effector Smad4 (Co-Smad). Consequently, the heteromeric complexes of R-Smads/Smad4 accumulate in the nucleus and engage in transcriptional complexes to regulate target gene, such as repression of *C-myc*, and activation of $p21^{Waf1/Cip1}$ and $p15^{Ink4b}$, all of which contribute to the inhibition of cell growth (Massague and Wotton, 2000; Pardali et al., 2000; Shi and Massague, 2003). TGF-β1 signaling generally has a negative effect on cell growth, inactivation of this pathway results in tumorigenesis (Blobe et al., 2000; de Caestecker et al., 2000; Harakeh et al., 2004; Massague, 2008). Tumor cells often escape from TGF-β1-induced growth arrest and apoptosis due to functional inactivation of Smads (Ramachandra et al., 2002; Ten Dijke et al., 2002). Functionally disruptive

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mutations of Smads exist in different types of cancers and associated with malignant progression (Fleming et al., 2013; Harradine and Akhurst, 2006; Miyaki and Kuroki, 2003). Furthermore, certain oncogene products have been shown to functionally inactivate Smads, such as Evi1 abrogating the DNA binding of Smad3 (Kurokawa et al., 1998) and oncogenic Ras abrogating R-Smad nuclear accumulation (Kretzschmar et al., 1999) and inducing Smad4 degradation (Saha et al., 2001) etc. All these alterations of Smads contribute to tumor cells resistance to TGF-β1-induced growth inhibition. In addition, homozygous knock-out mice of *Smad4* are embryonic lethal while *Smad4* heterozygous mice survive and develop malignant intestinal tumors accompanied with loss of heterozygosity (LOH) (Takaku et al., 1999; Xu et al., 2000).

BRCA1 was identified as the first breast and ovarian cancer susceptibility gene (Miki et al., 1994), which has been reported frequently mutated in hereditary human cancer (Hogervorst et al., 1995; Johannsson et al., 1996). As a tumor suppressor gene, BRCA1 is involved in many cellular processes including DNA damage repair, cell cycle control, apoptosis, as well as transcriptional control (Wu et al., 2010). BRCA1 relocates to DNA damage sites and forms nuclear foci following DNA double-strand breaks (DSBs) and co-localizes with Rad51, which is an essential protein in homologous recombination repair (Scully et al., 1997a, 1997b). In addition to its role in DNA repair, BRCA1 also participates in the entire cell cycle checkpoints to maintain the genome integrity, which ensure cell cycle proceeding normally (Mullan et al., 2006). The transcription ability of BRCA1 also contributes to the cell cycle regulation, which can stimulate the promoter activity and expression levels of growth inhibitory genes, such as $p21^{WAF1/Cip1}$, *Gadd45a* and $p27^{Kip1}$ (Fan et al., 2002; Somasundaram et al., 1997; Williamson et al., 2002). Therefore, the pivotal roles of BRCA1 in DNA damage response and cell cycle control might explain itself as an important tumor repressor. All these studies suggest that BRCA1 may play a key role in negative regulation of cell malignancy. However, the detailed mechanism(s) remains to be further elucidated. Here, we show that BRCA1 is involved in regulating TGF-β1/Smad pathway. Disruption of endogenous BRCA1 resulted in diminished TGF-β1 response of anti-proliferation in $BRCA1^{-/-}$ MEFs and MCF-7 cell line. Likely, BRCA1 maintains TGF-β1 responsiveness through its downstream gene Gadd45a by enhancing the protein stability of Smad4. Therefore the role of BRCA1 in regulating Smad4 might contribute to its tumor suppressor function.

2. Materials and methods

2.1. Plasmid clones and antibodies

Glutathione S-transferase (GST)-Gadd45a was constructed by inserting Gadd45a full-length cDNA into the XhoI site of the pGEX-5X-1 vector. The plasmid containing ORF of Smad4 and the control were purchased from Vigene Biosciences. The antibodies of BRCA1, Gadd45a, Smads and β-Trcp1 were commercially provided by Cell Signaling Technology. Actin antibody was commercially purchased from Sigma–Aldrich.

2.2. Cell culture and treatment

Immortalized $BRCA1^{+/+}$ and $^{-/-}$ MEFs were generously provided by Chu-Xia Deng (NIH, USA), and $Gadd45a^{+/+}$ and $^{-/-}$ MEFs were kindly given by Albert J. Fornace (University of Georgetown, USA). These MEFs were grown at 37 °C in a humidified atmosphere with 5% CO_2. Cell cultures were maintained in DMEM with 10% fetal bovine serum. The maintenance of Gadd45a tet-off inducible HeLa cell line and the induction of Gadd45a were performed as described previously (Jin et al., 2002). MCF-7 cell line was maintained in 1640 medium with 10% fetal bovine serum. For TGF-β1 (BD Biosciences, San Jose, CA) treatment, cells were seeded in a 96-well plate (8000 cells/well) and grown overnight. After 24 h synchronization in serum-free medium, cells were treated with 2.5 ng/ml TGF-β1 for 24 and 48 h till MTT analysis with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. For UV treatment, exponentially growing cells were rinsed with PBS and irradiated with UV-C at doses between 25 and 40 J/m^2 for indicated times. 25 μg/ml CHX (Sigma–Aldrich, St. Louis, MO), a translation blocking agent, was added to exponentially growing cells to inhibit protein synthesis and cells were incubated at 37 °C for different times till harvest. 20 μM MG132 (Sigma–Aldrich) was used to inhibit protein ubiquitin degradation.

2.3. MTT assay

For analysis of cell proliferation after TGF-β1 treatment, both wild-type and Gadd45a-null MEFs were treated with TGF-β1 for different times. Then cells were incubated with the 0.5 mg/ml MTT in a CO_2 incubator at 37 °C for 4 h. After removing all the media in the plates, 200 μl of DMSO was added to each well. Following incubation for a further 1 h, the absorbance at 490-nm in solution of every well was measured.

2.4. Proteins preparation and western blotting assay

For whole cell protein extraction, cells were lysed in 1 × PBS, 1% Nonidet P-40, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin and 50 μg/ml phenylmethylsulfonyl fluoride (PMSF). Cell lysates were kept on ice for 40 min and centrifuged at 14,000 g at 4 °C for 30 min. Then supernatants were collected as total cellular protein. Cellular protein extracted was separated by 12% SDS PAGE gel and transferred to Nitrocellulose membranes. Then, membranes were incubated with the indicated primary antibodies and anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase for enhanced chemiluminescence (Applygen, Beijing, China) detection of the signals.

2.5. RNA extraction and RT-PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Five micrograms of total RNA in each sample was used to

synthesize the first strand cDNA in 20 μl volume with SuperScriptTM First-Strand Synthesis System for RT-PCR Kit (Invitrogen). The single-strand cDNA synthesized was used as the template of PCR. The *VEGF* (Vascular Endothelial Growth Factor) and *GAPDH* (glyceraladehyde-3-phosphate dehydrogenase, a housekeeping gene) were amplified by PCR (forward of *VEGF*, 5'-gcc tcc gaa acc atg aac tt-3'; reverse of *VEGF*, 5'-tt aca cgt ctg cgg atc ttg-3'; forward of *GAPDH*, 5-gct gag aac ggg aag ctt gt-'; reverse of *GAPDH*, 5-gcc agg ggt gct aag cag tt-3').

2.6. GST-pull down and immunoprecipitation

The expression and extraction of GST-Gadd45a fusion protein was performed as described previously (Tong et al., 2005). Next, the glutathione-agarose beads (Sigma) were mixed with supernatant solution of lysed bacteria at 4 °C overnight. Then glutathioneagarose bead-conjugated GST fusion proteins were incubated with 3 mg lysates at 4 °C for 12 h. Then the mixtures were centrifuged and 10 μl supernatant was reserved to analyze Actin as control. For immunoprecipitation, cellular lysates were incubated with 10 μl of antibody and 20 μl of protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 8 h. The agarose beads-immunocomplexes were treated as described in GST-pull down assay.

2.7. Flow cytometry

Cells were removed from plates with trypsin and pelleted with centrifuge at 1000 g for 5 min. After washing 3 times with PBS, cell pellet were resuspended with 10% formaldehyde and incubated for 10 min. Next, cells were pelleted again and washed. At last cells were resuspended in PBS containing 3% serum and 70% ethanol. After at least 1 h at 4 °C, the cells were stained with propidium iodide and DNA contents were measured by Flow Cytometry.

2.8. Statistics

Each experiment was performed at least three times. Data were presented as the mean ± S.D. (standard deviation) and analyzed with SPSS software. A Student's *t*-test was used for statistical analysis of comparative data containing the differences of the MEFs' response to TGF-β1 and phases of cell cycle in different culture densities. The protein levels were quantified by Gel-Pro analyzer from multiple experiments and statistical analysis by SPSS software. Values of $p < 0.05$ were considered significant.

3. Result

3.1. Loss of BRCA1 attenuates TGF-β1-induced growth inhibition in MEFs and MCF-7

To elucidate whether BRCA1 plays a role in TGF-β1-induced growth inhibition, the TGF-β1 response of anti-proliferation was examined in MEFs derived from BRCA1 conditional knockout mice and wild-type mice. As shown in Figure 1A, following treatment with TGF-β1, proliferation of *BRCA1*$^{+/+}$ MEFs was inhibited and this growth suppression was significant at 48 h after cell exposure to TGF-β1 (about 15%, $p < 0.05$). Interestingly, the TGF-β1-induced growth inhibition was greatly abrogated in *BRCA1*$^{-/-}$ MEFs (Figure 1A). Cell cycle distributions were analyzed by Flow Cytometry after MEFs treated with TGF-β1. The results in Figure 1B showed that G1 arrest was induced in *BRCA1*$^{+/+}$ MEFs following TGF-β1 treatment and it was significant after 48 h exposure to TGF-β1 (about 10%, $p < 0.05$), but not in *BRCA1*$^{-/-}$ MEFs (Figure 1B). MCF-7, a cell line which had been reported sensitive to TGF-β1 (Mazars et al., 1995), was used to further confirm these results. The results in Figure 1C demonstrated that the TGF-β1-induced growth inhibition was about 19% ($p < 0.05$)in control siRNA transfected (siNC) cells, but the inhibition greatly attenuated after knocking down the expression of BRCA1 by siRNA. G1 arrest (about 8%, $p < 0.05$) was induced after TGF-β1 treatment in control siRNA transfected cells (siNC), while the failure of G1 arrest was found in BRCA1 siRNA transfected cells, shown in Figure 1D. These observations further confirm that BRCA1 might contribute to TGF-β1-induced growth inhibition.

3.2. Loss of BRCA1 results in the down-regulation of Smads proteins in TGF-β1 pathway

Considering the potential role of BRCA1 in TGF-β1-induced growth inhibition, the expression of TGF-β1/Smad pathway members was firstly examined in *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs. Strikingly, several TGF-β1/Smad pathway members, including Smad2, Smad3 and Smad4, were significantly down-regulated in *BRCA1*$^{-/-}$ MEFs (Figure 2A). To further confirm BRCA1 role in regulating Smads expression, MCF-7 cell line was employed. After knocking down the endogenous expression of BRCA1 by siRNA, the expression of these Smads were also decreased (Figure 2B). Taken together, all these findings indicate that BRCA1 regulates the protein level of cellular Smad2, Smad3 and Smad4, which might contribute to BRCA1 role in TGF-β1 responsiveness.

3.3. BRCA1 maintains the Smad4 protein level by increasing its protein stability indirectly

Since Smad4 is the co-Smad in TGF-β1/Smad pathway, we take Smad4 protein as the model to elucidate the mechanism of down-regulation of Smad members in TGF-β1 pathway in the BRCA1 deficient cells. As BRCA1 can function as a transcription factor (Rosen et al., 2006), we applied RT-PCR to check the transcriptional influence on Smad4 by BRCA1 and found no differential expression between *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs, and knockdown of BRCA1 in MCF-7 cells did not cause reduced expression of Smad4 (Figure 2C and 2D). Thus, effect of BRCA1 on Smad4 expression appears not on the transcriptional level, but is probably associated with its protein stability. To determine the half-life of Smad4 in these two cell lines, we added cycloheximide (CHX) into the culture medium, which is an inhibitor of protein biosynthesis in eukaryotic organisms. As shown in Figure 3A, after CHX was employed, the degradation of Smad4 protein occurred more quickly in *BRCA1*$^{-/-}$ MEFs than that in wild-type MEFs. The protein levels of Smad4 were quantified by Gel-Pro analyzer. The half-life of

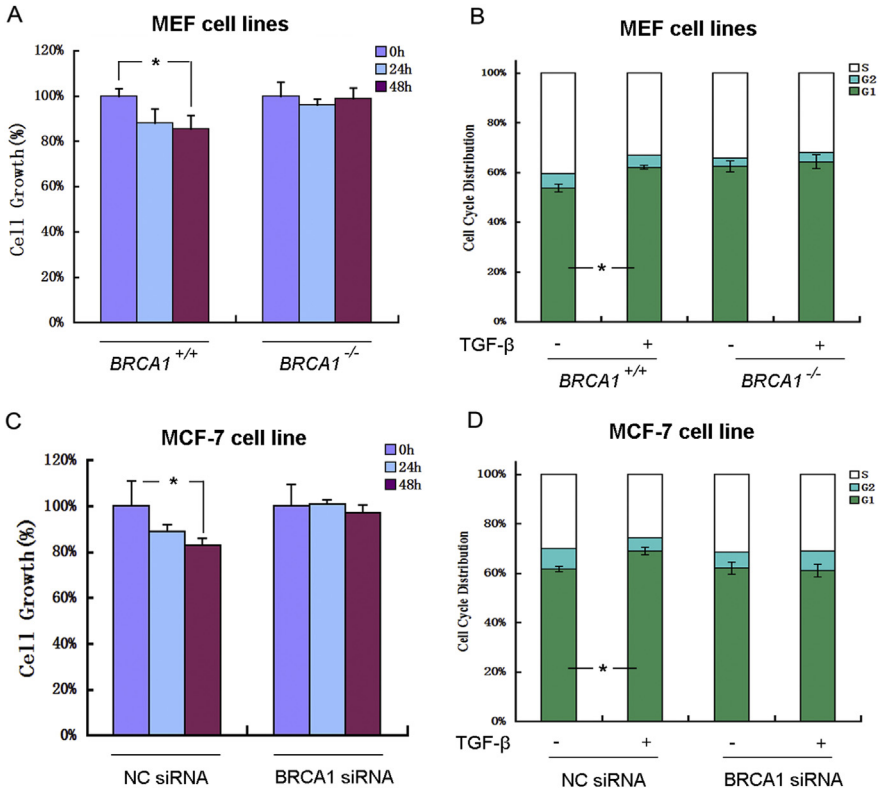

Figure 1 – The effect of TGF-β1 on the growth of *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs and MCF-7 cells treated with BRCA1 RNAi. (A) *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs were seeded on 96-well plates overnight to allow attachment. After 24 h of serum starvation, the cells were treated with 5 ng/ml TGF-β1 for 24 and 48 h, and cell growth was assessed by MTT assay. The cells without TGF-β1 treatment were used as control. The data were obtained from four individual experiments and are expressed as mean ± S.D. of percentage of the respective control. (B) Both MEF cells were treated with 5 ng/ml TGF-β1 for 24 and 48 h as described in (A). Then cells were harvested with trypsin for cell cycle analysis with Flow Cytometry. The data were obtained from three individual experiments and presented as the mean ± S.D. (C) MCF-7 cells were seeded on 96-well plates overnight to allow attachment after transfected with the control siRNA (siNC) or BRCA1 siRNA, and then treated with 10 ng/ml TGF-β1 for 24 and 48 has described in (A). (D) MCF-7 cells which had been transfected with siNC or BRCA1 siRNA were treated with 10 ng/ml TGF-β1 for 24 and 48 h as described in (B).

Smad4 in *BRCA1*$^{+/+}$ MEFs was longer than 10 h but decresed to about 2.5 h in *BRCA1*$^{-/-}$ MEFs (shown in Figure 3B), which suggest that BRCA1 might be involved in maintaining the stability of Smad4 protein. As an E3 ligase, BRCA1 is considered to mediate protein degradation (Starita and Parvin, 2006), but little is known about how BRCA1 maintains protein stability. We thus examined the association between BRCA1 and Smad4. As shown in Figure 3C, the results from immunoprecipitation (IP) assays demonstrated that there is no direct interaction between BRCA1 and Smad4. We also use an immunofluorescent approach to confirm that there is no co-localization of BRCA1 and Smad4 (Figure 3D). These observations indicate that BRCA1 is able to regulate the Smad4 protein level by maintaining its protein stability but does not physically bind to Smad4.

3.4. *Gadd45 maintains TGF-β1-induced growth inhibition by stabilization of Smad4 protein*

Simultaneously, we observed that Smad4 protein was obviously down-regulated in *BRCA1*$^{-/-}$ MEFs following the increased culture densities, but up-regulated in *BRCA1*$^{+/+}$ MEFs (Figure 4A). Interestingly, the expression pattern of Smad4 in *BRCA1*$^{-/-}$ MEFs was similar to Gadd45a, which is a growth arrest and DNA damaging inducible gene regulated by BRCA1 (Zhan, 2005). To better understand the role of Gadd45a in TGF-β1-induced growth inhibition, we knocked down the expression of Gadd45a by siRNA in MCF-7 cell line. As shown in Figure 4B, TGF-β1-induced growth inhibition was significant after 48 h cell exposure to TGF-β1 (about 18%, $p < 0.05$) in the control siRNA (siNC) transfected cells but not in Gadd45a siRNA transfected cells. In addition, we examined MEFs derived from Gadd45a knockout mice and wild-type mice, and found that the TGF-β1-induced growth inhibition was greatly abrogated in *Gadd45a*$^{-/-}$ MEFs while the growth suppression was significant in wild-type MEFs after cells exposure to TGF-β1 for 48 h (about 6%, $p < 0.05$). Consistently, cell cycle G1 arrest was induced in *Gadd45a*$^{+/+}$ MEFs and it was significant after 48 h exposure to TGF-β1 (about 11%, $p < 0.05$), but not in *Gadd45a*$^{-/-}$ MEFs (Figure 4C and 4D). These results suggest that loss of Gadd45a enables MEFs to escape from TGF-β1-induced growth inhibition. Next, increased cell culture density was employed to induce

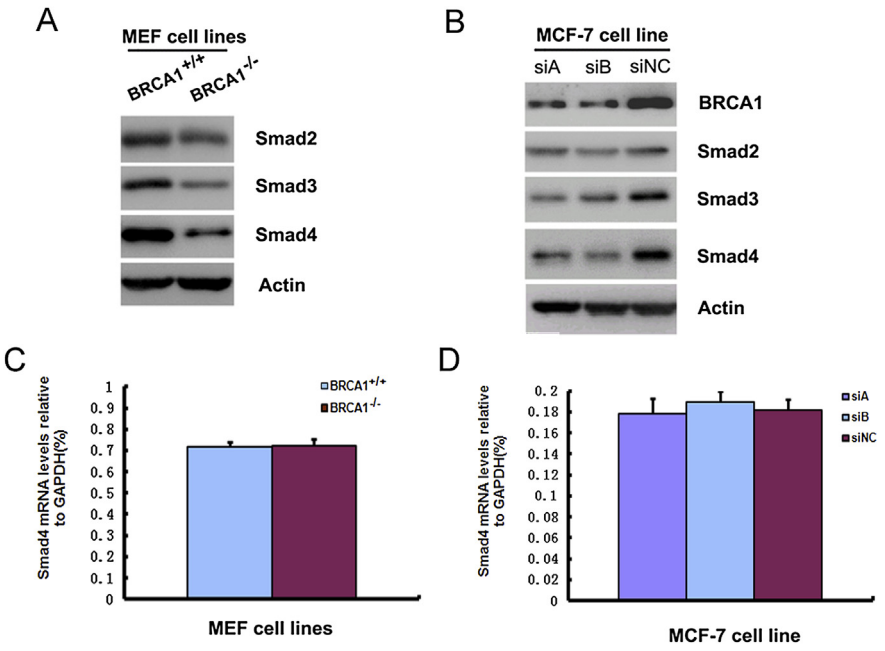

Figure 2 – BRCA1 regulates the protein level Smads protein. (A, C) Exponentially growing *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs were cultured with complete DMEM and cells were collected and lysates were prepared for immunoblotting assay with antibodies to Smad2, Smad3 and Smad4. Actin was used as the loading control. Total RNA was prepared for analyzing the expression of *smad4* transcripts by RT-PCR. (B, D) MCF-7 cells were transfected with BRCA1 siA and siB or control siRNA (siNC). After 48 h transfection, cells were collected and lysates were prepared for immunoblotting assay and total RNA was prepared for RT-PCR as described in (A).

Gadd45a expression, as Gadd45a is a growth arrest and DNA damage inducible gene.

As shown in Figure 5A, Smad4 was down-regulated in *Gadd45a*$^{-/-}$ MEFs following the increased culture densities, but up-regulated in *Gadd45a*$^{+/+}$ MEFs. To excluded the transcriptional influence on Smad4 by Gadd45a, we also checked the RNA levels of Smad4 in both of *Gadd45a*$^{+/+}$ and $^{-/-}$ MEFs. As expected, there was no difference between these two cell lines (Figure S1A).To further confirm Gadd45a role in regulating Smad4 expression, a tetracycline regulated (tet-off) Hela Gadd45a inducible cell line was used (Jin et al., 2002; Tong et al., 2005). After withdrawal of tetracycline, Gadd45a was highly induced and the expression of Smad4 was also increased (Figure 5B). These results go along with the observations in *BRCA1*$^{+/+}$ and $^{-/-}$MEFs. Besides cell culture density, Gadd45a can be induced by various kinds of DNA-damaging agents such as UV radiation (UV) and ionizing radiation (IR) etc (Zhan, 2005). Therefore, UV was used to induce the expression of Gadd45a in MEFs. The results in Figure 5C showed that following UV exposure, Smad4 protein was obviously down-regulated in *Gadd45a*$^{-/-}$ MEFs, while it kept similar level with Gadd45a induction in *Gadd45a*$^{+/+}$ MEFs, suggesting that the loss of Gadd45a might result in the down-regulation of Smad4. As in *BRCA1*$^{+/+}$ MEFs, UV radiation induced the expression of Gadd45a and Smad4, as well as BRCA1. But in *BRCA1*$^{-/-}$MEFs, the expression of Gadd45a and BRCA1 were rarely detectable, while Smad4 protein showed little up-regulation (Figure 5D). In addition, we also examined the Smad4 expression levels in *Gadd45a* knockout mice and wild-type mice, as shown in Figure 5E, Smad4 was down-regulated in *Gadd45a* knockout mice, both in liver and muscle tissues.

3.5. *Gadd45a-mediated inhibition of Smad4 ubiquitination is associated with β-trcp1*

As shown in Figure 6A, the degradation of Smad4 protein occurs more quickly in *Gadd45a*$^{-/-}$ MEFs than wild-type MEFs after CHX was added to the medium, which suggest that Gadd45 might be involved in maintaining the stability of Smad4 protein. The protein levels of Smad4 were quantified by the software mentioned above. The half-life of Smad4 in *Gadd45a*$^{+/+}$ MEFs was longer than 9 h but decresed to about 5 h in *BRCA1*$^{-/-}$ MEFs (Figure 6B). We then employed MG132, an inhibitor of ubiquitin proteasome, to examine the effect of Gadd45 on ubiquitin degradation of Smad4. As shown in Figure 6C, the degradation of Smad4 was significantly inhibited in both MEFs when MG132 was added, suggesting that the enhanced stability of Smad4 might be due to Gadd45 suppression on its ubiquitin-degradation. To further verify this result, both MEFs were treated with MG132 when exposure to UV. We found Smad4 was down-regulated after UV in *Gadd45a*$^{-/-}$ MEFs, but its down-regulation was obviously rescued after the addition of MG132 (Figure 6D), suggesting that the down-regulation of Smad4 in Gadd45 deficient cells is likely due to abnormal ubiquitin-proteasomal degradation. In addition, following treatment of both UV and MG132 the poly-ubiquitination of Smad4 was observed in *Gadd45a*$^{-/-}$ MEFs, but not in wild-type MEFs (Figure 7A). All of these data indicate

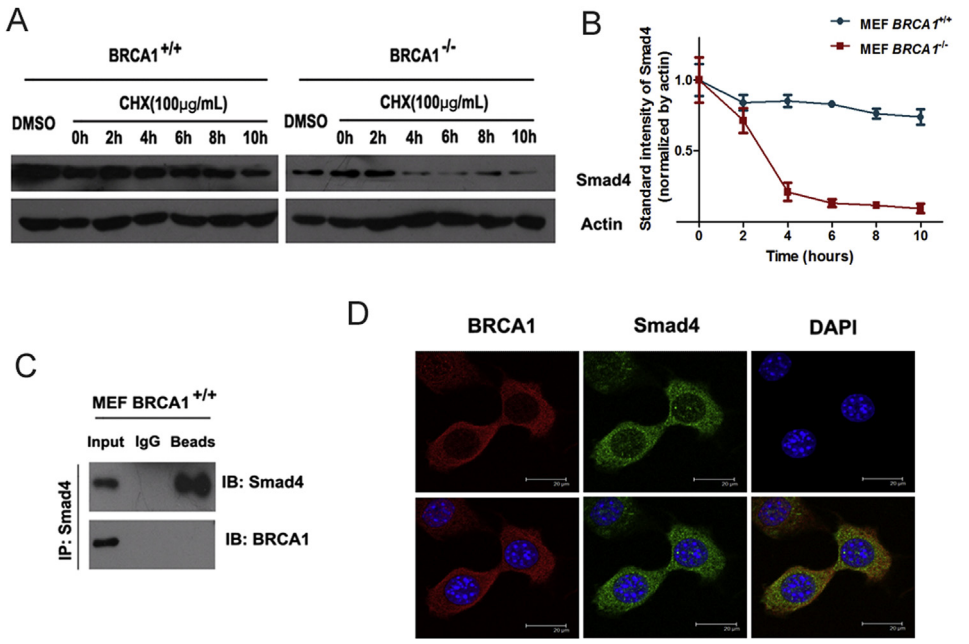

Figure 3 – BRCA1 maintains the Smad4 protein level by increasing its protein stability indirectly. (A) Exponentially growing *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs were treated with 25 μg/ml CHX for the indicated times. MEFs treated with DMSO were used as the control as this agent was used to dissolve CHX. The immunoblotting analyses were conducted with antibodies to Smad4. (B) Smad4 protein levels were quantified by ImageJ software and normalized by the intensity of Actin. The data were obtained from three individual experiments and are expressed as mean ± S.D. (C) The cell lysates of *BRCA1*$^{+/+}$ MEF were prepared for Co-immunoprecipitation (Co-IP). Co-IP with Smad4 antibody was performed and the precipitates were then subjected to immunoblotting assays with antibodies to Smad4 and Brca1. (D) *BRCA1*$^{+/+}$ MEF cell lines were stained with anti-BRCA1 and anti-Smad4 antibody, and then incubated with TRITC-conjugated goat anti rabbit IgG and FITC- conjugated goat anti mouse IgG. DAPI was used to visualize nuclei. BRCA1 (red spots), Smad4 (green spots) and nuclei (blue spots) were visualized by the laser-scanning confocal microscope.

that Gadd45a contributes to the stability of Smad4 protein through suppressing its ubiquitination. It has been demonstrated that Smad4 can be proteasomaly degraded after poly-ubiquitination by the E3 ligases such as Jab1, CHIP and SCF$^{\beta\text{-trcp1}}$ (Wan et al., 2004). We thus examined the expressing levels of Samd4 after knocking down the endogenous expression of β-trcp1, which is an F-box protein serving as the substrate recognition submit of SCF (Deshaies, 1999). As expected, the depletion of β-trcp1 prevented Smad4 degradation in both of *Gadd45a*$^{+/+}$ and $^{-/-}$ MEFs (Figure 7B). Furthermore, we examined the association between Gadd45a and β-trcp1. As shown in Figure 7C, the results from GST-pull down assays demonstrated that Gadd45a can interact with Smad4 and β-trcp1. Following induced expression of Gadd45a in Hela Gadd45a-inducible cells, β-Trcp1 was able to co-immunoprecipitate with more Gadd45a protein (Figure 7D). Similarly, an increased interaction between Gadd45a and β-Trcp1 was seen in *Gadd45a*$^{+/+}$ MEFs after UV treatment (Figure 7E). These results indicate that physical interaction exists among Smad4, β-trcp1 and Gadd45a. We further analyzed whether the interaction of Gadd45a with β-Trcp1 interrupts the degradation of Smad4 by SCF$^{\beta\text{-trcp1}}$. As shown in Figure 7D and 7E, following Gadd45a induction, β-Trcp1 interacted with similar amounts of Smad4 protein, but an increased β-Trcp1 interaction with Gadd45a was observed. These observations suggest that increased interaction between Gadd45a and Smad4 decreases the ubiquitin-degradation of Smad4 by SCF$^{\beta\text{-trcp1}}$.

3.6. *BRCA1 contributes to TGF-β1-induced growth inhibition through its downstream gene Gadd45a*

We notice that Gadd45a physically bind to the Smad4 protein to protect it from ubiquitin-degradation, meanwhile, Gadd45a is a downstream gene regulated by BRCA1, which also can maintain the Smad4 expression level. To elucidate whether BRCA1 maintenance of the Smad4 stability is mediated through Gadd45a, we transfected myc-tagged Gadd45a to both of *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs. As expected, over-expression of Gadd45a largely rescued the Smad4 protein level compared with that transfected myc alone (Figure 8A). Furthermore, knock-down of Gadd45a by siRNA in *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs exhibit down-regulation of Smad4 protein, especially in *BRCA1*$^{-/-}$ MEF cells (Figure 8B). To better understand synergistic effects of BRCA1 and Gadd45a in TGF-β1-induced growth inhibition, we knocked down the expression of Gadd45a by siRNA in *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs and treated these cells with TGF-β1. As shown in Figure 8C, TGF-β1-induced growth inhibition was significant after 48 h cell exposure to TGF-β1 (about 10%, $p < 0.05$) in siNC transfected *BRCA1*$^{+/+}$

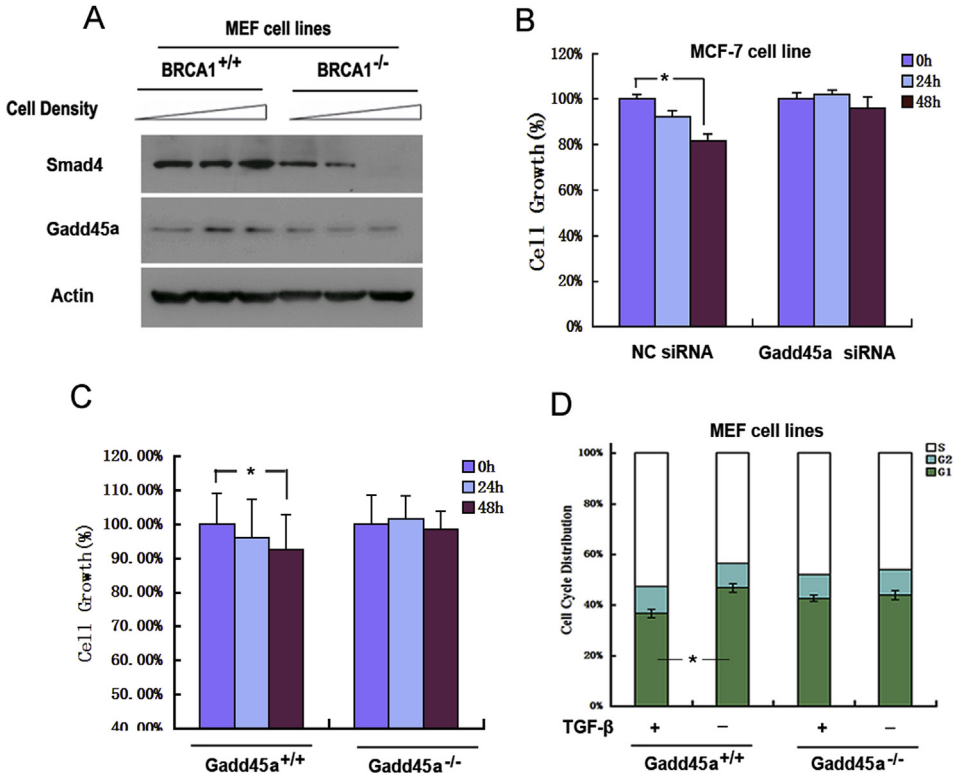

Figure 4 – Gadd45 maintains TGF-β1-induced growth inhibition. (A) *BRCA1*$^{+/+}$ and *BRCA1*$^{-/-}$ MEFs were seeded at different densities and harvested till the cells with highest-density reached confluent. Cell lysates were prepared for analyzing the expression of Gadd45a, Smad4 and Actin. (B) MCF-7 cells were seeded on 96-well plates overnight to allow attachment after transfected with the control siRNA(siNC) or Gadd45a siRNA, and then treated with 10 ng/ml TGF-β1 for 24 and 48 h as described before. (C) *Gadd45a*$^{+/+}$ and $^{-/-}$ MEFs were seeded on 96-well plates and treated with 2.5 ng/ml TGF-β1 for 24 and 48 h, and cell growth was assessed by MTT assay as described before. (D) Both MEF cells were treated with 2.5 ng/ml TGF-β1 for 24 and 48 h as described above. Then cells were harvested with trypsin for cell cycle analysis with Flow Cytometry. The data were obtained from three individual experiments and presented as the mean ± S.D.

MEFs, but substantially abrogated in Gadd45a siRNA transfected-*BRCA1*$^{+/+}$ MEFs. However, no growth inhibition was seen in either Gadd45a siRNA or siNC transfected *BRCA1*$^{-/-}$ MEFs. Furthermore, we transfected Smad4 plasmid to both of *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs to elucidate whether exogenously expression of Smad4 could rescue the phenotypes caused by BRCA1 absence. As expected, TGF-β1-induced growth inhibitions were significant after 48 h both in Smad4-transfected *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs, the inhibition rates were 30% and 26% ($p < 0.05$), respectively (Figure 8D). We also performed this assay in *Gadd45a*$^{+/+}$ and $^{-/-}$ MEFs, the inhibition rates were 54% and 36% ($p < 0.05$), respectively (Figure 8E). These results demonstrates that exogenously expression of Smad4 could rescue the phenotypes in BRCA1 or Gadd45a deficient cells. In summary, BRCA1 contributes to TGF-β1-induced growth inhibition mainly through the inhibitory function of Gadd45a in Smad4 ubiqitin-degradation.

4. Discussion

BRCA1 has been found to broadly regulate gene transcription by interacting directly or indirectly with numerous molecules. It can transcriptionally regulate the Gadd45a gene via its interaction with transcription factors Oct-1 and NF-YA (Fan et al., 2002; Jin et al., 2000b). A number of investigations indicate that Gadd45a, as a downstream gene of BRCA1 and p53, plays roles in the control of cell cycle checkpoints, DNA repair, and apoptosis through its interactions with p21$^{Cip1/Waf1}$, Cdc2/CyclinB1 complex, proliferating cell nuclear antigen (PCNA), MTK1, EF-1a and core histone protein (Jin et al., 2000a; Tong et al., 2005; Wang et al., 1999; Zhan et al., 1999). It has been well accepted that most tumor cells show the resistance to TGF-β1-mediated inhibition of proliferation. Inhibition of cell proliferation is central to TGF-β1 response in many types of cells, and inactivation of components in TGF-β1/Smad pathway contributes to tumorigenesis.

In the current study, we demonstrate that the loss of BRCA1 predisposes MEFs to escape from TGF-β1-induced growth inhibition and cell cycle G1 arrest. Therefore, tumor suppressive function of BRCA1 may be partially mediated through its role in regulating TGF-β1/Smad pathway. As demonstrated in Figure 2, BRCA1 greatly maintains stability of Smad4 protein, and the loss of BRCA1 results in Smad4 down-regulation. Inactivation of Smad4 is a frequent event of tumor cells escaping from TGF-β1-induced growth arrest

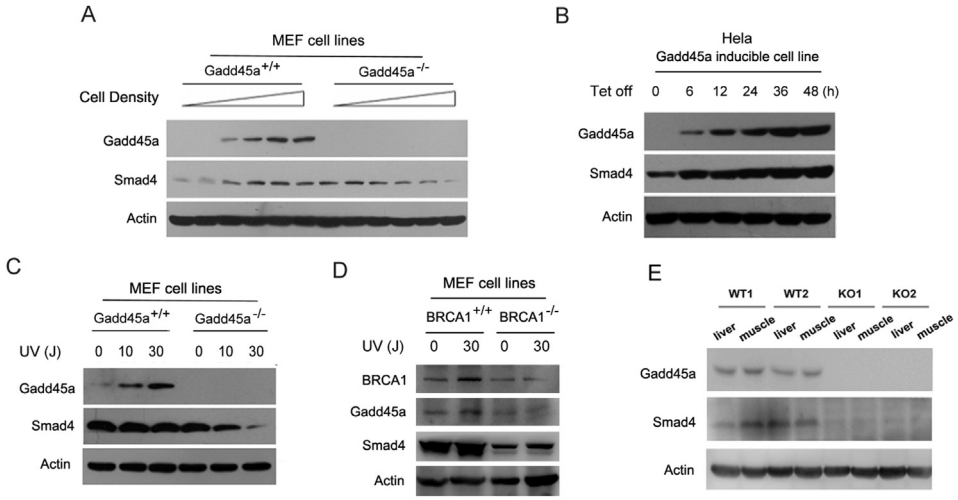

Figure 5 – Gadd45a regulates the protein level Smads protein. (A) *Gadd45a*$^{+/+}$ and *Gadd45a*$^{-/-}$ MEFs were seeded at different densities and harvested till the cells with highest-density reached confluent. (B) HeLa Gad45a-inducible tet-off cell line was grown in DMEM in the presence of tetracycline at a concentration of 2 μg/ml. Following the withdrawal of tetracycline, cells were harvested at the different times. Cell lysates were subjected to immunoblotting analysis with antibodies against Gadd45a, Smad4 and Actin. (C)After exposing to UVR, both exponentially growing *Gadd45a*$^{+/+}$ and *Gadd45a*$^{-/-}$ MEFs were cultured with fresh complete DMEM for 6 h. Cell lysates of C were prepared for analyzing the expression of Gadd45a, Smad4 and Actin. (D) After exposing to UVR, both exponentially growing *BRCA1*$^{+/+}$ and *BRCA1*$^{-/-}$ MEFs were cultured with fresh complete DMEM for 6 h and and prepared for analyzing the expression of BRCA1, Gadd45a, Smad4 and Actin. (E) The liver and muscle tissues of *Gadd45a* knockout (KO1 and KO2) and wildtype mice (WT1 and WT2) were lysed and prepared for analyzing the expression of Gadd45a, Smad4 and Actin.

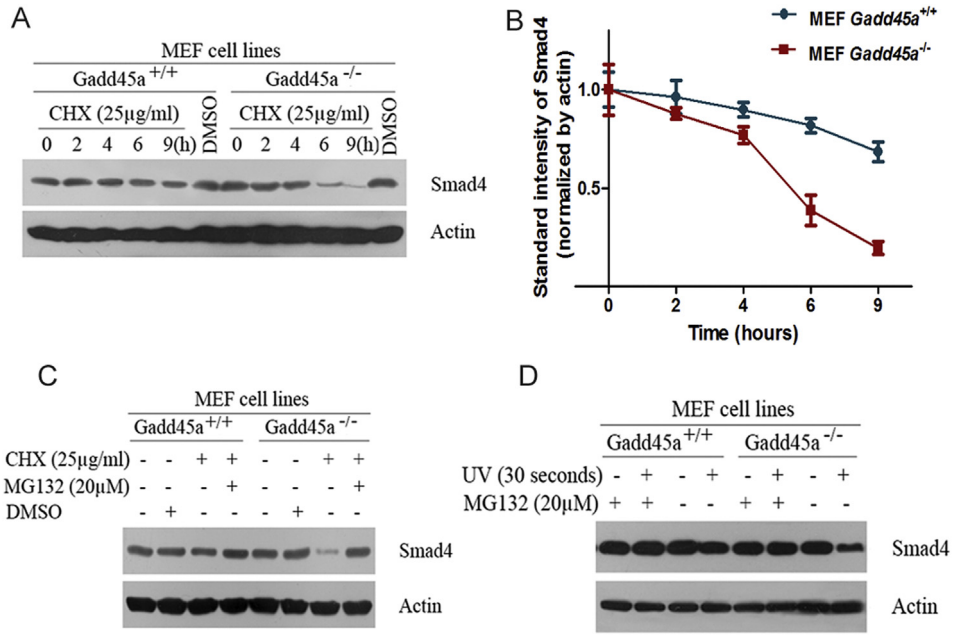

Figure 6 – Gadd45 maintains the stabilization of Smad4 protein. (A) Exponentially growing *Gadd45a*$^{+/+}$ and $^{-/-}$ MEFs were treated with 25 μg/ml CHX for the indicated time. MEFs treated with DMSO were used as the control. The immunoblotting analyses were conducted with antibodies to Smad4. (B) Smad4 protein levels were quantified by ImageJ software and normalized by the intensity of Actin. The data were obtained from three individual experiments and are expressed as mean ± S.D. (C) *Gadd45a*$^{+/+}$ and *Gadd45a*$^{-/-}$ MEFs were exposed to 25 μg/ml CHX alone or CHX accompanied with 20 μM MG132 for 9 h. The cell lysates were prepared for analyzing the expression of Smad4 and Actin. (D) Both exponentially growing MEFs were exposed to UV alone or UV accompanied with MG132 for 6 h. Cell lysates were subjected to immunoblotting with antibodies against Smad4 and Actin.

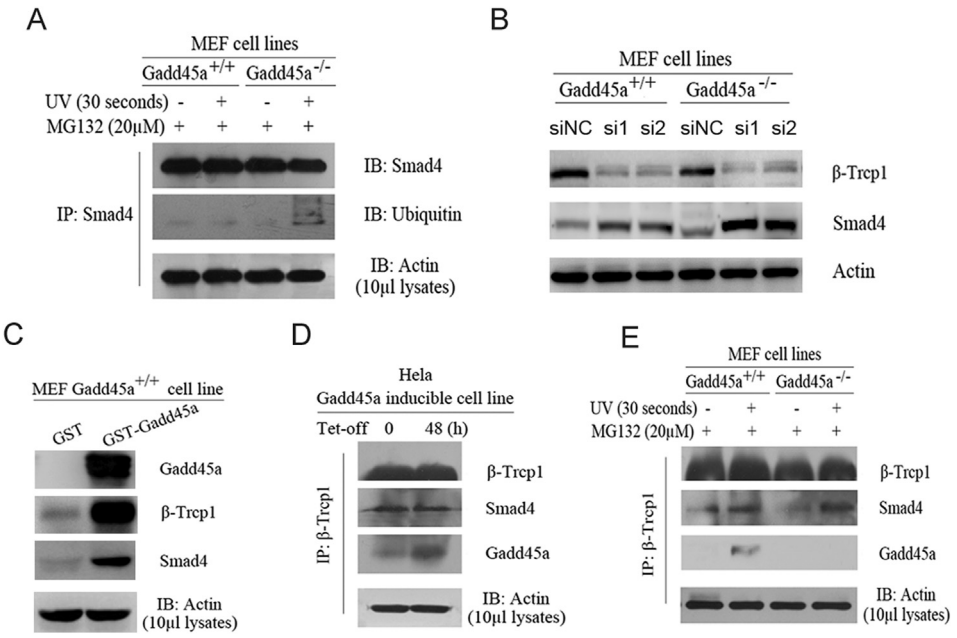

Figure 7 – Gadd45a enhances Smad4 protein stability by inhibition β-trcp1-mediated ubiquitin-dependent degradation. (A) Both exponentially growing MEFs were exposed to UV and MG132 for 6 h. Cell lysates were prepared for Co-immunoprecipitation (Co-IP). Co-IP with Smad4 antibody was performed and the precipitates were then subjected to immunoblotting assays with antibodies to Smad4 and Ubiquitin. Actin levels in 10 μl total material were used for input control. (B) *Gadd45a*$^{+/+}$ and $^{-/-}$ MEFs were transfected with β-Trcp1 si1 and si2 or control siRNA (siNC). After 48 h transfection, cells were collected and lysates were prepared for immunoblotting assay with antibodies to Smad4 and β-Trcp1. (C) Total cellular proteins were extracted from exponentially growing *Gadd45a*$^{+/+}$ MEFs for GST-pull down assay. GST-pull down assays was performed with GST alone and GST-Gadd45a. GST-immuno-complexes were subjected to immunoblotting analysis using antibodies to Gadd45a, β-Trcp1 and Smad4. (D) Hela Gadd45a-inducible cells were harvested 48 h after tetracycline withdrawal. (E) Both exponentially growing MEFs were harvested after treatment with UV and MG132. (D, E) Co-IP with β-Trcp1 antibody was performed and the precipitates were then subjected to immunoblotting with antibodies to Gadd45a, Smad4 and β-Trcp1.

and its functional expression in cells can rescue cell growth inhibition (Ramachandra et al., 2002). Interestingly we have found that the down-regulation of Smad4 in BRCA1 deficient cells is likely related to its downstream gene Gadd45a, as the *Gadd45a*$^{-/-}$ MEFs also show resistance to TGF-β1-induced growth inhibition and decreased stability of Smad4 protein. The key regulation of Smad4 protein is its ubiquitin-proteasomal degradation (Shi and Massague, 2003). Smad4 can be proteasomally degraded after poly-ubiquitination by some E3 ligase complexes such as Jab1 (Wan et al., 2002), CHIP (Li et al., 2004), and SCF complex that contains β-Trcp1 as the F-box protein (Wan et al., 2004). SCF$^{\beta\text{-trcp1}}$ induces the degradation of Smad4 through the interaction between β-trcp1 and Smad4 (Wan et al., 2004). Our results demonstrate that there is a physical interaction between β-trcp1 and Gadd45a and this interaction is enhanced with Gadd45a induction. Furthermore, this interaction appears to suppress Smad4 ubiquitination. When endogenous Gadd45a is abrogated, the abundance of both Smad4 interaction with β-trcp1 and ubiquitination of Smad4 are enhanced. These observations indicate that the interaction between Gadd45a and β-trcp1 interrupts the degradation of Smad4 after poly-ubiquitination by SCF$^{\beta\text{-trcp1}}$.

Recently, Smad4 has become an important tumor suppressor gene and is involved in the tumor suppression via inhibition of cell proliferation such as reduction of S phase or p21 induction and anti-angiogenesis such as the down-regulation of VEGF transcripts (Duda et al., 2003; Fink et al., 2001; Ramachandra et al., 2002; Schwarte-Waldhoff et al., 2000). Consistently, we have found that following Gadd45a induction, S phase population is significantly decreased and p21 expression is obviously up-regulated, which goes along with increased Smad4 protein (Figure S2 and S3). These findings further support the role of Gadd45a in regulating Smad4. We have also demonstrated that the exogenous expression of Gadd45a can largely rescue the protein level of Smad4 in BRCA1 deficient cells. All above suggest that BRCA1 might be involved in the TGF-β1 response of anti-proliferation at least in part through its downstream gene Gadd45a, which greatly inhibits the degradation of Smad4 by interacting with β-trcp1. In addition, we also show that knock-down of Gadd45a in BRCA1 deficient cells lead to much more down-regulation of Smad4 protein rather than in BRCA1 wide type cells, which indicates BRCA1 and Gadd45a may serve as double insurance in regulating TGF-β1/Smad pathway. Meanwhile, exogenously expression of Smad4 could rescue the phenotypes in BRCA1 or Gadd45a deficient cells. The observations from the current study demonstrated that BRCA1 also regulates other proteins in TGF-β1/Smad pathway. Since β-trcp1 is also the E3 ubiquitin ligase of Smad3, we can hypothesize that BRCA1 may

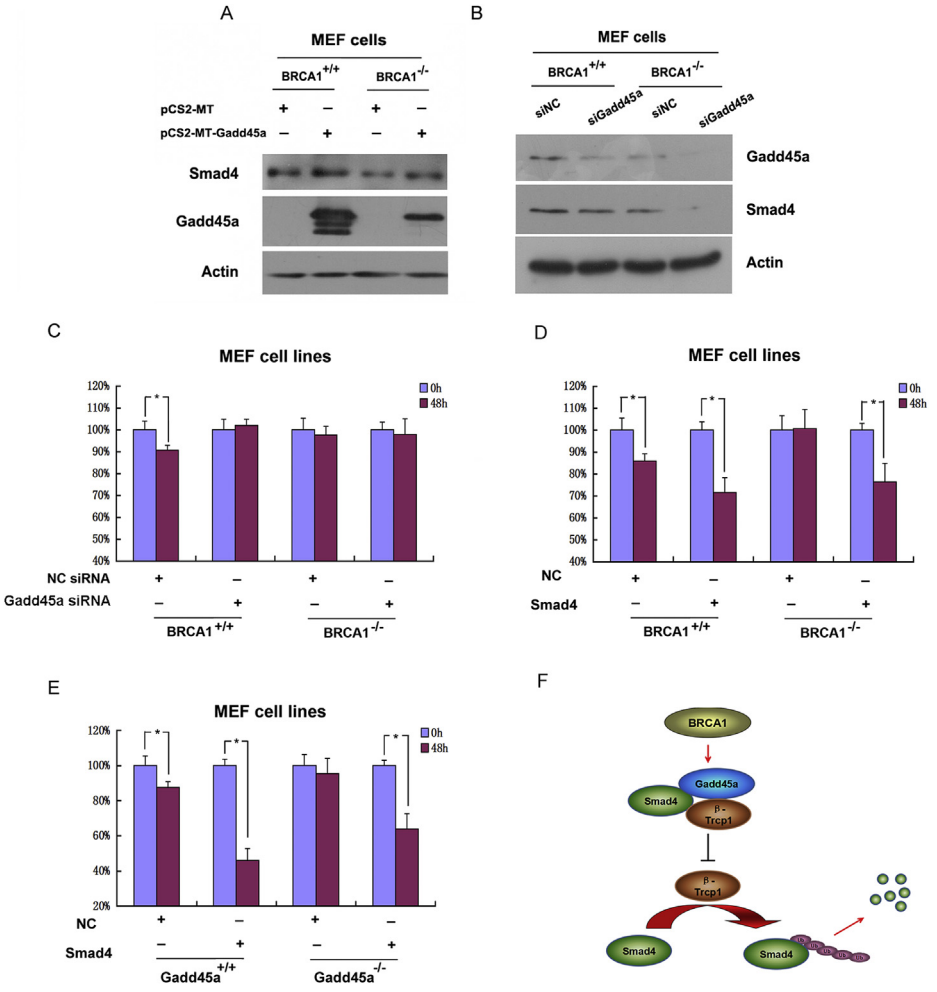

Figure 8 – BRCA1 enhances the protein stability of Smad4 through Gadd45a. (A) *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs were transfected with pCS2-MT or pCS2-MT. After 48 h transfection, cells were collected and lysates were prepared for immunoblotting assay with antibodies to Gadd45a and Smad4. Actin was used as the loading control. (B, C) *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs were transfected with Gadd45a siRNA or control siRNA (siNC). After 48 h transfection, cells were collected and lysates were prepared for immunoblotting assay with antibodies to Gadd45a and Smad4. Actin was used as the loading control. (C) *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs were transfected with Gadd45a siRNA or control siRNA (siNC). The cells were treated with 5 ng/ml TGF-β1 for 48 h, and cell growth was assessed by MTT assay as described before. (D, E) *BRCA1*$^{+/+}$ and $^{-/-}$ MEFs or *Gadd45a*$^{+/+}$ and $^{-/-}$ MEFs were transfected with Smad4 plasmid or control plasmid. The cells were treated with 5 ng/ml TGF-β1 for 48 h, and cell growth was assessed by MTT assay as described before. (F) Proposed model for BRCA1 regulating TGF-β1 pathway. BRCA1 maintains TGF-β1 responsiveness through its downstream gene Gadd45a by enhancing the protein stability of Smad4.

stabilize Smad3 through the interaction of Gadd45a and β-trcp1 in the same way. As for Smad2, protein stability is modulated by other ubiquitin ligases, such as Smurf2 or Nedd4-2. It is likely that BRCA1 may influence the stability of smad2 by Gadd45a or other downstream genes.

In summary, we have shown that BRCA1 protein is involved in regulating the TGF-β1 response of growth inhibition through stabilizing Smad4 indirectly. And its downstream gene Gadd45a interacts with β-Trcp1 and consequently prevents the Smad4 ubiquitin-degradation by $SCF^{\beta-Trcp1}$. The increasing steady-level of Smad4 by Gadd45a not only rescues the TGF-β1 responsiveness, but also inhibits the cell proliferation and angiogenesis. These findings have provided a novel insight into the role of BRCA1 in the control of cell growth. Particularly, BRCA1 and Gadd45a might play important roles in negative regulation of carcinogenesis and tumor malignancy through TGF-β1 responsiveness.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.05.002.

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