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HOXB9 inhibits proliferation in gastric carcinoma cells via suppression of phosphorylated-Akt and NF-κB-dependent Snail expression

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ABSTRACT

Background: HOXB9 is a homeobox transcription factor which plays an important role in carcinoma development. This protein has been shown to inhibit cancer cell proliferation. However, the mechanisms that underpin HOXB9-mediated inhibition of cellular proliferation remain to be elucidated.
Methods: In this study, two gastric cancer cell lines, SGC7901 and MKN45, were transfected with plasmids pLVX-HOXB9 and shHOXB9. These transfections resulted in the over-expression of the HOXB9 gene in the SGC7901/HOXB9 cells and knockdown of the HOXB9 gene in the MKN45/shHOXB9 cells.
Results: Over-expression of the HOXB9 gene in the SGC7901/HOXB9 cells caused an increase in the apoptotic rate and a concomitant reduction in metastatic ability compared with the knocked-down MKN45/shHOXB9 cells. Moreover, a reduction in the expression of the phosphorylated-Akt protein was observed in the SGC7901/HOXB9 cells, while an increase in expression of the same protein was observed in the MKN45/shHOXB9 cells. We also observed that HOXB9 mediated a reduction in both NF-κB and N-cadherin and Snail protein expression. Conversely, HOXB9 caused an increase in the expression of E-cadherin.
Conclusions: In summary, this study reports that HOXB9 can suppress both phosphorylated-Akt expression and NF-κB activity. The latter phenomenon affects Snail protein expression and the inhibition of gastric carcinoma proliferation.
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1. Introduction

Gastric carcinoma (GC) is one of the most common and lethal malignancies worldwide with a relatively high prevalence in East Asia. Despite the availability of curative therapeutic options including surgical resection and chemoradiotherapy, the 5-year survival rate and prognosis for gastric carcinoma patients remain poor. It has been reported to be heterogeneous at the molecular level as a consequence of genetic alternations [1]. Studies have indicated that invasiveness and metastasis are important determinants in GC patient treatment efficacy. Therefore, characterization of the mechanisms that underlie GC invasion and metastasis should help us to better understand this condition while also allowing us to more optimally diagnose the occurrence of this disease in patients.

Homeobox (HOX) genes encode a family of transcription factors that cooperatively regulate angiogenesis and cellular fate in embryogenesis and organogenesis [2,3]. In mammals, 39 identified HOX genes are organized into four clusters (HOXA, B, C and D), according to their locations on different chromosomes (7p15, 17p21, 12p13, 2q3) [4]. HOX gene mutations are reported to be involved not only in disorders pertaining to limb formation, but also exhibit oncogenic potential with involvement in leukemia chromosomal translocations [5]. Down-regulation of HOXB9 expression is correlated with malignancy, metastasis and poor survival in gastric carcinoma patients [6,7]. Conversely, HOXB9 over-expression

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in gastric carcinoma cells can induce mesenchymal-to-epithelial transition (MET), a phenomenon that results in opposite effects to those caused by epithelial-to-mesenchymal transition (EMT). However, other studies have reported that HOXB9 over-expression results in poor survival in colorectal cancer, hepatocellular carcinoma, ovarian cancer and head and neck squamous cell carcinoma cancer [8–11].

Protein kinase B (PKB/Akt) is known as a serine/threonine protein kinase that regulates the downstream of both the activated receptor tyrosine kinase (RTK) and phosphatidylinositol-3-kinase (PI3K) signaling pathways [12]. The PI3K/Akt signaling pathway is involved in cell proliferation and metastasis, and Akt activation can promote the proliferation and metastasis of human gastrointestinal cancer [13–16]. Akt/PI3K-dependent signaling can activate NF-κB, a nuclear transcription factor [17], which also plays an important role in regulating cell proliferation. Previous studies have demonstrated that NF-κB can modulate Snail expression which promotes EMT (epithelial–mesenchymal transition) progression in various cell types [18,19]. Although the Akt/NF-κB signaling pathway promotes cells proliferation and inhibits apoptosis, the mechanism by which HOXB9 regulates gastric carcinoma cell proliferation and induces MET (mesenchymal-to-epithelial transition) progression via the Akt/NF-κB pathway remains to be elucidated.

Thus, the goal of this study was to elucidate the mechanisms underpinning HOXB9-mediated gastric carcinoma cell proliferation. To facilitate this we constructed the SGC7901/HOXB9 cell line that over-expressed HOXB9 along with a knocked-down HOXB9 gene cell line, MKN45/shHOXB9. These cell lines were used to analyze the role of HOXB9 in gastric carcinoma. We observed that HOXB9 suppressed the growth of gastric carcinoma cells while stimulating MET progression following inhibition of p-Akt and Snail protein expression. Meanwhile, the activation of NF-κB was inhibited in HOXB9 over-expression cells.

2. Materials and methods

2.1. Cell culture and plasmid construction

Wild-type HOXB9 and HOXB9 mutations were cloned into a pLVX-IRES-mCherry vector according to a previously published method [7]. Gastric carcinoma cell lines SGC7901 and MKN45 were procured from the Shanghai Digestive Surgery Institute (Shanghai, China), and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Lentivirus was used as the gene delivery vector for transfection, and transfected cells were selected with puromycin and identified by Western blotting. HOXB9 shRNA and the siRNA plasmids were designed by Genepharma Company (Shanghai, China) and transfected using lipofectamine 2000 (Invitrogen, NY, USA).

2.2. Western blot and immunohistochemistry assays

Western blots were performed using previously published methods, and the relevant antibodies including antibodies against HOXB9, Ki67, GAPDH (Santa Cruz Biotechnology, CA, USA), E-cadherin, N-cadherin, Snail, T-AKT, p-AKT and p-GSK3β (Cell Signaling Technology, Boston, MA, USA) were used according to the manufacturers' protocols.

For immunohistochemistry (IHC), tissues were treated according to published protocols and stained tissues were scored as previously described [7]. Scoring was performed based on staining intensity and positive tumor cell percentage as follows: Intensity scores: 0—negative staining, 1—weak staining, 2—moderate staining, and 3—strong staining. Positive tumor cell percentage scores were 0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, ≥75% positive cells. Final scores were derived by multiplying the intensity scores by the positive tumor cell percentages: final scores of 0–4 indicated weak expression, whereas 4–12 represented strong expression.

2.3. Real time quantitative PCR (qPCR)

Total RNA of tissues or cells was isolated using a Trizol kit (Invitrogen, NY, USA). The RNA was subsequently reverse-transcribed using an AMV RT kit (Invitrogen, NY, USA) according to the manufacturer's instructions. cDNA was amplified using a 2 × SYBR Green PCR mix (QIAGEN, CA, USA) using the primers listed in Supplementary Table S1. A melt curve analysis was performed to confirm specificity in relation to the amplified PCR products. GAPDH gene expression was used as an internal control to facilitate normalization. Each experiment was repeated three times.

2.4. Cell proliferation assay

GC cells were seeded in 96-well plates (2×10^3 cells/well) and incubated for 5 days. Cellular proliferation was measured using a water-soluble tetrazolium salt assay (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions and experiments were repeated in triplicate.

2.5. Cell migration assay

Migration assays were performed using the Boyden chamber technique and a previously published method [7]. For the migration assay, 1.5×10^5 cells in 100 μL serum-free medium were placed in the upper transwell chamber (Corning Costar, NY, USA), while 600 μL of the same medium containing 10% FBS was placed in the lower transwell chamber. After 24 h, migrated cells were fixed with methanol and stained with 0.5% crystal violet solution. The cells were subsequently counted under the Olympus microscope using five random fields of the counting chamber (magnification: 100×).

2.6. Tumorigenicity and metastasis assays

Animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Shanghai Jiao Tong University, and experiments were conducted according to guidelines from the Animal Research Ethics Board of Shanghai Jiao Tong University, Shanghai, China. Nude male mice (Institute of Zoology Chinese Academy of Sciences, 3-week-old) were housed in the Animal Laboratory Unit, Shanghai Ruijin Hospital.

For the experiments, five mice were used per group. For the tumor xenograft model, cells (1×10^6) were inoculated subcutaneously into right flanks of mice. Next, tumor length (L) and width (W) were measured every 5 days. Tumor volume (V) was then calculated as follows: $V = 1/2 \times L \times W^2$. This value was plotted as a function of time for growth curves. Animals were euthanized one month after cell inoculation and then tumor specimens were collected, imaged and weighed. For the peritoneal metastasis study, 100 μL (1×10^6 cells) of cell suspension/mouse were injected into the abdominal cavity. After one month, mice were killed using the cervical dislocation method, and the metastatic masses were observed and nodules counted.

2.7. NF-κB reporter assay

The capacity of HOXB9 to induce NF-κB activation was measured using SGC7901 cells and the Dual Luciferase Reporter Assay System (Promega, Southampton, UK). Briefly, 1.5 μg of pNF-κB-luc (Firefly luciferase reporter for NF-κB activity) and 1.5 μg of pRL-TK (Renilla luciferase reporter as a control) were independently

transfected into a total of 8×10^6 Jurkat T-cells using Amaxa with solution T and program T16 (Amaxa, Cologne, Germany). The transfected cells were pipetted into 6–8 wells of a 24-well plate, left for 20 h to recover and then treated with 10 μg/mL LPS or vehicle for 6 h. The cells were harvested, washed with PBS, lysed and assayed for luciferase activity using a VICTOR3 luminometer (PerkinElmer, Beaconsfield, UK) following the manufacturer's instructions.

2.8. Flow cytometry and TUNEL analysis assays

To analyze the apoptotic nature of the gastric carcinoma cells, the apoptotic dyes Annexin-V APC and Propidium Iodide were used. 2.5 μL Annexin-V APC and 5 μL Propidium Iodide dyes in 100 μL binding buffer were incubated with 1×10^6 SGC7901/HOXB9 cells and the SGC7901/vector cells, along with the MKN45/shHOXB9 cells and MKN45/NC cells for 15 min in the dark, and then washed with phosphate-buffered saline (PBS) and resuspended with 200 μL binding buffer. Flow cytometry was used for testing the apoptosis (Becton Dickinson, USA).

The paraffin section was used for TUNEL kits (Beyotime, China), according to the manufacturer's instructions. Briefly, the paraffin section was incubated with 50 μL TUNEL reaction mixture for 60 min at 37 °C. Then the paraffin section was washed by phosphate-buffered saline (PBS) for three times. At least 1000 cells per section were examined in three random fields by fluorescent microscopy.

2.9. Statistical analysis

Statistical analyses were performed using SPSS 22.0 software. Results were summarized as means ± SD. The distributions of quantitative variables were tested. Continuous variables were compared between two groups using an unpaired t-test and a paired t-test within each group.

3. Results

3.1. HOXB9 attenuates gastric carcinoma cell proliferation and tumor growth

In order to investigate the correlation between HOXB9 expression and gastric carcinoma cell proliferation, the SGC7901/HOXB9 (over-expressing HOXB9) and MKN45/shHOXB9 (knocked-down HOXB9) cell lines were generated. A western blot analysis revealed that the SGC7901/HOXB9 cells over-expressed HOXB9 protein, while the MKN45/shHOXB9 cells expressed reduced levels of HOXB9 protein (Fig. 1A). A HOXB9 mRNA analysis revealed that SGC7901/HOXB9 contained significantly increased levels of HOXB9 mRNA compared with the SGC7901/vector cells. Conversely, the expression of HOXB9 was reduced in the MKN45/shHOXB9 cell line; once more there was a significant difference with the control cell mRNA levels ($p < 0.01^{**}$) (Fig. 1A). We subsequently monitored gastric carcinoma cell proliferation, and we observed that SGC7901/HOXB9 exhibited a significant reduction in proliferation compared with the control cells. On days 3, 4 and 5, SGC7901/HOXB9 proliferation was significantly reduced ($p < 0.01^{**}$). Conversely, MKN45/shHOXB9 proliferation was significantly increased compared with the control cells ($p < 0.01^{**}$) (Fig. 1B). Thus, stimulation of HOXB9 expression could suppress the proliferation of gastric carcinoma cells *in vitro*.

The afore-mentioned results demonstrate that the HOXB9 gene can affect the growth of SGC7901 and MKN45 cells *in vitro*. However, to investigate whether the HOXB9 gene could also inhibit gastric carcinoma growth *in vivo*, SGC7901/HOXB9 and MKN45/shHOXB9 were injected into the subcutaneous tissue of nude mice and tumor volumes were monitored at days 7, 14, 21, 28 and 35, respectively. When tumor growth was monitored at 28 and 35 days, the tumor volumes of the SGC7901/HOXB9 group were significantly different than those of the SGC7901/vector group ($p < 0.05^{*}$, $p < 0.01^{**}$) (Fig. 1C). After 35 days, the mice were euthanized and the tumor weights were recorded. In the SGC7901/HOXB9 group, tumor weight was significantly reduced compared with the SGC7901/vector group ($p < 0.01^{**}$) (Fig. 1C). In the MKN45/shHOXB9 group, the final tumor weights were significantly heavier than for the MKN45/NC group ($p < 0.01^{**}$). Finally, we monitored the proliferation of the SGC7901/HOXB9 group and MKN45/shHOXB9 group using the proliferation marker, Ki67. The expression of Ki67 was monitored in tumor tissues using immunohistochemical (IHC) techniques (Fig. 1D). In the SGC7901/HOXB9 group, a reduced number of Ki67-stained cells was observed compared with the SGC7901/vector group (percentage of positively stained cells: 42.2 ± 2.9% vs 72.2 ± 2.9% ($p < 0.01^{**}$)). Meanwhile in the MKN45/shHOXB9 group, an increase in the number of Ki67-stained cells was observed (percentage of positively stained cells: 56.8 ± 2.2% vs 41.6 ± 2.1% ($p < 0.01^{**}$)). These results demonstrate that HOXB9 can attenuate gastric carcinoma proliferation.

3.2. HOXB9 expression inhibits gastric carcinoma metastasis

Transwell migration assays were used to examine the effect of the HOXB9 gene on gastric carcinoma metastasis. The results showed that the migration ability of cells over-expressing HOXB9 (SGC7901/HOXB9 cell line) were significantly reduced *in vitro* compared with the control group (SGC7901/vector cell line) ($p < 0.05^{*}$), while the migration ability of the HOXB9 knocked-down cells (MKN45/shHOXB9 cell line) was increased compared with the control group (MKN45/NC cell line) ($p < 0.01^{**}$) (Fig. 2A). SGC7901/HOXB9 and MKN45/shHOXB9 cells were injected into the peritoneum of nude mice, respectively. After 60 days, the mice were euthanized and the tumors that developed in the peritonea of the mice were recorded. The number of peritoneal tumors observed in the HOXB9 over-expression group (SGC7901/HOXB9 cell line) ($p < 0.05^{*}$) was reduced compared with the control group (SGC7901/vector cell line) while an increase in tumor numbers was observed in the HOXB9 knocked-down group (MKN45/shHOXB9 cell line) compared with the control group (MKN45/NC cell line) ($p < 0.01^{**}$) (Fig. 2B). Thus, both *in vitro* and *in vivo* data revealed that the expression of HOXB9 negatively correlates with gastric carcinoma metastasis.

3.3. HOXB9 can induce apoptosis in gastric carcinoma cells

A previous study demonstrated that apoptosis is capable of influencing malignant phenotypes associated with carcinoma [20]. Thus, we investigated the apoptotic rates of gastric carcinoma cells in response to changes in HOXB9 expression. The apoptotic dyes Annexin V and Propidium Iodide were used to simultaneously stain the HOXB9 over-expression cells (SGC7901/HOXB9 cell line) and the SGC7901/vector cells, along with the HOXB9 knocked-down cells (MKN45/shHOXB9 cell line) and MKN45/NC cells. Flow cytometry revealed that the apoptotic rate of the SGC7901/HOXB9 cells was 5.63% compared with 2.45% for the SGC7901/vector cells; this difference was significant ($p < 0.01^{**}$). The apoptotic rate was 1.9% and 3.89% for HOXB9 knocked-down cells (MKN45/shHOXB9 cell line) and MKN45/NC cells, respectively (Fig. 3A); the difference was significant ($p < 0.05^{*}$). These results indicate that the HOXB9 gene can enhance gastric carcinoma cell apoptosis (Fig. 3A). To investigate the apoptotic status during gastric carcinoma, a TUNEL assay was employed to detect tumor tissue apoptosis (Fig. 3B). Tumor xenografts from nude mice were examined for 35 days using the TUNEL assay following subcutaneous implantation. We

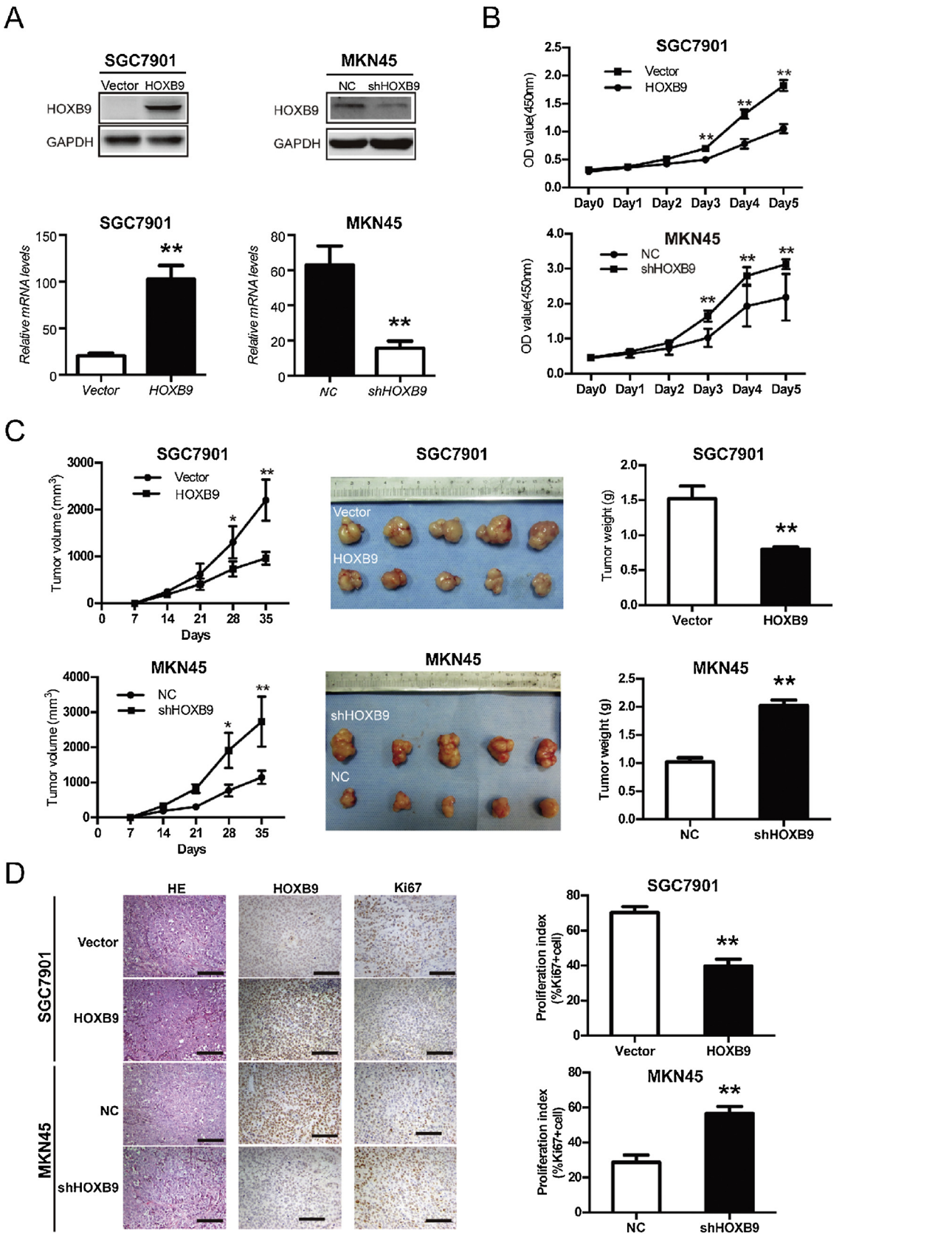

Fig. 1. The proliferation of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells. (A) The western blot results and relative mRNA levels of the HOXB9 expression in SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells. (B) The proliferation of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells ($p < 0.01^{**}$). (C) Tumor growth of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells. (D) Ki67 stain of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells ($p < 0.01^{**}$). NC: normal control.

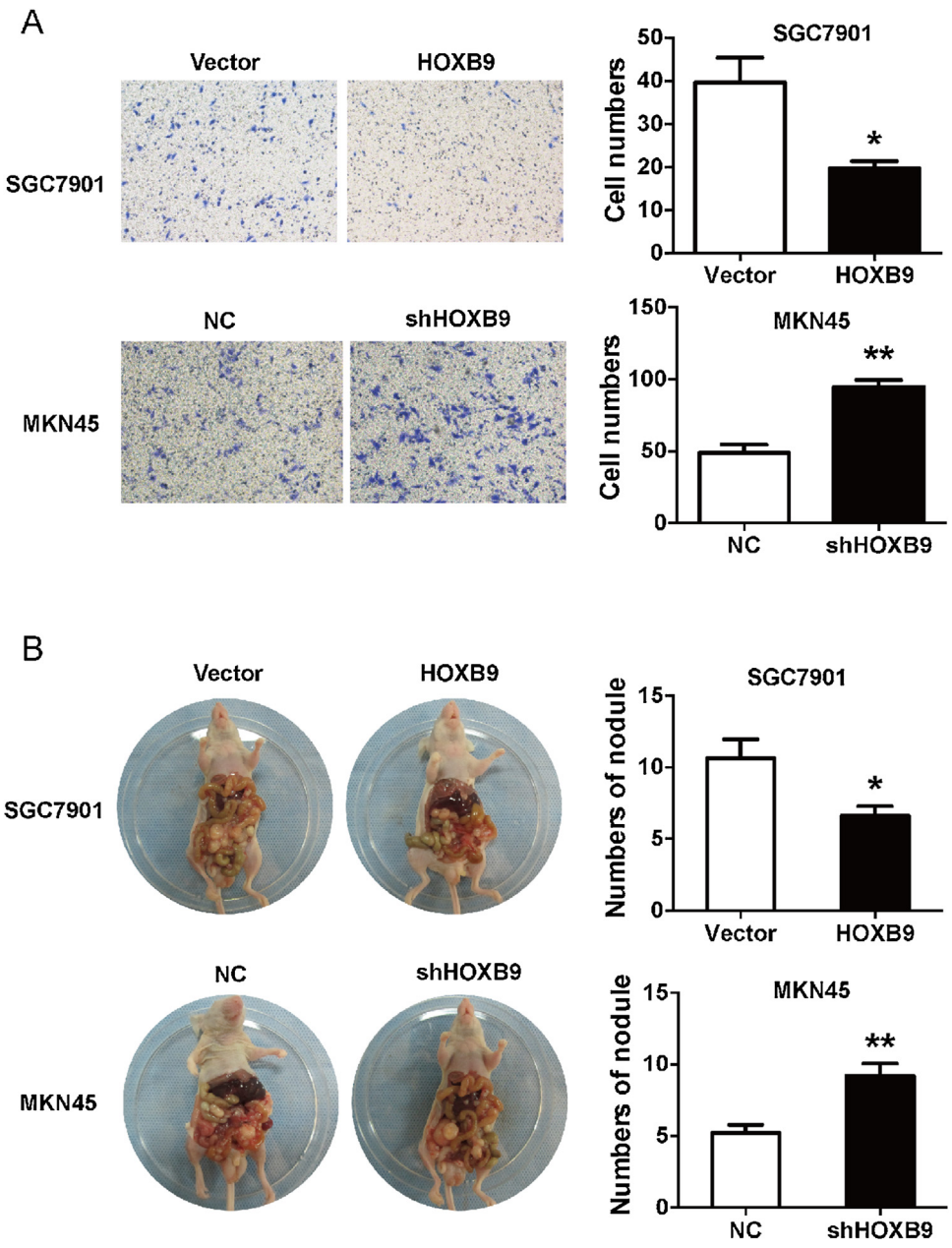

Fig. 2. The metastatic results of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells. (A) The transwell results of the SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells ($p < 0.05^*$) ($p < 0.01^{**}$). (B) The tumor nodules of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells in nude mice ($p < 0.05^*$) ($p < 0.01^{**}$).

observed an increase in the number of TUNEL-positive cells in the HOXB9 over-expression cell line (SGC7901/HOXB9 cells) compared with the SGC7901/vector cells; the difference was significant ($p < 0.01^{**}$). As part of this analysis nuclear cells were stained with Hoechst 33342 dye. The number of TUNEL-positive cells in the HOXB9 knocked-down cell line (MKN45/shHOXB9 cell line) was less than that for the MKN45/NC cells; this difference was significant ($p < 0.01^{**}$) (Fig. 3B). Collectively, these results indicate that expression of HOXB9 can suppress gastric carcinoma cell growth through the induction of apoptosis.

3.4. HOXB9 suppresses the expression of the Snail protein by reducing p-Akt expression

HOXB9 is capable of inducing mesenchymal-to-epithelial transition (MET) through downregulation of mesenchymal markers including Snail, E-cadherin and N-cadherin [7]. In the SGC7901/HOXB9 cells, Snail protein expression was reduced in comparison with the SGC7901/vector control cells, while in the MKN45/shHOXB9 cells, expression of the Snail protein was increased compared with the MKN45/NC control cells ($p < 0.05^*$) (Fig. 4A). Because E-cadherin is also an important protein in MET progression, we simultaneously monitored the expression of E-cadherin in both the SGC7901 and MKN45 cell lines. We observed that the expression of the E-cadherin protein in the SGC7901 cells (over-expressing the HOXB9 gene) was higher than for the SGC7901 cells (transfected with the empty plasmid vector) ($p < 0.05^*$) (Fig. 4B). Conversely, expression of the E-cadherin protein in the knocked-down HOXB9 cell line (MKN45 cells) was lower than for the MKN45/NC cells ($p < 0.05^*$). N-cadherin protein expression in the SGC7901 cells was decreased compared with the SGC7901/vector cells ($p < 0.05^*$). However, N-cadherin protein

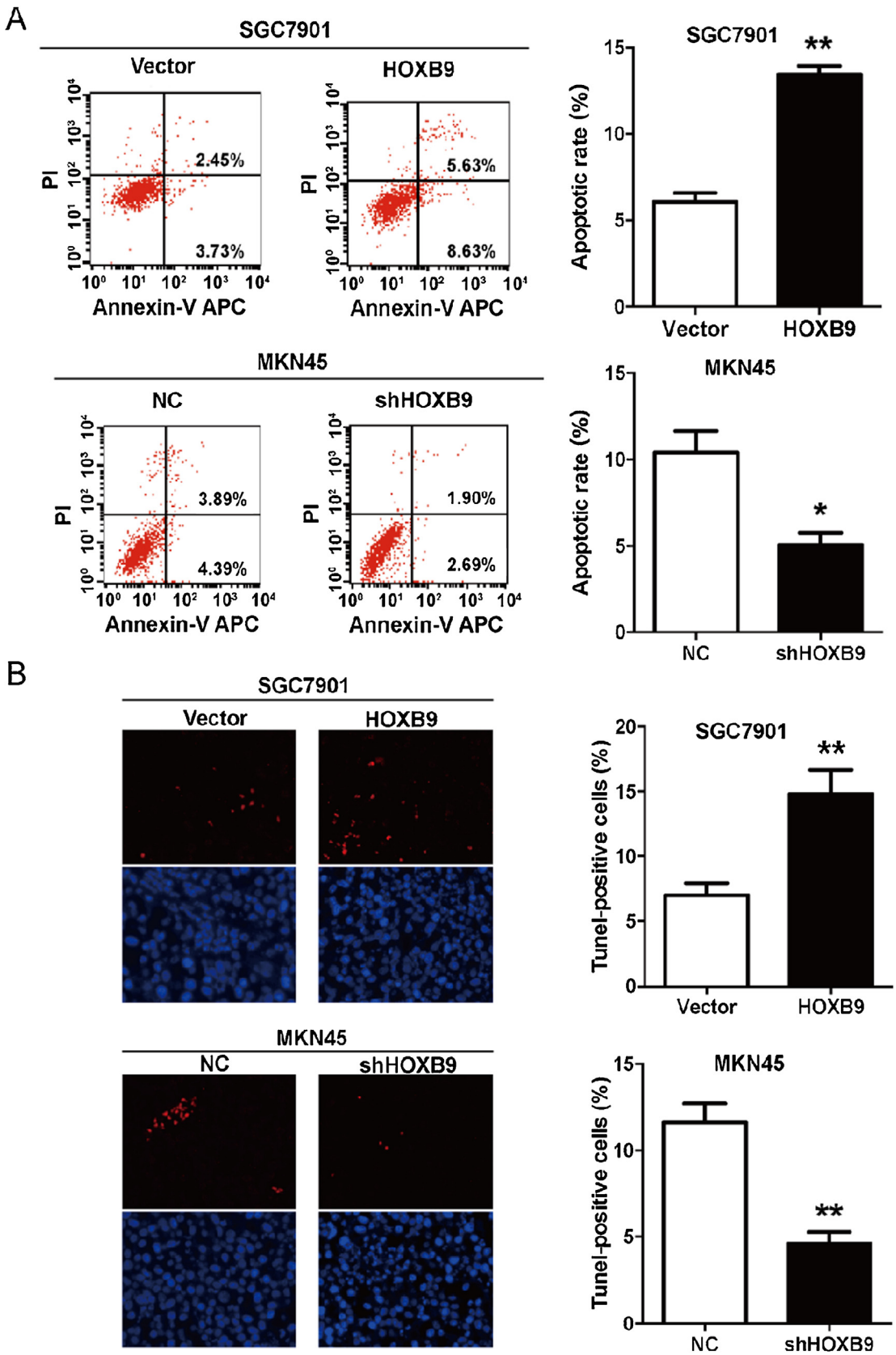

Fig. 3. Flow cytometry results of Annexin-V APC and Propidium Iodide stains in SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells. (A) Annexin-V APC and Propidium Iodide double stained SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells. (B) The TUNEL stains of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells.

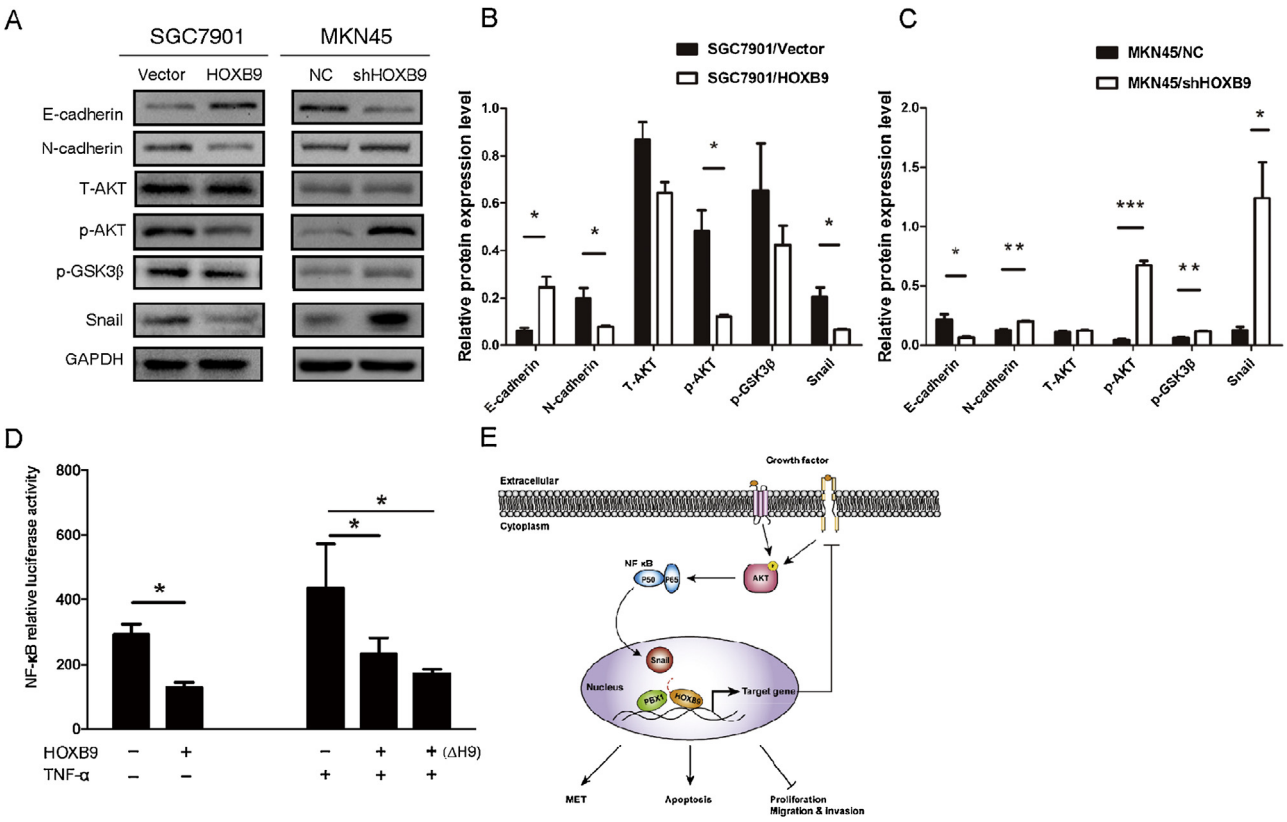

Fig. 4. Relative proteins expression in SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells, respectively. (A) Western blot results show E-cadherin, N-cadherin, T-Akt, p-Akt, p-GSK3β, and Snail proteins expression in SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells, respectively. (B–C) The statistical analysis of the Western blot results ($p < 0.05^*$) ($p < 0.01^{**}$) ($p < 0.001^{***}$). (D) NF-κB activities of SGC7901/HOXB9 cells, SGC7901/vector cells, MKN45/shHOXB9 cells and MKN45/NC cells under TNF-α treatment, respectively ($p < 0.05^*$). (E) The sketch map of the HOXB9 expression signaling pathway in the cells.

expression in the MKN45/shHOXB9 cells was increased compared with the MKN45/NC cells ($p < 0.01^{**}$). We concluded from these results that the HOXB9 can induce MET progression.

In order to determine which signaling pathway is involved in the HOXB9-mediated MET progression, we examined the Akt signaling pathway. We monitored molecular changes pertaining to the Akt signaling pathway in HOXB9-transfected or HOXB9 knocked-down gastric carcinoma cells. In the SGC7901/HOXB9 cell line, although total Akt (T-Akt) expression remained relatively stable, the expression of phosphorylated-Akt was reduced ($p < 0.05^*$). The reduction in the expression of p-Akt resulted in a concomitant decrease in the expression of p-GSK3β. Conversely, in the MKN45/shHOXB9 cell line, the expression of p-Akt was increased ($p < 0.001^{***}$), and the p-GSK3β was also increased ($p < 0.01^{**}$). GSK3β is a major downstream molecule of Akt pathway; when the expression of protein p-Akt was increased, the expression of p-GSK3β protein was also increased [21]. These results suggest that HOXB9 gene can suppress the the Akt signaling pathway of gastric carcinoma cells.

3.5. The expression of HOXB9 can suppress the NF-κB signaling pathway

A previous study indicated that Akt can activate the NF-κB signaling pathway in breast cancer and squamous cell carcinoma [22,23]. The activation of this pathway results in the further induction of the EMT process in carcinogenesis [22,24]. In order to investigate the effect of HOXB9 expression on NF-κB activity and MET induction, we tested NF-κB activity in the SGC7901/HOXB9 cells (Fig. 4D). It has previously been shown that the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α), can stimulate tumor initiation, proliferation and invasion [25], and that tumor invasion is mediated by NF-κB activity. We used TNF-α to stimulate the NF-κB pathway. From these results, we observed that when the SGC7901/HOXB9 cells were treated with TNF-α, NF-κB activity was reduced significantly compared with the SGC7901 cells transfected with empty plasmid vector ($p < 0.05^*$) (Fig. 4D). The HOXB9 gene has a hexapeptide motif, and our previous study confirmed that this motif can enhance HOXB9 tumor suppression activity while inducing mesenchymal-to-epithelial transition (MET) progress [7]. We observed that when this hexapeptide motif was knocked out (ΔH9 gene) in SGC7901/HOXB9 cells, NF-κB activity was significantly reduced. From these results, we concluded that the HOXB9 gene can suppress SGC7901 cell growth through inhibition of NF-κB activity; we also observed that NF-κB was stimulated by TNF-α activity.

4. Discussion

HOXB9 is a homeobox domain-containing transcriptional factor, which plays crucial roles in both embryonic development and numerous human cancers [26]. Hayashida and Chiba et al. observed that HOXB9 can promote tumorigenicity and metastasis in breast cancer [27,28]. When HOXB9 was overexpressed in breast cancer cells, E-cadherin and β-catenin protein expressions were decreased. However, we observed that HOXB9 expression inhibits the gastric carcinoma metastasis. When over-expressing HOXB9 in gastric carcinoma cells (SGC7901/HOXB9), the expression of E-cadherin protein was increased, and Snail protein expression was decreased. Lin Sha et al. reported that HOXB9 can also promote EMT in hepatocellular carcinoma cells [29]. Furthermore, HOXB9 can up-regulate EMT-related molecules, N-cadherin and vimentin, in lung adenocarcinoma cells. In contrast, knockdown

of HOXB9 using siRNAs, led to up-regulation of E-cadherin protein [30]. However, we found that HOXB9 can induce MET in gastric cancer; the effect of MET opposes that of EMT with suppression of the migration and invasion capacities of gastric cancer cells (Fig. 2). In cells over-expressing HOXB9 (SGC7901/HOXB9), the N-cadherin protein expression was decreased, and the expression of E-cadherin protein was increased. Moreover, there was a reduction in the expression of Snail in SGC7901 cells over-expressing HOXB9 (SGC7901/HOXB9) (Fig. 4A and B). Conversely, in the knocked-down HOXB9 cells (MKN45/shHOXB9), the expression of E-cadherin protein was decreased; N-cadherin and Snail protein expressions were increased (Fig. 4A–C). These results were in accordance with the MET phenotype; this demonstrated that over-expression of HOXB9 induces MET progress in gastric carcinoma cells.

Akt is reported to be associated with gastric cancer cell proliferation, chemo-resistance and radio-resistance [31]. Previous studies discovered that patients had abnormal expressions of the Akt pathway. Immunohistochemical stain results showed that Akt protein expression in the patients gastric carcinoma tissue was higher than adjacent noncancer tissue [32]. However, the mechanisms by HOXB9 gene affects the Akt pathway have not yet been elucidated. Hayashida and Chiba et al. found that phosphorylated-Akt protein was activated in the overexpression HOXB9 in breast cancer cells. Nonetheless, our finding supported that HOXB9 was over-expressed in gastric cancer cells (SGC7901/HOXB9), and phosphorylated-Akt was decreased, while the total Akt protein expression remains at the same level (Fig. 4A). This suggests that HOXB9 could modulate the Akt signaling pathway. A previous study investigated the Akt protein activation involved in NF-κB stimulation [33]. Here, we also suggested that overexpressing HOXB9 in SGC7901 cells can decrease the p-Akt and NF-κB activation. Moreover, the hexapeptide motif of HOXB9 was knocked out, the inhibitory effects of HOXB9 towards tumor formation were enhanced, while NF-κB activity was also reduced (Fig. 4D). It has previously been reported that GSK3β is involved in regulating the activity of NF-κB during inflammation [34]; however, our western blot results showed that there was no difference in p-GSK3β expression when NF-κB levels were reduced in the SGC7901/HOXB9 cells. Nonetheless, we observed an increase in p-GSK3β expression when NF-κB levels were increased in the MKN45/shHOXB9 cells (Fig. 4A–D).

HOXB9 is a tumor suppressor in gastric carcinoma with an important role in inhibiting EMT progression during gastric cancer. This protein also stimulates an increase in the expression of E-cadherin and a reduction in the expression of N-cadherin, while concomitantly suppressing Snail protein expression (Fig. 4A). In our previous study, we demonstrated that expression of HOXB9 could decrease and suppress the growth of gastric carcinoma [7]; PBX1 protein is a product of a proto-oncogene, which interacts with HOXB and can promote melanoma and breast cancer cell proliferation [35,36]. Upon complexing with PBX1, HOXB9 is capable of stimulating gastric carcinoma growth. However, when HOXB9 is uncoupled from PBX1, HOXB9 can target genes involved in the activation of cellular apoptosis and MET progression; additionally, cellular proliferation, migration and invasion were inhibited (Fig. 4E). We hypothesized that upon detachment of HOXB9 from PBX1, phosphorylation of Akt occurs resulting in the activation of NF-κB. A previous study found that Akt strongly stimulates the expression and activation of the NF-κB transfactor, while NF-κB can induce Snail transcription in breast cancer and prostate cancer [37], but our results also confirmed that HOXB9 gene could decrease the expression of p-Akt, NF-κB, and Snail protein in gastric carcinoma cells.

In this study, we constructed a cell line over-expressing HOXB9 (SGC7901/HOXB9) along with a HOXB9 knocked-down cell line (MKN45/shHOXB9) to investigate the role of the HOXB9 gene in gastric carcinoma. We observed that HOXB9 is capable of inhibiting tumorigenesis and gastric carcinoma migration, while also increasing the rate of cellular apoptosis. We also observed that HOXB9 can inhibit both p-Akt expression and NF-κB activation, thus inhibiting the Akt-signaling pathway. HOXB9 can also inhibit the expression of both the Snail protein and N-cadherin, while increasing the expression of E-cadherin, and these results demonstrate that HOXB9 can induce the gastric carcinoma cell MET phenotype.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.dld.2018.08.018.

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