

Keywords: Smad4; colorectal cancer; 5-fluorouracil; Akt; mouse model; tissue microarray; angiogenesis; Bcl-2; TGF- β

Loss of Smad4 in colorectal cancer induces resistance to 5-fluorouracil through activating Akt pathway

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Background: Higher frequency of Smad4 inactivation or loss of expression is observed in metastasis of colorectal cancer (CRC) leading to unfavourable survival and contributes to chemoresistance. However, the molecular mechanism of how Smad4 regulates chemosensitivity of CRC is unknown.

Methods: We evaluated how the loss of Smad4 in CRC enhanced chemoresistance to 5-fluorouracil (5-FU) using two CRC cell lines *in vitro* and *in vivo*. Immunoblotting with cell and tumour lysates and immunohistochemical analyses with tissue microarray were performed.

Results: Knockdown or loss of Smad4 induced tumorigenicity, migration, invasion, angiogenesis, metastasis, and 5-FU resistance. Smad4 expression in mouse tumours regulated cell-cycle regulatory proteins leading to Rb phosphorylation. Loss of Smad4 activated Akt pathway that resulted in upregulation of anti-apoptotic proteins, Bcl-2 and Bcl-w, and Survivin. Suppression of phosphatidylinositol-3-kinase (PI3K)/Akt pathway by LY294002 restored chemosensitivity of Smad4-deficient cells to 5-FU. Vascular endothelial growth factor-induced angiogenesis in Smad4-deficient cells might also lead to chemoresistance. Low levels of Smad4 expression in CRC tissues correlated with higher levels of Bcl-2 and Bcl-w and with poor overall survival as observed in immunohistochemical staining of tissue microarrays.

Conclusion: Loss of Smad4 in CRC patients induces resistance to 5-FU-based therapy through activation of Akt pathway and inhibitors of this pathway may sensitise these patients to 5-FU.

Colorectal cancer (CRC) is one of the most prevalent malignancies in the United States and the second leading cause of cancer deaths (Siegel *et al*, 2012). The majority of colon cancer deaths is due to their sporadic origin and often diagnosed at an advanced stage. Therefore, understanding the molecular basis of genetic and epigenetic changes that promote CRC progression is important for new therapeutic strategies and for overcoming drug resistance.

TGF- β signalling has a critical but complex role in CRC progression depending on tumour types and stages (Massague, 2008). TGF- β functions as either tumour promoter or tumour suppressor depending on the status of Smad and non-Smad pathways. We previously showed that Smad4 is important in the functional shift of TGF- β from tumour promoter to tumour suppressor (Zhang *et al*, 2010). Higher frequency of Smad4 inactivation is observed in liver metastasis leading to unfavourable

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Received 3 July 2013; revised 12 November 2013; accepted 15 November 2013; published online 2 January 2014

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survival (Losi *et al*, 2007). Colorectal cancer patients with tumours expressing high Smad4 levels have significantly better survival than patients with low level (Alazouzi *et al*, 2005; Alhopuro *et al*, 2005). Interestingly, loss of heterozygosity is observed in 95% invasive and metastatic CRCs with Smad4 mutations.

5-Fluorouracil (5-FU)-based adjuvant chemotherapy is routinely given to the great majority of patients with CRC. However, chemoresistance is a major obstacle for CRC treatment, and as high as 50% of metastatic CRC patients are resistant to 5-FU-based chemotherapy (Douillard *et al*, 2000; Giacchetti *et al*, 2000; Alhopuro *et al*, 2005). Smad4 deficiency upregulates VEGF expression in CRC cells (Papageorgis *et al*, 2011). Higher VEGF expression is associated with increased vascular density, development of metastasis and drug resistance, indicating a worse prognosis in CRC (Samuel *et al*, 2011). Low Smad4 expression in human CRC predicts early recurrence after curative therapy, less response to 5-FU (Ahn *et al*, 2011; Baraniskin *et al*, 2011), and shorter survival (Roth *et al*, 2012). However, the molecular mechanism for the role of Smad4 in promoting resistance to 5-FU-based therapy and how that can be circumvented are not known. Phosphatidylinositol-3-kinase (PI3K)/Akt pathway has been shown to be critical in promoting cell survival, inhibiting apoptosis, and creating chemoresistance (You *et al*, 2009; Shin *et al*, 2010; Chen *et al*, 2012). Although the crosstalk between Smad and non-Smad pathway exists (Conery *et al*, 2004), nothing is known about how the modification of non-Smad pathway, including PI3K/Akt pathway, by the loss of Smad4 in CRC might contribute to chemosensitivity.

In this study, we provide the molecular evidence that loss of Smad4 expression in CRC promotes progression and leads to 5-FU resistance. We have observed that Smad4 deficiency in CT26 cells induces tumour progression in an orthotopic model through the activation of Akt and upregulation of VEGF. Our results suggest that loss of Smad4 in CRC cells has an important role in conferring resistance to 5-FU-based therapy through the activation of PI3K/Akt pathway and the regulation of cell cycle and apoptosis-related proteins. Moreover, in CRC patients, no Smad4 expression in the tumour is correlated with higher Bcl-2 and Bcl-w expression and poor survival. These findings imply that Smad4 may be a candidate biomarker for the combination use of PI3K inhibitor and/or anti-angiogenic therapy with 5-FU-based chemotherapy for patients with CRC.

MATERIALS AND METHODS

Cell lines and animals. CT26 cells, an undifferentiated murine adenocarcinoma-derived cell line from Balb/c mouse, and human lymph-node metastasis-derived cell line SW620 were maintained in RPMI containing 7% FBS. Smad4 knockdown stable single cell clones were generated from CT26 cells by transfecting lentiviral shRNA construct targeting Smad4 (Sigma, St Louis, MO, USA, NM_008540) using puromycin ($5 \mu\text{g ml}^{-1}$) selection. SW620 stable clones expressing Smad4 were established as described previously (Zhang *et al*, 2010). One parental, two vector control clones, and three transfected single clones from each cell line were used for the following experiments. Female Balb/c mice (8 weeks old) and athymic nude mice (6 weeks old) were used for the experiments. All of animal works were performed in accordance with IACUC and state and federal guidelines for the humane treatment and care of laboratory animals.

Reagents and antibodies. TGF- β 1 was purchased from R&D Systems (Minneapolis, MN, USA). The TGF- β receptor kinase inhibitor, SB431542, was purchased from Tocris Cookson, Inc. (Ellisville, MO, USA). 5-Fluorouracil was obtained from Sigma. LY294002 was obtained from CalBiochem (San Diego, CA, USA).

Antibodies were purchased as follows: Santa Cruz Biotechnology (Santa Cruz, CA, USA): anti-Smad4, anti-p21^{Cip1}, anti-p27^{Kip1}, anti-Cyclin D1, anti-Survivin, anti-Bcl-2, anti-VEGF; Cell Signaling (Denver, MA, USA): anti-PARP, anti-cleaved-Caspase3, anti-p-Akt, anti-Akt, anti-Bcl-w, anti-Bcl-xL, anti-Bad, anti-Bim, anti-Bax, anti-PUMA; Zymed Laboratories Inc. (San Francisco, CA, USA): anti-c-Myc.

Transcriptional response assay. CT26 cells (2000 per well) were seeded into 12-well plates and transiently transfected with p3TP-Lux, (GAGA)₉ MLP-Luc, and CMV- β gal reporter plasmids for 5 h. Transfected cells were treated with serum-free media with 5 ng ml^{-1} TGF- β 1 for 24 h. Cell lysates were used to measure both luciferase and β -gal activities, and normalised luciferase activity was presented as the mean \pm s.d. of triplicate measurements.

Cell counting assay. CT26 cells (2000 per well) were seeded into 12-well plates. Cells were then cultured in media with 7% FBS, or treated with TGF- β 1 (5 ng ml^{-1}) in the presence of SB-431542 ($10 \mu\text{M}$) for 5 days. Media containing TGF- β 1 and/or SB431542 was replaced every other day. Cells were counted each day and the average cell numbers of triplicate wells were plotted.

Wound healing assay. Confluent CT26 cells were pretreated with Mitomycin C ($1 \mu\text{g ml}^{-1}$) for 3 h. A straight wound line was made in the monolayer of the cells. The cells were treated with 5 ng ml^{-1} TGF- β , and wound line was monitored for 48 h. Pictures of cells along the wound line were taken by phase-contrast microscope.

MTT cell proliferation assay. CT26 and SW620 cells (5×10^3) were plated in 96-well flat-bottomed microtitre plates and incubated overnight. Cells were treated with indicated concentrations of 5-FU with or without LY294002 for 72 h. The number of viable cells was determined by MTT following the kit protocol (Millipore, Billerica, MA, USA, CT01). The percent viability was calculated as ((absorbance of drug-treatment)/(control absorbance)) \times 100% from three wells.

Flow-cytometric analysis. Apoptosis detection was performed by flow cytometry as previously described (Halder *et al*, 2005). Cells (1×10^5 per well) were seeded into 6-well plates for overnight, and treated with LY294002 and/or different concentrations of 5-FU for 48 h. Cells were harvested for FITC Annexin V and Propidium Iodide staining, following the Detection Kit (BD Pharmingen, San Diego, CA, USA).

Cell migration and invasion assay. Cell migration and invasion assays were performed as described previously (Zhang *et al*, 2009, 2010). Briefly, 2×10^4 cells in $100 \mu\text{l}$ serum-free media were seeded in the upper chamber of $8\text{-}\mu\text{m}$ pore transwells coated with collagen or matrigel (for invasion assay). Media containing 7% FBS was added in the lower chambers. For migration assay, cells were allowed to migrate for 5 h, and for invasion 24 h through either collagen or matrigel barrier. Cells that passed through the transwells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Five random fields ($200 \times$) in each well were counted, and the average number/field from triplicated wells was plotted.

Anchorage-independent growth assay. Soft agarose assay was performed as previously described (Zhang *et al*, 2010). After 48 h growth, cells were treated with media containing $10 \mu\text{M}$ or $20 \mu\text{M}$ 5-FU. Media was replaced every other day. Picture of colonies was taken by phase-contrast microscope 10 days after cell plating and colonies were counted using the GELCOUNT software (Oxford Optronix Ltd., Abingdon, UK).

Immunohistochemical analysis. Formalin-fixed paraffin-embedded tissue sections were subjected to standard immunostaining for H&E, Ki67, cleaved-Caspase3, and CD31. Microscopy images and the Image-Pro Plus software were used for Vessel evaluation. Vessel number corresponds to the number of

continuous CD31-positive structures per field. Vessel density was determined by the percentage of CD31-positive signal occupied in each field ($\times 200$). Four samples from each indicated group were randomly selected for vessel evaluation, and five random 0.159 mm^2 fields were examined at $\times 200$ magnification for each slide.

Western blotting. Lysates from CT26 or SW620 cells after treatment, and from tumours in mice or tissue from patients with CRC were used for western blot analyses as described previously (Halder *et al*, 2005).

Tumorigenicity assay. CT26 cells (8×10^4) suspended in $100 \mu\text{l}$ PBS were injected subcutaneously behind the anterior forelimb of Balb/c mice ($n = 5$) as described previously (Zhang *et al*, 2010). Mice were euthanised 24 days after injection, when the biggest tumour led to health problem for the mice. For the drug treatment experiment, CT26 (1×10^5) or SW620 cells (2×10^6) were injected subcutaneously to Balb/c mice or athymic nude mice ($n = 5$), respectively. 5-Fluorouracil treatment was started as soon as the tumour had reached palpable size. For SW620 tumour xenograft, 5-FU (50 mg kg^{-1}) was administered i.p. on a three times per week basis for 3 consecutive weeks, and for CT26 allograft, 5-FU (100 mg kg^{-1}) was administered once a week in the first 2 weeks and 50 mg kg^{-1} once a week thereafter as reported previously (Fanciullino *et al*, 2007; Wagner *et al*, 2009). Tumour volume was calculated as previously reported (Zhang *et al*, 2010). For survival assay, animals were euthanised according to the IACUC protocol. The day of kill was considered as the day of death for survival evaluation.

Spontaneous CRC tumour growth and metastasis. The Balb/c mice were anaesthetised with isoflurane inhalation, and caecum was exposed through a lower abdomen incision. CT26 cells (1×10^7) suspended in $50 \mu\text{l}$ PBS were injected subserosally using a 30-G needle, under the stereo microscope. Mice were monitored and euthanised 35 days after caecal injection when some mice became moribund. The tumours in caecum, livers, and spleens were examined for primary tumour and metastatic growth.

Assessment of CRC TMAs. A TMA from 92 patients with CRC was used for immunohistochemistry with anti-Smad4, -Bcl-2, and -Bcl-w antibodies. This TMA contains carcinoma samples in triplicate, with normal control colonic mucosa. Excess tumour samples were used and this study was exempt from IRB review. Immunostaining was assessed independently by two pathologists in a blinded manner. Each marker was scored as previously reported (Galvano *et al*, 2006).

Statistics. Statistical analyses including Student's *t*-test, Chi-square tests, and GLM ANOVA followed by Tukey's *post hoc* test and pre-planned contrasts were performed with SAS version 9.3 (Cary, NC, USA). Chi-square tests and *t* tests were used to assess the associations between baseline characteristics and Smad4 expression. A log-rank test and Kaplan–Meier survival curves were used for survival analysis. The results were considered as statistically significant if the *P*-value is < 0.05 .

RESULTS

Smad4 deficiency promotes TGF- β induced tumorigenicity, migration, and invasion. To determine the role of Smad4 expression in CRC tumorigenicity and chemosensitivity, we used two model cell lines: (1) CT26 cells that express Smad4 and are sensitive to 5-FU and (2) SW620 cells that lack Smad4 expression and are not sensitive to 5-FU. We have previously shown that stable expression of Smad4 in SW620 cells decreases tumorigenicity and metastatic potential of these cells and reverses TGF- β from tumour promoter to suppressor (Zhang *et al*, 2010). To test the role of the loss of Smad4 in CRC cells on tumorigenicity and

chemosensitivity, we first knocked down Smad4 from CT26 cells and characterised the tumorigenic properties. Stable knockdown of Smad4 in clones was tested by western blotting (Figure 1A, upper panel). In an attempt to assess the functional outcome of Smad4 knockdown, we observed that TGF- β induced p3TP-Lux and (GAGA)₉ MLP-Luc reporter activities in vector control cells, but not in Smad4 knockdown clone (Figure 1A, lower panel). To determine the effect of Smad4 deficiency on CRC, we examined cell growth, migration, and invasion using these knockdown clones. As the endogenous TGF- β level is high (Zhang *et al*, 2009), we first evaluated the growth of Smad4-deficient clones without TGF- β treatment and observed that Smad4 deficiency promoted cell growth (Supplementary Figure S1A). We next examined the effect of exogenous TGF- β on Smad4-deficient CT26 cells. Smad4 deficiency blocks the growth suppression effects of exogenous TGF- β in CT26 cells (Supplementary Figure S1B). The TGF- β receptor kinase inhibitor, SB431542 treatment blocked the growth suppression effect of TGF- β in CT26 vector cells, whereas it had no significant effect in the Smad4-deficient clones (Supplementary Figure S1C).

We next examined the effects of the loss of Smad4 expression on tumorigenicity of these cells using anchorage-independent growth assay. Knockdown of Smad4 in CT26 cells increased the size and number of colonies compared with control cells (Figure 1B). To determine the effect of Smad4 on cell motility and invasion, we performed wound closure, migration, and invasion assays. Smad4-deficient clones showed more motile cells in the wounded line, and this effect was enhanced by exogenous TGF- β (Supplementary Figure S1D). Smad4-deficient clones showed significantly increased migration and invasion compared with control group (Figure 1C and D). Therefore, these data suggest that loss of Smad4 in CT26 cells induces proliferation, migration, invasion, and tumorigenicity.

Smad4 reduces Akt phosphorylation, and regulates cell cycle and apoptosis-related proteins. To gain insight into the molecular mechanism by which loss of Smad4 contributes to tumorigenicity of CRC, we checked the expression of cell cycle and apoptosis-related proteins. We observed increased Akt phosphorylation (p-Akt) in Smad4-deficient cell clones compared with Smad4 expressing (CT26) or overexpressing cell clones (SW620) (Figure 1E). The p38 Mitogen-Activated Protein Kinase (p38-MAPK) phosphorylation was activated when Smad4 was deficient in both cell lines (Supplementary Figure S2). Cell cycle-related proteins and pro- or anti-apoptotic proteins were examined to elucidate the mechanism of Smad4-mediated regulation of proliferation and apoptosis. In both cell lines, Smad4 downregulated c-Myc, Cyclin D1, while upregulated p27^{Kip1} (Figure 1E). p21^{Cip1} was absent in SW620 cells and was not upregulated by overexpression of Smad4, whereas it was downregulated mostly in Smad4 knockdown CT26 clones. We did not observe any change in the level of CDK4 irrespective of Smad4 status in both cell lines, but CDK2 level went down in SW620 cell clones stably expressing Smad4. Changes in the levels of Cyclin D1, CDK2, and CDK inhibitors in these two cell lines resulted in the phosphorylation of Rb protein, when Smad4 levels are less or lost leading to cell proliferation. The anti-apoptotic Bcl-w was downregulated by Smad4 in both cell lines, whereas Bcl-2 is absent in CT26 cells and downregulated by Smad4 in SW620 cells (Figure 1F). On the other hand, the pro-apoptotic Bim and Bad were upregulated by Smad4 in CT26 cells (Figure 1F). However, Bim was not expressed and Bad was not changed by Smad4 in SW620 cells. These data provide a mechanism by which Smad4 inhibits cell proliferation through cell-cycle arrest and promotes apoptosis by regulating anti- and pro-apoptotic proteins, and inactivation of Akt may have a role in these processes.

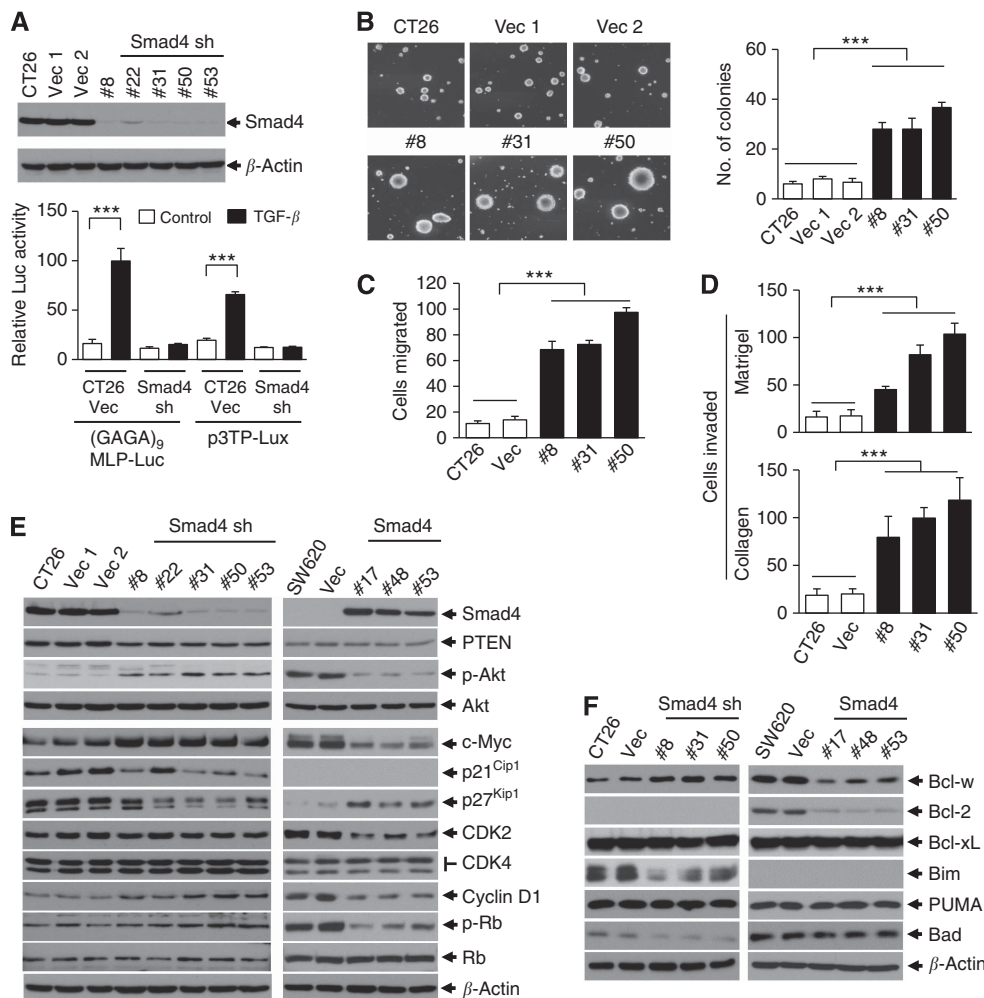


Figure 1. Smad4 inactivation promotes CRC malignancy *in vitro*. (A) Expression of Smad4 in stable clones generated by lentiviral Smad4 shRNA transfection was examined by western blotting. CT26 cell clones were transfected with TGF- β responsive reporters, treated with TGF- β , luciferase activity was normalised to β -gal activity and expressed as the mean \pm s.d. Result from vector clone and one of the Smad4 knockdown clones are shown. (B) CT26 cell clones were cultured in soft agarose for 10 days. Picture of colonies is shown, and number of colonies is shown as the mean \pm s.d. of triplicate wells. (C) Cell migration and (D) invasion assay. CT26 cells were allowed to migrate through transwells (C), and pass through a matrigel barrier or a collagen layer (D) in the transwell chambers. Five random fields in each well were counted. Each data point represents the mean \pm s.d. from three wells. (E and F) Lysates from CT26 and SW620 clones were used for western blot analyses. Each experiment was repeated at least three times. *** $P < 0.0001$.

Loss of Smad4 expression enhances tumorigenicity and liver metastasis in an orthotopic model. As orthotopic models accurately represent the progression and metastasis of CRC, an orthotopic caecal injection model was used to evaluate the effect of Smad4 on CRC tumorigenicity and liver metastasis. We injected the parental CT26 cells, one vector control clone, and three Smad4 knockdown clones into the distal caecum of syngeneic Balb/c mice to generate primary tumours and metastasis. We observed significantly increased primary tumour growth by Smad4-deficient clones, in which 9 out of 15 mice generated visible liver metastasis and 2 mice presented splenic metastasis. On the contrary, only 1 out of 10 mice injected with control cells presented portal vein metastasis, while no splenic or liver metastasis was observed (Figure 2A and B). In the control group, primary tumours in the caecum were small, and located between the submucosa and the subserosa (Figure 2D). No intrahepatic or intrasplenic micro-metastasis was presented in the control group. In contrast, the Smad4-deficient group showed aggressive primary tumour and specific metastasis in the liver and the spleen. The structure of the submucosa and the subserosa was disrupted owing to the invasion of the primary tumour cells (Figure 2D).

Consistent with the data from orthotopic model, we also observed significantly increased tumour growth and liver metastasis in mice injected with Smad4 knockdown clones compared with control cells, using subcutaneous injection and splenic injection model, respectively (Supplementary Figure S3). p21^{Cip1} and p27^{Kip1} were downregulated, while c-Myc and Cyclin D1 were upregulated in the tumours from Smad4-deficient clones (Figure 2C; Supplementary Figure S3B). Tumours showed significant higher levels of cleaved-PARP and cleaved-Caspase3 in control group compared with Smad4 inactivation group. These data indicate that Smad4 inactivation induces cell proliferation and inhibits cell apoptosis *in vivo*, which is consistent with the *in vitro* results.

Smad4 deficiency in CRC cells induces resistance to 5-FU through the regulation of downstream apoptotic pathway. To investigate the mechanism of function of Smad4 in drug sensitivity, we treated CT26 and SW620 cells with 5-FU, and performed MTT assay. Smad4 expressing or overexpressing cell clones showed significantly higher sensitivity to 5-FU, when compared with that of Smad4-deficient cells (Figure 3A and B).

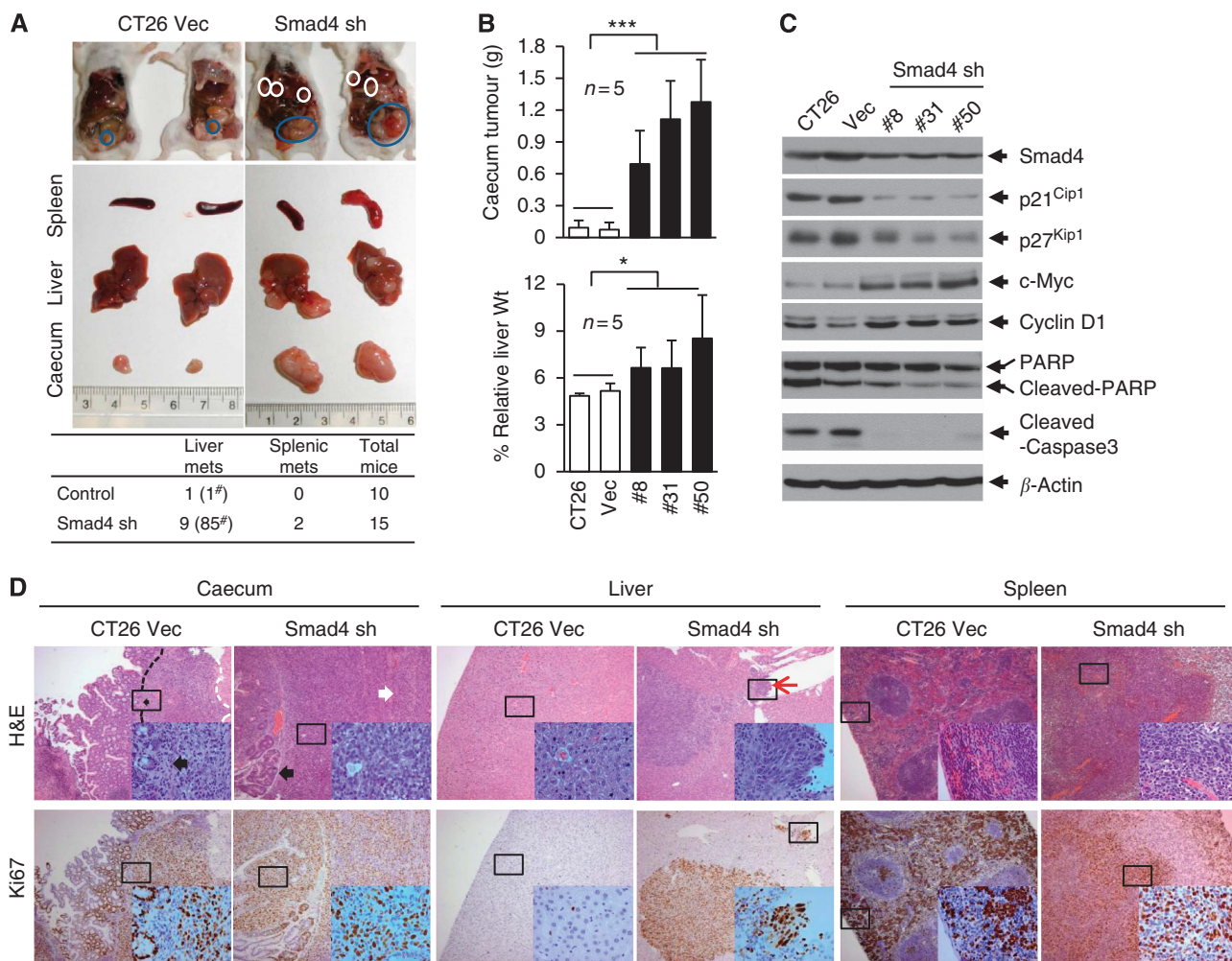


Figure 2. Smad4 inactivation in CT26 cells increases tumorigenicity, and promotes spontaneous liver metastasis. **(A)** An orthotopic CRC animal model was established by injecting CT26 cells, one vector control, and three Smad4 knockdown clones into the caecum of mice ($n=5$). Representative pictures of the tumour mass in caecum, liver, and spleen are shown. The number of mice with liver or splenic metastasis was determined. #Total number of visible liver metastases. **(B)** The primary tumours and livers described above were weighed. Liver weight was compared with the total body weight to present liver metastasis. Each bar shows the mean \pm s.d. values from five mice in each group. * $P<0.01$, *** $P<0.0001$. **(C)** Lysate from primary tumours in caecum was used for western blotting analyses. **(D)** Tissue sections from caecum, liver, and spleen were subjected to immunostaining for H&E and Ki67. Black line and arrows show the submucosa, while white line and arrows show the subserosa of the caecum. The white arrow shows the structure of the subserosa that was disrupted by tumour cells. The red arrow in the liver section shows that the tumour cells metastasise to liver parenchyma through the portal vein. Magnification: $\times 50$ and $\times 400$.

The sensitivity of CT26 control clones to 5-FU was more than double compared with Smad4-deficient CT26 clones based on 50% inhibiting concentration (IC_{50}) value (Figure 3A). In contrast, SW620 clones expressing Smad4 were three-fold more sensitive to 5-FU treatment compared with control. More evidence of 5-FU resistance in Smad4-deficient cells was obtained in soft agarose assay in both cell lines (Figure 3C). Treatment with 5-FU decreased the number of colonies by 85% and 89% in Smad4 expressing CT26 and SW620 clones, respectively, and by only 18% and 46% in Smad4-deficient CT26 and SW620 clones, respectively. With 5-FU treatment, the percentage of apoptotic cells in CT26 vector control was increased by $15.3 \pm 3.8\%$, while in Smad4-deficient clone by $6.4 \pm 1.6\%$ (Figure 3D). In SW620 cells, 5-FU increased apoptosis by $26.3 \pm 3.4\%$ in control clone, while by $47.3 \pm 6.2\%$ in Smad4 expressing clones (Figure 3D). We observed similar effects with other Smad4 knockdown and overexpressing clones (data not shown). These results suggest that loss of Smad4 enhances resistance to 5-FU treatment of CRC cell lines through attenuating apoptosis.

Studies have shown that in cancer cells 5-FU sensitivity is linked with the activation of PI3K/Akt pathway (Nicholson and Anderson, 2002; Knuefermann *et al*, 2003; Kodach *et al*, 2006; Taiyoh *et al*, 2011; Zheng *et al*, 2012). Our study showed an inverse relationship between Smad4 expression and activation of Akt in both CRC cell lines (Figure 1E). We hypothesised that loss of Smad4 might result in the resistance to 5-FU through activating PI3K/Akt pathway. Expression of Smad4 in both cell lines decreased the activation of Akt that was not altered with 5-FU treatment (Figure 3E). Bcl-w expression in both cell lines and Bcl-2 in SW620 were positively correlated with the phosphorylation of Akt and correlated inversely with Smad4 expression. Bim, a downstream pro-apoptotic target of Akt pathway, was continuously increased after 5-FU treatment with time in CT26 control cells. On the contrary, Bim was increased after 72 h of 5-FU treatment in Smad4-deficient cells (Figure 3E). Interestingly, another anti-apoptotic protein, Survivin was upregulated in Smad4-deficient CT26 and SW620 cells, compared with Smad4 expressing cells. 5-Fluorouracil induced PARP cleavage as early as 24 h in Smad4 expressing clones, whereas, with 48 or 72 h

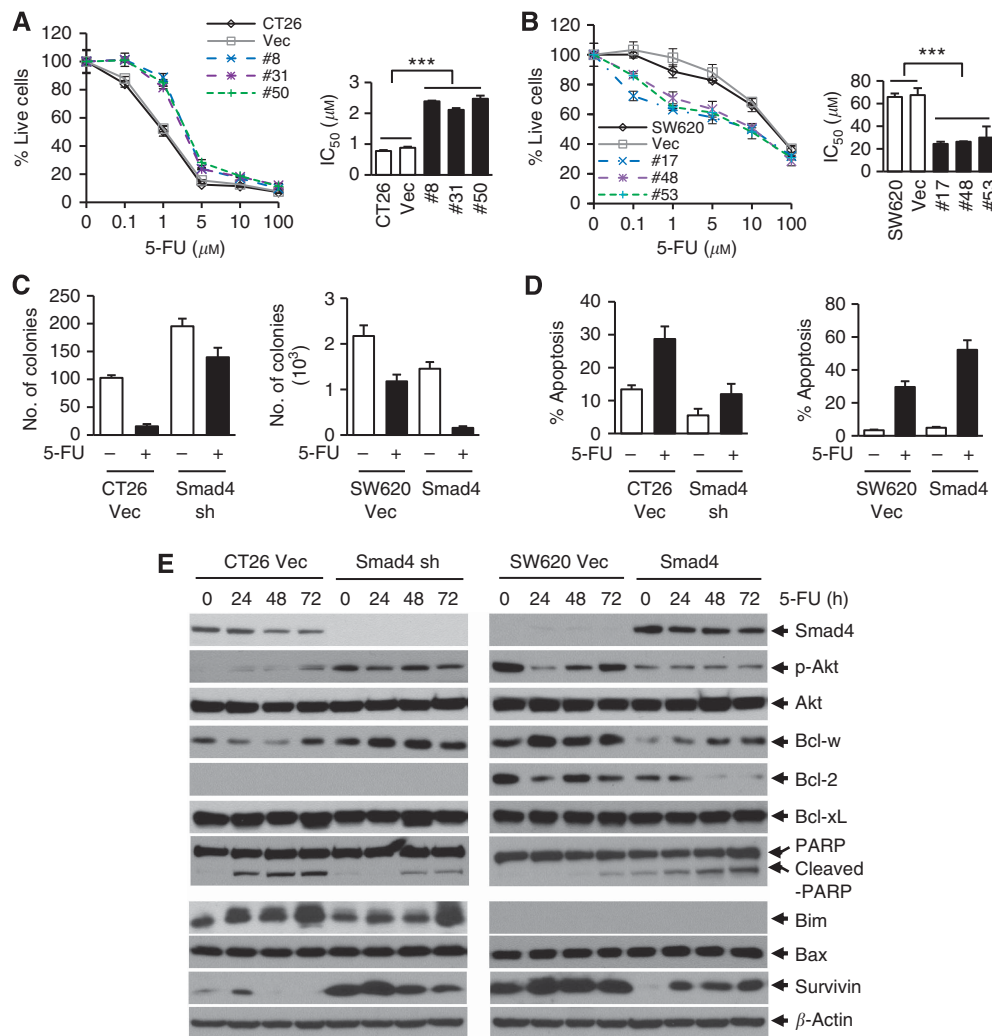


Figure 3. Smad4 promotes chemosensitivity to 5-FU *in vitro*. (A) CT26 and (B) SW620 cells were treated with indicated concentrations of 5-FU for 72 h. The number of viable cells was determined by the MTT. Relative percentages of live cells were analysed by comparing with cells without 5-FU treatment. IC₅₀ values were calculated using linear or logarithmic regression ($R^2 > 0.9$). Values were presented as the mean \pm s.d. from three wells. *** $P < 0.0001$. (C) Soft agarose assay was performed to examine 5-FU sensitivity, and the number of colonies was evaluated 10 days after cell plating. The data are presented as the mean \pm s.d. from three wells. (D) Cell apoptosis was examined by FACS analysis 48 h after 5-FU treatment. The percentages of apoptotic cells are shown as the mean \pm s.d. from three wells. (E) Western blotting assay was performed using lysates from CT26 and SW620 clones after treating with 5-FU for different time points. All of the experiments were performed for one parental, one vector, and three stable clones from each cell line, and triplicated.

treatment, the cleaved-PARP in Smad4-deficient clones was significantly less when compared with the Smad4 expressing clones. These results suggest that loss of Smad4 desensitises CRC cells to 5-FU through activation of Akt pathway and regulation of downstream apoptotic proteins.

Loss of Smad4 in CRC cells promotes resistance to 5-FU and decreases overall survival. To get a mechanistic insight into the role of Smad4 in 5-FU sensitivity *in vivo*, we treated mice bearing subcutaneous tumours with 5-FU. Mice bearing tumours from Smad4-deficient CT26 or SW620 cell clones showed resistance to 5-FU (Figure 4A and B) and shorter survival (Figure 4C and D), when compared with those from Smad4 expressing or over-expressing clones. We observed similar effects of 5-FU on tumour from other clones (data not shown). In addition, 5-FU significantly improved overall survival of mice bearing tumours with high Smad4, while it had almost no effect on those mice bearing Smad4-deficient tumours.

We next examined two tumours from two mice of each group of CT26 vector and Smad4 knockdown clones by western blot

analysis. The upregulation of p-Akt and Bcl-w in CT26-deficient clones was maintained *in vivo*, which is consistent with the *in vitro* study (Figure 4E). Interestingly, while the expression of Survivin in control tumours was decreased by 5-FU, its expression was increased in knockdown tumours by 5-FU. The cleaved-PARP and cleaved-Caspase3 were sharply increased in the CT26 vector control tumours after 5-FU treatment, but there were low or no levels of these in Smad4-deficient tumours even after 5-FU treatment. Immunostaining showed Smad4 suppressed Ki67 but increased cleaved-Caspase3 expression in CT26 vector control tumours, and these differences were amplified by 5-FU treatment (Figure 4F). 5-Fluorouracil treatment did not have much effect on tumours from Smad4 knockdown clone. Taken together, these observations suggest a mechanism by which loss of Smad4 results in drug resistance and poor survival of mice bearing CRC tumours.

LY294002 restores chemosensitivity to 5-FU in Smad4-deficient CRC cells. To elucidate the role of PI3K/Akt pathway in promoting 5-FU resistance in cells lacking Smad4 expression, we performed cell viability assay after treating CT26 and SW620 cell

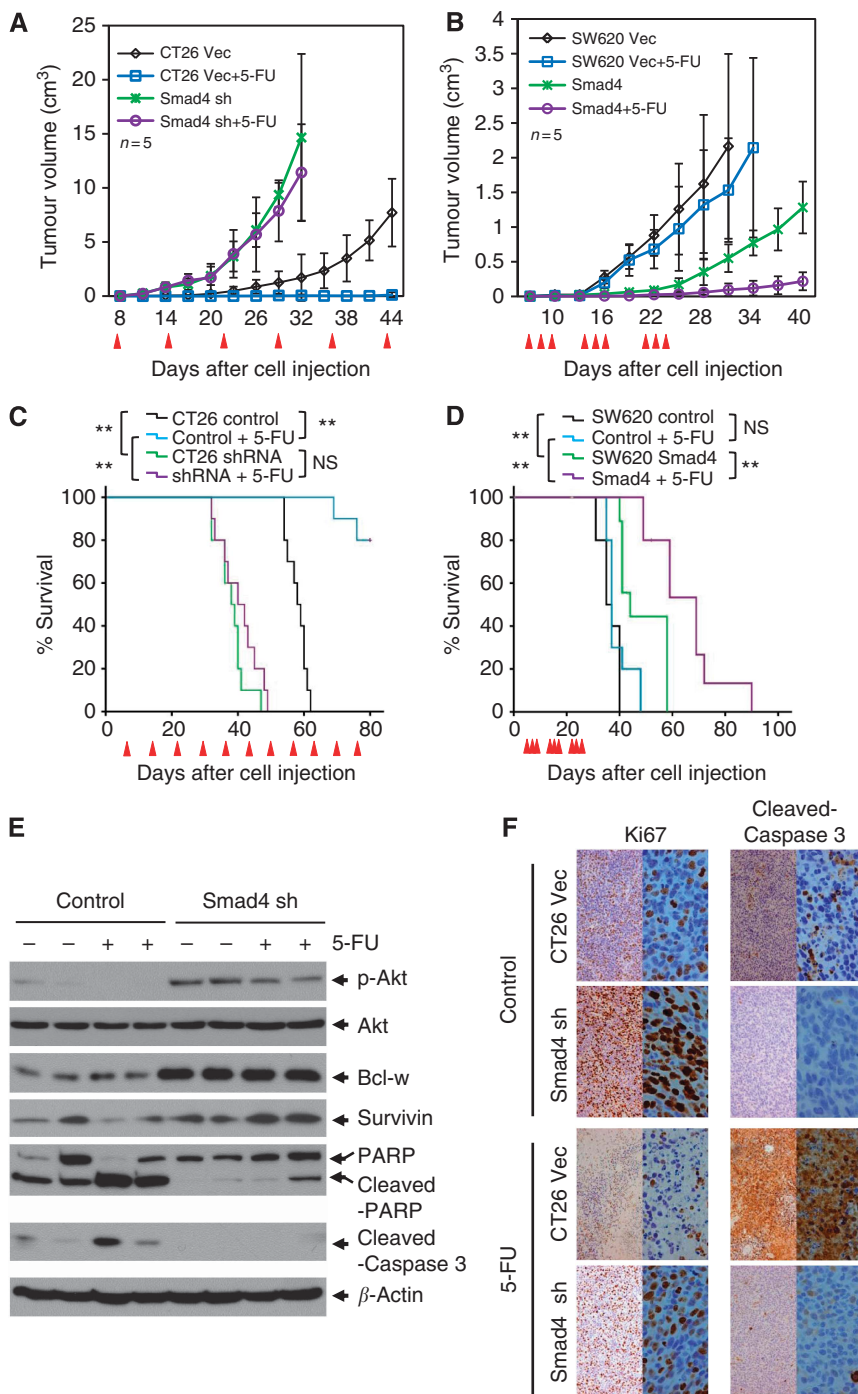


Figure 4. Smad4 promotes chemosensitivity to 5-FU and prolongs overall survival. (A) CT26 and (B) SW620 cells were injected subcutaneously to Balb/c mice and athymic nude mice, respectively. Red triangles indicate the day of 5-FU treatment. Each plot presents the mean \pm s.d. volumes of tumours from five mice in each group. Survival of mice treated with 5-FU or vehicle ($n = 10$) was assayed after injecting CT26 (C) or SW620 (D) cells subcutaneously to the mice. $**P < 0.001$, NS: not significant. (E) Subcutaneous tumour lysates from two mice of each group injected with CT26 clones were used for western blot analyses. (F) Subcutaneous tumours from CT26 clones were used for Ki67 and Cleaved-Caspase3 immunostaining ($\times 100$ and $\times 400$). All of the experiments were performed for one parental, one vector, and two stable clones from each cell line, and repeated twice.

clones with 5-FU and/or the PI3K inhibitor. The Smad4 inactivated clones showed significantly higher sensitivity to 5-FU in the presence of LY294002 (Figure 5A and B). The effect of LY294002 on decreasing IC₅₀ of 5-FU in Smad4-deficient CT26 and SW620 cell clones was much stronger than that in Smad4-expressing cell clones (Figure 5A and B, right panels). LY294002 alone had marginal effect on cell apoptosis in both CT26 and SW620 cells. However, it significantly amplified the 5-FU-induced

apoptosis in Smad4-deficient cells compared with that in Smad4 expressing cells (Figure 5C and D). In these experiments (Figure 5A–D), we observed similar effects with other Smad4 knockdown or overexpressing clones (data not shown). Interestingly, the extent of apoptosis in both Smad4-deficient cell lines treated with 5-FU and LY294002 was similar to that in the corresponding Smad4 expressing counterparts treated with both. These results suggest that LY294002 with 5-FU can sensitise the

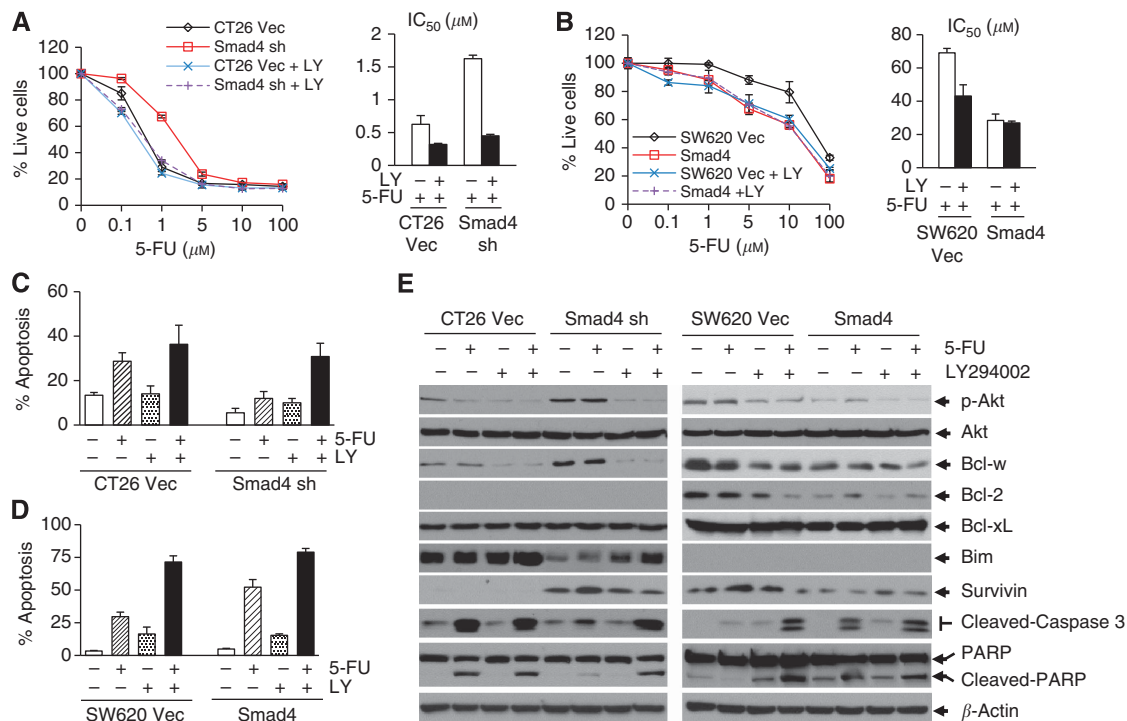


Figure 5. LY294002 restores chemosensitivity to 5-FU in Smad4-deficient cells. **(A)** CT26 and **(B)** SW620 clones were treated with indicated concentrations of 5-FU with or without LY294002 for 72 h. Relative percentages of live cells were analysed by MTT assay. IC_{50} values were calculated using linear or logarithmic regression ($R^2 > 0.9$). Data are presented as the mean \pm s.d. from triplicate wells. **(C and D)** Cell apoptosis was examined by FACS analysis 48 h after 5-FU and/or LY294002 treatment. The percentages of apoptotic cells are shown as the mean \pm s.d. from three wells. **(E)** CT26 and SW620 clones were treated with 5-FU and/or LY294002 for 24 h and cell lysates were used for western blot analyses. All of the experiments were performed for one parental, one vector, and three stable clones from each cell line, and triplicated.

Smad4-deficient cells up to the same extent as 5-FU alone does to the Smad4 expressing cells.

LY294002 inhibited Akt activation in Smad4-deficient cells to the similar level as that in Smad4 expressing cells (Figure 5E). Interestingly, the expression of Bcl-w in both cell lines and Bcl-2 in SW620 cells was positively correlated with the activation of Akt. Bim was mostly restored in Smad4 knockdown cells by LY294002. In addition, Survivin expression was downregulated by LY294002 in Smad4-deficient cells, though its expression was increased by 5-FU alone. As mentioned before, there was not much change in the cleavage of Caspase3 or PARP in Smad4-deficient cells by 5-FU. However, treatment of these cells by LY294002 and 5-FU synergistically induced cleaved-Caspase3 and cleaved-PARP. Taken together, these results suggest that LY294002 restores 5-FU sensitivity in Smad4-deficient cells through the inhibition of PI3K/Akt pathway.

VEGF upregulation by Smad4-deficient CRC cells induces angiogenesis. Angiogenesis has a role in drug sensitivity, and Smad4 has been shown to downregulate VEGF expression in the *in vitro* model (Papageorgis *et al*, 2011). We observed that VEGF expression was upregulated in both CT26 and SW620 cell clones lacking Smad4 expression (Figure 6A). We also observed that VEGF expression was significantly increased in subcutaneous allografts, orthotopic primary tumours in the caecum and liver metastases generated by Smad4-deficient clones (Supplementary Figure S4).

To investigate whether the upregulation of VEGF in Smad4-deficient tumours induced angiogenesis and contributed to the chemoresistance *in vivo*, we examined the tumour samples after 5-FU treatment macroscopically and microscopically. We observed that tumour masses from Smad4-deficient clones presented significantly more visible vessels, whereas tumours from Smad4

expressing cells had less visible vessels (Figure 6B). CD31 immunostaining showed significantly higher in number and density of vessels in the tumours generated by CT26 Smad4-deficient clones when compared with those from vector control clone (Figure 6C and D). However, 5-FU did not have any effect on vessel number and density in tumours lacking Smad4 expression. These findings suggest that loss of Smad4 in CRC cells induces angiogenesis through upregulation of VEGF and may promote 5-FU resistance.

Loss of Smad4 correlates with higher Bcl-2 and Bcl-w levels and predicts poor survival in patients with CRC. To support our hypothesis, we determined the protein expression in paired normal and tumour specimens of eight CRC patients by western blotting and performed TMA (92 tumour samples) immunostaining. Smad4 expression in tumour was significantly decreased in six out of eight patients (Figure 7A). Interestingly, p-Akt was upregulated in five of those tumour specimens with significantly lower Smad4 expression, whereas Survivin expression was increased in all tumour specimens. Consistent with western blot data, Smad4 nuclear score was 4.4 times lower in CRC samples (34.3 average) compared with normal colon tissue (150 average). Bcl-w and Bcl-2 expression inversely correlated with Smad4 nuclear staining significantly (Figure 7B and C). Patients with positive Smad4 nuclear staining showed significantly longer overall survival, when compared with Smad4-negative group (Figure 7D). No significant correlations were observed between the expression levels of Smad4 and other clinicopathologic features including age, sex, tumour stage, tumour site, grade, and depth of invasion (Supplementary Figure S5). These results further support the conclusions from our *in vitro* and animal studies that loss of Smad4 in CRC contributes to poor prognosis and overall survival through the activation of Akt pathway, and regulation of apoptotic proteins and VEGF.

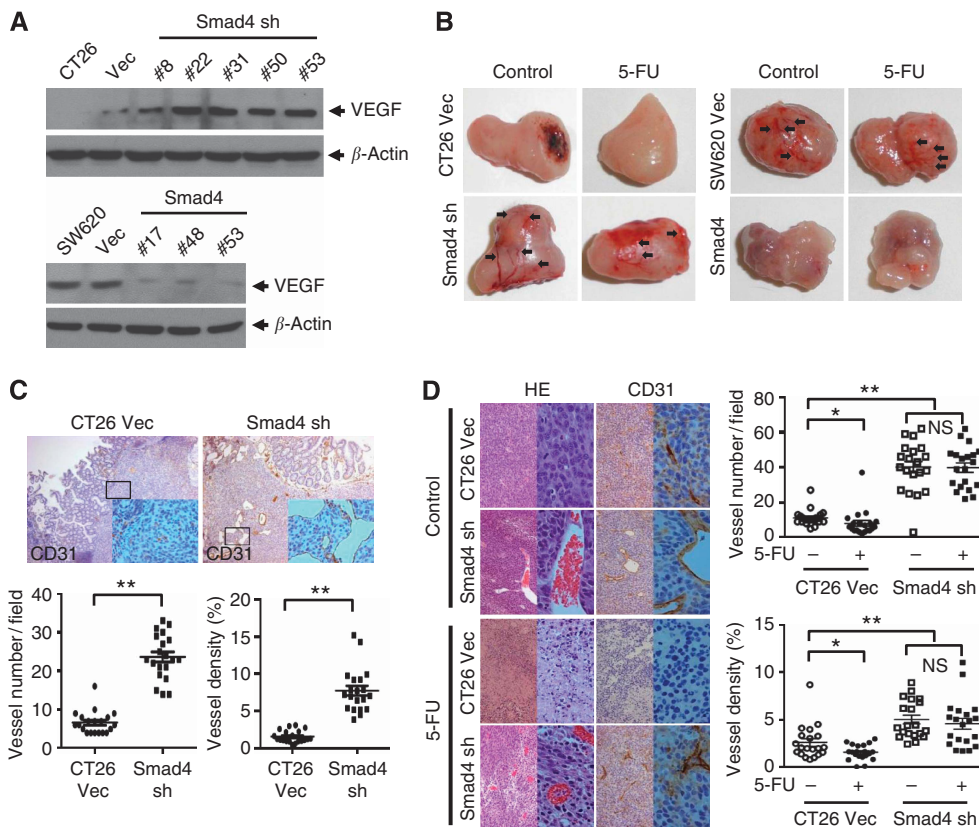


Figure 6. Smad4 downregulates VEGF and inhibits angiogenesis. **(A)** VEGF expression in CT26 and SW620 cell clones was examined by western blot analyses. **(B)** Representative tumour masses with or without 5-FU treatment are shown. Visible vessels are marked by black arrows. Formalin-fixed paraffin-embedded tissue sections from **(C)** orthotopic tumours and **(D)** subcutaneous tumours were subjected to H&E and CD31 staining. Representative pictures are shown ($\times 100$ and $\times 400$) with vessel density and vessel number. * $P < 0.05$, ** $P < 0.001$, NS: not significant.

DISCUSSION

5-Fluorouracil, a potent inhibitor of thymidylate synthase (TS), is widely used in the treatment of a variety of solid tumours. However, tumour cells find ways to evade drug-induced death signals, and as a result, chemoresistance is the major impediment to the 5-FU-based therapy. Therefore, the combination of other anticancer agents with 5-FU has been the focus of considerable investigation over the past several years to improve response rates. Smad4 mutation and its downregulation in CRC are correlated directly with poor prognosis and survival, and increased metastasis (Miyaki *et al*, 1999; Losi *et al*, 2007). Moreover, low Smad4 levels in CRC are associated with poor prognosis after surgery and 5-FU-based adjuvant therapy (Alhopuro *et al*, 2005). In contrast, the Smad pathway has been shown to mediate the pro-metastatic function of TGF- β in breast cancer bone and lung metastases (Azuma *et al*, 2005; Kang *et al*, 2005). Therefore, the role of Smad4 in CRC metastasis is not fully understood, and the molecular mechanism by which loss of Smad4 expression in CRC contributes to resistance to 5-FU-based therapy is not known. In this study, the salient features are (1) to our knowledge, this is the first study, to determine the role of Smad4 in CRC progression using an orthotopic model that mimic spontaneous human metastasis; (2) to study the molecular mechanism of the function of Smad4 in natural metastasis; (3) to investigate how the loss of Smad4 in CRC induces resistance to 5-FU *in vivo*; and (4) to find way to circumvent this resistance to 5-FU.

TGF- β signalling has a bilateral role in CRC progression and prognosis, depending on the condition of the intracellular Smad pathway and non-Smad pathway (Derynck and Zhang, 2003).

Smad4, a pivotal signal transducer of the TGF- β /Smad pathway, acts as a tumour suppressor in CRC (Cancer Genome Atlas Network, 2012; Freeman *et al*, 2012). The Smad pathway has an indirect role in regulating the activation of the extracellular signal-regulated kinase (ERK), p38-MAPK, C-Jun N-terminal kinase (JNK), and PI3K/Akt. Smad4 inactivation selectively abrogates the tumour suppressive axis of the pathway, allowing TGF- β to preferentially overactivate the Smad-independent pathways in cooperation with other pro-oncogenic pathways including activated K-Ras (Massague, 2008). Oncogenic K-Ras mutation (40–50%) is thought to be critical for colorectal carcinogenesis due to its ability to activate growth pathways and inhibit the tumour suppressor functions of TGF- β (Jin *et al*, 2003). To investigate the effect of the loss of Smad4 expression in the presence of K-Ras activation in CRC progression and metastasis, and on chemoresistance, we used two K-Ras activated CRC cell lines, (1) CT26 cells that express Smad4 and are sensitive to 5-FU and (2) SW620 cells that lack Smad4 expression and are not sensitive to 5-FU because of the overexpression of TS. Endogenous depletion of Smad4 in CT26 cells promoted tumorigenicity, migration, invasion, and metastasis in an orthotopic model through inhibiting tumour suppression activity of TGF- β coupled with the activation of K-Ras (Figures 1 and 2). Caecal injection is the most accurate orthotopic representation of tumour growth and spontaneous metastasis for CRC (Donigan *et al*, 2010). To our knowledge, this is the first study to examine the role of Smad4 in tumour progression and metastasis using an orthotopic caecal inoculation model that can accurately mimic the human tumour growth and metastasis. In the present work, Smad4 knockdown group showed significantly bigger primary tumour size in the caecum, and higher percentage of liver metastasis (60%) when compared with those from vector

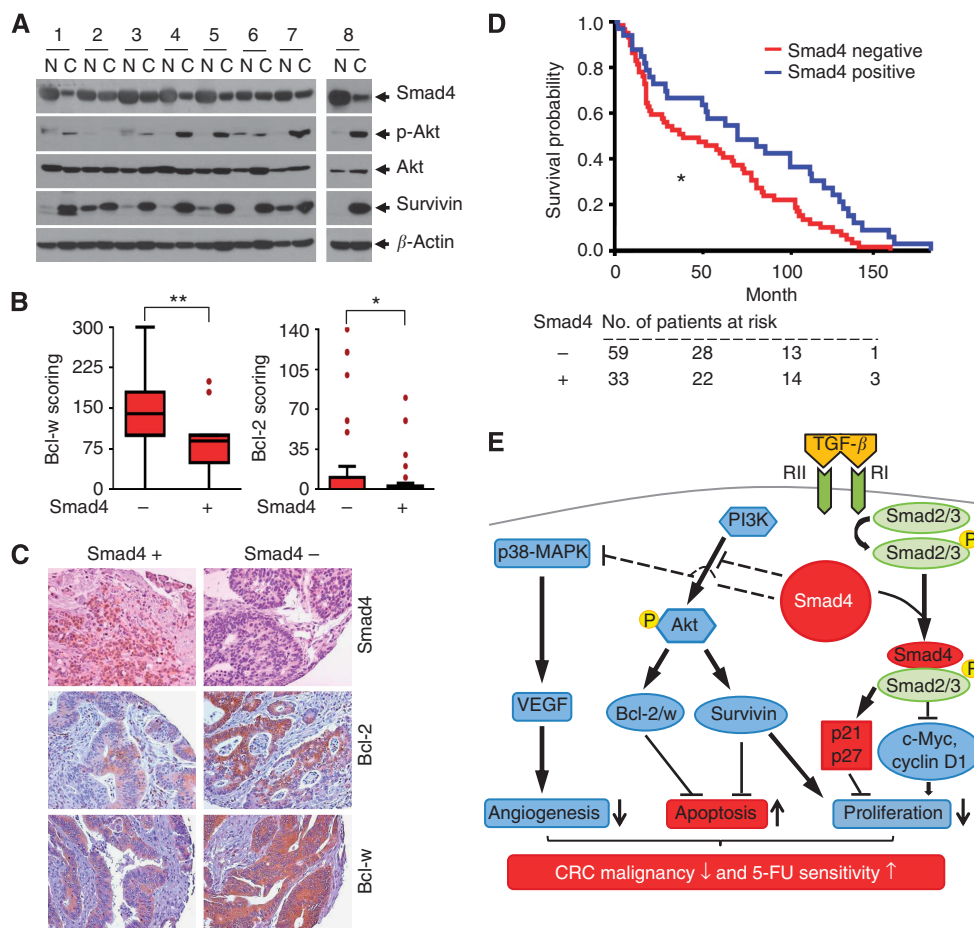


Figure 7. Loss of Smad4 correlates with higher Bcl-2 and Bcl-w expression and shows poor survival of CRC patients. (A) Paired normal and cancer specimen from eight patients with CRC were examined by western blot analyses. (B–D) TMA with 92 CRC tissues was used for immunostaining of Smad4, Bcl-2, and Bcl-w. (B) The Bcl-2 and Bcl-w scoring in Smad4-negative group (n = 59) and Smad4-positive group (n = 33) was plotted, and the mean and 95% CI values are shown. (C) Representative pictures are shown for Smad4, Bcl-2, and Bcl-w expressions. (D) Overall survival of patients with negative or positive nuclear Smad4 staining was analysed. *P < 0.05, **P < 0.01. (E) A model showing the role of Smad4 in CRC progression and chemosensitivity. TGF-β activates the heteromeric complex of receptor II (RII) and receptor I (RI), leading to the phosphorylation and activation of Smad2 and Smad3, which combine with Smad4 to regulate downstream targets. Smad4 inhibits tumour proliferation through TGF-β/Smad pathway, by upregulating p21^{Cip1} and p27^{Kip1}, and downregulating c-Myc and Cyclin D1. Crosstalk between Smad pathway and non-Smad pathway exists. Anti-apoptotic PI3K/Akt/Bcl-2 and PI3K/Akt/Survivin pathways are inhibited by Smad4. Smad4 can also reduce VEGF-induced angiogenesis through inhibiting p38-MAPK pathway.

control cells. These studies provide an interesting model system to determine the aggressive behaviour of the CRC tumours lacking Smad4 expression coupled with the activation of K-Ras, a scenario common for aggressive CRC.

In an attempt to gain insight into the molecular mechanism underlying the aggressive CRC tumour behaviour, we observed that PI3K/Akt (Figure 1E) and p38-MAPK (Supplementary Figure S2) pathways were activated in both types of Smad4-deficient cell clones with oncogenic K-Ras mutation. Loss of Smad4 expression was associated with upregulation of c-Myc and Cyclin D1, and downregulation of p21^{Cip1} and p27^{Kip1}. Changes in the expression of these cell-cycle regulatory proteins might decrease the phosphorylation of Rb that results in G1-S transition and cell proliferation. Activation of PI3K/Akt pathway in conjunction with activated Ras is a key to protect cells from apoptosis through regulating apoptosis-related proteins (Garofalo *et al.*, 2008). Consistent with this, we observed that the anti-apoptotic Bcl-2 and Bcl-w were upregulated and the pro-apoptotic Bim and Bad were downregulated following activation of Akt by the loss of Smad4 expression (Figure 1F) that is also supported by experiments with mice and CRC tumours. Together, these observations provide a mechanism by which Smad4-deficient CRC tumours

with activating K-Ras become aggressive and finally metastasise to distant organs.

Resistance to 5-FU-based therapy is still an obstacle owing to great polymorphism in drug metabolising enzymes, as well as the status of several signalling pathways (Ischenko *et al.*, 2008; Noda *et al.*, 2009). Although clinical studies showed the role of Smad4 in 5-FU-induced chemoresistance, the mechanism is far from clear. One of our important findings was that mice bearing subcutaneous tumours from CT26 and SW620 clones lacking Smad4 were resistant to 5-FU treatment and the overall survival of these mice was poor (Figure 4A–D). In addition, activation of Akt pathway in these Smad4-deficient and K-Ras activated CRC cell lines (Figure 3E) and in the corresponding tumours (Figure 4E) induced the expression of anti-apoptotic Bcl-2, Bcl-w, and Survivin and reduced pro-apoptotic Bim that might be involved in the acquisition of resistance to 5-FU-based therapy. Interestingly, blockade of PI3K/Akt pathway by LY294002 in Smad4-deficient cell clones drastically enhanced 5-FU-based cell death, but not in their Smad4 expressing counterparts (Figure 5A–D). Mechanistically, inhibition of PI3K/Akt was associated with the downregulation of Bcl-2, Bcl-w, and Survivin, and the upregulation of Bim in Smad4-deficient cells (Figure 5E). Taken together, these

results indicate that LY294002 restores 5-FU sensitivity in Smad4-deficient cells through the inhibition of PI3K/Akt pathway and the regulation of apoptotic proteins. Furthermore, activation of p38-MAPK in these cell lines by the loss of Smad4 induced the expression of VEGF (Figure 6A; Supplementary Figure S4), which is in agreement with a previous *in vitro* study (Papageorgis *et al*, 2011). As a result, we observed an increase in angiogenesis *in vivo* as evidenced by vessel number and density (Figure 6). It is also possible that loss of Smad4 promotes chemoresistance to 5-FU through the induction in VEGF-mediated angiogenesis. The combination of anti-angiogenic therapy with 5-FU-based chemotherapy in first- or second-line treatment of advanced CRC has been shown to improve prognosis (Ferrara *et al*, 2004; Hurwitz *et al*, 2004). Therefore, future clinical studies should address the combination of anti-angiogenic therapy and 5-FU-based chemotherapy in patients with CRC with low Smad4 expression.

It is well known that 5-FU targets TS. Therefore, factors that affect TS expression may contribute to 5-FU sensitivity. Other chemotherapeutic drugs including oxaliplatin, irinotecan, folinic acid, and bevacizumab have been used in combination with 5-FU to overcome drug resistance. As 5-FU and oxaliplatin are commonly used chemotherapeutic drugs for CRC, we performed experiments to determine the role of Smad4 in the sensitivity of these two drugs in this study. However, Smad4 did not affect the chemosensitivity of oxaliplatin in both cell lines (data not shown). Future studies will address whether the loss of Smad4 is a general phenomenon for creating resistance to any cytotoxic drug or it is specific for inducing resistance to only 5-FU. This study indicates that combination of LY294002 and 5-FU might overcome 5-FU resistance in patient with CRC with lower Smad4 expression.

In summary, the present investigation provides molecular mechanism demonstrating how the loss of Smad4 expression in CRC promotes progression and metastasis, and leads to 5-FU resistance (Figure 7E). Smad4 deficiency-induced activation of PI3K/Akt pathway and upregulation of VEGF in CRC cells have an important role in conferring resistance to 5-FU-based therapy. Moreover, in CRC patients, no Smad4 expression in the tumour is correlated with higher Bcl-2 and Bcl-w expression that might contribute to poor survival. Collectively, Smad4 may be a biomarker for the therapeutic strategy in CRC, and the combination of PI3K/Akt inhibitor and/or anti-angiogenic therapy with 5-FU-based chemotherapy may improve treatment of CRC patients with low Smad4 expression.

ACKNOWLEDGEMENTS

This study was supported by R01 CA95195, NCI SPORE grant in lung cancer (5P50CA90949, project#4), and Veterans Affairs Merit Review Award (to PK Datta). We thank VDDRC for supporting core services (NIH P30DK058404). We also thank Dr Frank Revetta for immunohistochemical analyses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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