

Over-Expression of FoxM1 Leads to Epithelial–Mesenchymal Transition and Cancer Stem Cell Phenotype in Pancreatic Cancer Cells

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ABSTRACT

FoxM1 is known to play important role in the development and progression of many malignancies including pancreatic cancer. Studies have shown that the acquisition of epithelial-to-mesenchymal transition (EMT) phenotype and induction of cancer stem cell (CSC) or cancer stem-like cell phenotypes are highly inter-related, and contributes to drug resistance, tumor recurrence, and metastasis. The molecular mechanism(s) by which FoxM1 contributes to the acquisition of EMT phenotype and induction of CSC self-renewal capacity is poorly understood. Therefore, we established FoxM1 over-expressing pancreatic cancer (AsPC-1) cells, which showed increased cell growth, clonogenicity, and cell migration. Moreover, over-expression of FoxM1 led to the acquisition of EMT phenotype by activation of mesenchymal cell markers, ZEB1, ZEB2, Snail2, E-cadherin, and vimentin, which is consistent with increased sphere-forming (pancreatospheres) capacity and expression of CSC surface markers (CD44 and EpCAM). We also found that over-expression of FoxM1 led to decreased expression of miRNAs (let-7a, let-7b, let-7c, miR-200b, and miR-200c); however, re-expression of miR-200b inhibited the expression of ZEB1, ZEB2, vimentin as well as FoxM1, and induced the expression of E-cadherin, leading to the reversal of EMT phenotype. Finally, we found that genistein, a natural chemo-preventive agent, inhibited cell growth, clonogenicity, cell migration and invasion, EMT phenotype, and formation of pancreatospheres consistent with reduced expression of CD44 and EpCAM. These results suggest, for the first time, that FoxM1 over-expression is responsible for the acquisition of EMT and CSC phenotype, which is in part mediated through the regulation of miR-200b and these processes, could be easily attenuated by genistein. *J. Cell. Biochem.* 112: 2296–2306, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: FOXM1; EMT PHENOTYPE; MIRNAS; CSC-SELF-RENEWAL; GENISTEIN

Forkhead box protein M1 (FoxM1) is one of the family member of evolutionarily conserved transcriptional factors characterized by the presence of a DNA-binding domain called the forkhead box or winged helix domain [Laoukili et al., 2005; Laoukili et al., 2007; Wang et al., 2010a]. It has been recognized that FoxM1 is involved in cell proliferation and apoptosis which regulates the developmental function of many organs in the body [Kato and Kato, 2004]. Many studies have reported that FoxM1 is a key cell-cycle regulator of both the transition from G1 to S phase and the progression to mitosis [Leung et al., 2001]. Moreover, FoxM1 has been demonstrated to regulate the transcription of cell-cycle regulatory molecules, such as Cdc25A, Cdc25B, cyclin B, cyclin D1, p21cip1, and p27kip1, all of which are recognized as the essential

regulators of G1-S and G2-M cell cycle progression [Wang et al., 2001; Wang et al., 2002; Kalin et al., 2006; Laoukili et al., 2007].

Recent studies have shown that FoxM1 signaling plays important roles in cellular developmental pathways including the maintenance of homeostasis between cell proliferation and apoptosis. Emerging evidences suggest that FoxM1 also plays a significant role in tumor aggressiveness by increasing drug resistance and cancer cell metastasis, and alterations in FoxM1 signaling pathway have been reported to be associated with tumorigenesis [Kalin et al., 2006; Laoukili et al., 2007]. Moreover, studies have shown that FoxM1 signaling pathway is frequently deregulated in human malignancies with increased expression of FoxM1 found in glioblastomas, lung cancer, prostate cancer, hepatocellular carcinoma, breast cancer,

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basal cell carcinomas, and pancreatic cancer [Kalin et al., 2006; Kim et al., 2006; Laoukili et al., 2007; Wang et al., 2007]. Interestingly, higher expression of FoxM1 was found to be associated with poor prognosis of breast cancer patients [Bektas et al., 2008]. Furthermore, recent studies have shown that FoxM1b expression could be used as an independent predictor of poor survival in patients with gastric cancer [Li et al., 2009a]. These results strongly suggest that FoxM1 may have a crucial role in the development and progression of human cancers; however, the molecular mechanism(s) by which FoxM1 signaling induces tumor growth and progression is poorly understood.

Emerging evidences suggest that the acquisition of epithelial-to-mesenchymal transition (EMT) that is reminiscent of cancer stem cells (CSCs) or cancer stem-like cells contributes to tumor cell aggressiveness. The EMT is an important process whereby epithelial cells with a cobblestone phenotype acquire mesenchymal cell characteristics with a spindle-shaped fibroblast-like morphology. This process involves a disassembly of cell-cell junctions, such as down-regulation and relocation of E-cadherin and zonula occludens-1 (ZO-1), which are epithelial cell phenotype markers, and down-regulation and translocation of β -catenin from the cellular membrane to the nucleus, reorganization of actin cytoskeleton, and up-regulation of mesenchymal cell phenotype markers (vimentin, fibronectin, and N-cadherin) [Christiansen and Rajasekaran, 2006]. This allows mesenchymal phenotypic cells to have less cell adhesion capacity, which leads to increased cell migration and invasion capacity, resulting in tumor aggressiveness [Hugo et al., 2007].

During the acquisition of EMT phenotype, several transcription factors such as zinc-finger E-box binding homeo-box 1 (ZEB1) and ZEB2/SIP1, and Snail1, Snail2/Slug, Twist, and E47. ZEB1 has been shown to be a critical mediator of EMT phenotype induced by various inducers in different cell lines including cancer cells [Graham et al., 2008; Gregory et al., 2008a; Korpala et al., 2008]. ZEB1 has been demonstrated to regulate the expression of genes by binding to ZEB-type E-boxes (CACCTG) within the promoter region of target genes, resulting in chromatin condensation and gene inactivation [Peinado et al., 2007]. The expression of E-cadherin, a marker for epithelial cell phenotype is repressed by ZEB1 through its binding to ZEB-type E-boxes in the E-cadherin gene promoter, which is fundamental for the acquisition of EMT phenotype [Eger et al., 2005]. Recent studies have also demonstrated that ZEB1 promotes cell migration and tumor metastasis by the inhibition in the expression of cell polarity factors [Spaderna et al., 2008]. ZEB2/SIP1 has been shown to be involved in the down-regulation in the expression of many genes coding for crucial proteins of the epithelial cell phenotype, including E-cadherin [Vandewalle et al., 2005], concomitant with up-regulation in the expression of vimentin, a marker for mesenchymal phenotype [Bindels et al., 2006]. Snail2/Slug has been shown to play a key role in the induction of EMT phenotype by growth factors such as TGF [Park et al., 2008a], which suggest that ZEB1, ZEB2, and Snail2 are important gene transcription factors in controlling the induction of EMT phenotype.

Emerging evidences suggest that the expression of genes that are fundamental to the acquisition of EMT and CSC phenotype consistent with tumor cell aggressiveness are regulated by microRNAs (miRNAs), which are small (19–24 nucleotides) non-

coding RNA molecules known to regulate gene expression by interacting with specific sequences in the 3' untranslated region of multiple target mRNAs, which results in either translational repression or degradation of target mRNAs [Garzon et al., 2006; Cano and Nieto, 2008]. Moreover, it has been suggested that miR-200 family could regulate the processes of EMT by targeting ZEB1 and ZEB2 [Gregory et al., 2008a; Korpala et al., 2008; Park et al., 2008b; Kong et al., 2009; Kong et al., 2010]. However, the molecular mechanism(s) by which FoxM1 leads to the acquisition of EMT phenotype through deregulation of miRNAs has not been investigated. In this study, we showed for the first time that forced over-expression of FoxM1 led to the acquisition of EMT phenotype consistent with increased CSC-self-renewal capacity in pancreatic cancer cells (AsPC-1 cells). Forced over-expression of FoxM1 showed decreased expression of let-7 and miR-200, and interestingly re-expression of miR-200b reversed the EMT phenotype and showed decreased expression of FoxM1 in AsPC-1 cells. We further demonstrated that FoxM1-mediated cell growth, migration and invasion, acquisition of EMT phenotype, and CSC self-renewal capacity of pancreatic cancer cells could be easily attenuated by genistein treatment.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Antibodies against human CD44, and EpCAM, Snail2, and vimentin were purchased from Cell Signaling Technology (Beverly, MA). Antibody against human FoxM1, VEGF, Hes1, NF- κ B, cyclin D1, ZEB1, ZEB2, Snail1, and E-cadherin were purchased from Santa Cruz (Santa Cruz, CA). Antibody against human β -actin was acquired from Sigma Chemicals (St. Louis, MO). Alexa Fluor 488 goat anti-mouse IgG for CD44 and EpCAM staining were purchased from Invitrogen. The miRNA reverse transcription primers and PCR probes were purchased from Applied Biosystems (Carlsbad, CA). Genistein was purchased from Sigma (St. Louis, MO). RT-PCR assay kits were purchased from Applied Biosystems. Growth supplements B-27 and N-2 were purchased from Invitrogen.

CELL CULTURE

Human pancreatic cancer cell line AsPC-1 was chosen for forced over-expression of FoxM1 by stable transfection of FoxM1 cDNA especially because AsPC-1 cells showed lowest expression of FoxM1 among many pancreatic cancer cells tested. AsPC-1 cells have been authenticated through Applied Genomics Technology Center Core Facility of the Barbara Ann Karmanos Cancer Institute at Wayne State University, Detroit, MI, on March 13, 2009. These authenticated cells were frozen in liquid N₂ tank for subsequent use. The method used for testing was short tandem repeat profiling by using the PowerPlex 16 System from Promega. AsPC-1 cells was transfected with the corresponding empty vector pCDNA3 control or pCDNA3-FoxM1 vector by using ExGen-500 transfection reagent (Fermentas, Germany) following the manufacturer's protocol, as previously described [Kong et al., 2009; Ali et al., 2010; Kong et al., 2010], which are referred to as AsPC-1-control or AsPC-1-FoxM1 cells, respectively, in this study. Over-expressing FoxM1 stable cell line was cloned by antibiotics gentamycin and confirmed by Western blot analysis, as described below.

CELL SURVIVAL ASSAY

MTT assay was conducted to examine the effect of genistein treatment on cell viability of AsPC-1-control and AsPC-1-FoxM1 cells, as described previously [Ali et al., 2010]. Different concentrations of genistein ranging from 0–60 $\mu\text{mol/L}$ were tested in both cell lines. After 72 h of incubation in 96-well plates, MTT assay was performed as described previously [Ali et al., 2010].

CLONOGENIC ASSAY

Clonogenic assay was performed to examine the effect of genistein treatment on cell growth in AsPC-1-control and AsPC-1-FoxM1 cells, as described previously [Ali et al., 2010]. 5×10^4 cells were plated in a six-well plate. After 72 h of exposure to 0, 30, and 60 $\mu\text{mol/L}$ of genistein, the cells were trypsinized, and 1,000 single viable cells were plated in 100-mm culture dishes. The cells were then incubated for 10–12 days at 37°C in the condition of 5% CO_2 /5% O_2 /90% N_2 . Colonies were stained with 2% crystal violet, washed with water, and counted.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Whole cell lysates from different experiments were obtained by lysing the cells in the protein lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM Na_3VO_4 , 1 mM EDTA, 1 mM EGTA, and 1 \times protease inhibitor cocktail. Western blotting analysis was performed as previously described [Kong et al., 2007].

WOUND HEALING ASSAY

Wound healing assay was performed to examine the capacity of cell migration and invasion, as described previously [Mahato et al., 2010]. Briefly, after the cells grew in 90–95% confluence in six-well plates, the wound was generated by scratching the surface of the plates with a 0–200 μl pipette tip. The cells were then incubated in 0, 30, and 60 $\mu\text{mol/L}$ of genistein for 18 h, and then photographed using a microscope (Nikon ECLIPSE TS100).

SPHERE FORMATION ASSAY

Sphere formation assay was performed to assess the capacity of CSC self-renewal. Single cell suspensions of both AsPC-1-control and AsPC-1-FoxM1 cells were plated on ultra low adherent wells of six-well plates (Corning, Lowell, MA) at 1,000 cells/well in 1.5 ml of sphere formation medium (1:1 DMEM/F12 medium supplemented with 50 units/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, B-27, and N-2). One milliliter of sphere formation medium was added every 3–4 days. After 7 days of incubation with different concentrations of genistein, the formed spheres termed as “pancreatospheres” were collected by centrifugation at 300 g for 5 min and counted with a Nikon TS100 microscope. The proportion of sphere-generating cells was calculated by dividing the number of pancreatospheres formed by the number of cells seeded. The sphere formation assay of secondary pancreatospheres was performed by using parental pancreatospheres, as described above. Briefly, primary pancreatospheres of AsPC-1-FoxM1 cells were harvested, washed with PBS, and incubated with accutase solution (Sigma) at 37°C for 5–10 min. Five hundred single suspension cells were plated in each well of ultra low adherent wells of six-well plates in 1.5 ml of sphere formation

medium. One milliliter of the medium was added to each well every 3–4 days. After 1 or 3 weeks of incubation with different concentrations of genistein, secondary pancreatospheres were harvested for counting, as described above.

IMMUNO-FLUORESCENT STAINING ASSAY AND CONFOCAL MICROSCOPY

Single cell suspensions of AsPC-1-control and AsPC-1-FoxM1 cells were plated in ultra low adherent wells of six-well plate at 5,000 cells/well in sphere formation medium, as described above. After 7 days of incubation with different concentrations of genistein, the pancreatospheres were collected by centrifugation, washed with PBS, and fixed with 3.7% paraformaldehyde for immuno-fluorescence staining, as described previously [Kong et al., 2008]. Antibodies against human CD44 and epithelial cell adhesion molecule (EpcAM) were applied for immunostaining assay, following the manufacturer's protocol. Alexa Fluor 488 goat anti-mouse IgG was used for secondary immunostaining reaction. The CD44 or EpcAM-labeled pancreatospheres were mixed with Prolong Gold antifade reagent (Invitrogen), mounted in the slides, and photographed under confocal microscope (Leica TCS SP5) using software LAS AF 1.2.0 Build 4316 in the MIRC Core Facility of Wayne State University School of Medicine.

REAL-TIME RT-PCR OF MIRNAS

To determine the expression of miRNAs (let-7a, b, c, miR-21, and miR-200a, b, c) in AsPC-1-control and AsPC-1-FoxM1 cells, we used TaqMan MicroRNA Assay kit (Applied Biosystems, Foster City, CA) following manufacturer's protocol. Five nanogram of total RNA was subjected for reverse transcription in 15 μl of reaction mixture and real-time PCR reactions were performed in 25 μl of reaction mixture as described previously [Li et al., 2009b], by using a Smart Cycler II thermocycler (Cepheid). Data were analyzed using C_t value and were normalized by the expression of control miRNA RNU6B in each sample.

REAL TIME RT-PCR OF MRNAS

To measure the relative expression of mRNAs in AsPC-1-FoxM1 cells, 2 μg of total RNAs of each sample were used for RT reaction in 20 μl of reaction volume, using a reverse transcription system (Invitrogen) as described previously [Kong et al., 2010]. CybrGreen Assay kit (Applied Biosystems) was used for real time PCR reaction, following manufacturer's protocol. Sequences of PCR primers were described previously [Kong et al., 2010]. Real-time PCR reactions were performed in 25 μl of reaction mixture as described previously [Li et al., 2009c] using a Smart Cycler II thermocycler. Data were analyzed using C_t value and were normalized by the expression of control mRNA GAPDH in each sample.

TRANSFECTION OF MIRNA PRECURSOR MIR-200B

2×10^5 Cells/well of AsPC-1-FoxM1 cells were seeded in six-well plates and transfected with pre-miR-200b or miRNA-negative control #2 (Ambion, Austin, TX) at a final concentration of 20 nM using DharmaFECT #3 transfection reagent (Dharmacon), following the manufacturer's protocol, as described previously [Kong et al., 2009; Ali et al., 2010]. After 3 days of transfection, the transfected

cells were harvested for total RNA isolation or protein extraction. The relative levels of mRNAs, miRNAs, and proteins were measured, as described above.

STATISTICAL METHODS

The mean and SD were calculated by using Graph Pad Prism software (version 4.03). Statistical difference of each treatment was compared by the paired *t* test. The *p* value equal to or less than 0.05 was considered as statistical significance.

RESULTS

OVER-EXPRESSION OF FOXM1 INCREASED CELL GROWTH IN ASPC-1 CELLS

To investigate whether or not forced over-expression of FoxM1 promotes cell growth, the growth was assessed by MTT assay. After

4 days of incubation, the cell growth was assessed as presented in Figure 1A. The data shows that after 4 days of incubation, over-expression of FoxM1 increased cell growth to a moderate degree in AsPC-1 cells, suggesting that FoxM1 over-expression could lead to increased cell growth to some degree in AsPC-1 cells.

FORCED OVER-EXPRESSION OF FOXM1 ALTERED PROTEIN EXPRESSIONS AND MORPHOLOGY IN ASPC-1 CELLS

The Figure 1B shows the comparative expression of FoxM1 mRNA in AsPC-1-control and AsPC-1-FoxM1 cells, which indicates that AsPC-1-FoxM1 cells had higher level of FoxM1 mRNA. To examine the effect of FoxM1 over-expression on protein expressions, we measured the relative protein expression of NF- κ B p65 subunit, cyclin D1, Hes-1, VEGF, ZEB1, CD44, and EpCAM in FoxM1-over-expressed AsPC-1 cells by Western blot analysis. The results show that forced over-expression of FoxM1 increased the relative levels of NF- κ B p65 subunit, cyclin D1, Hes-1, VEGF, ZEB1, CD44, and

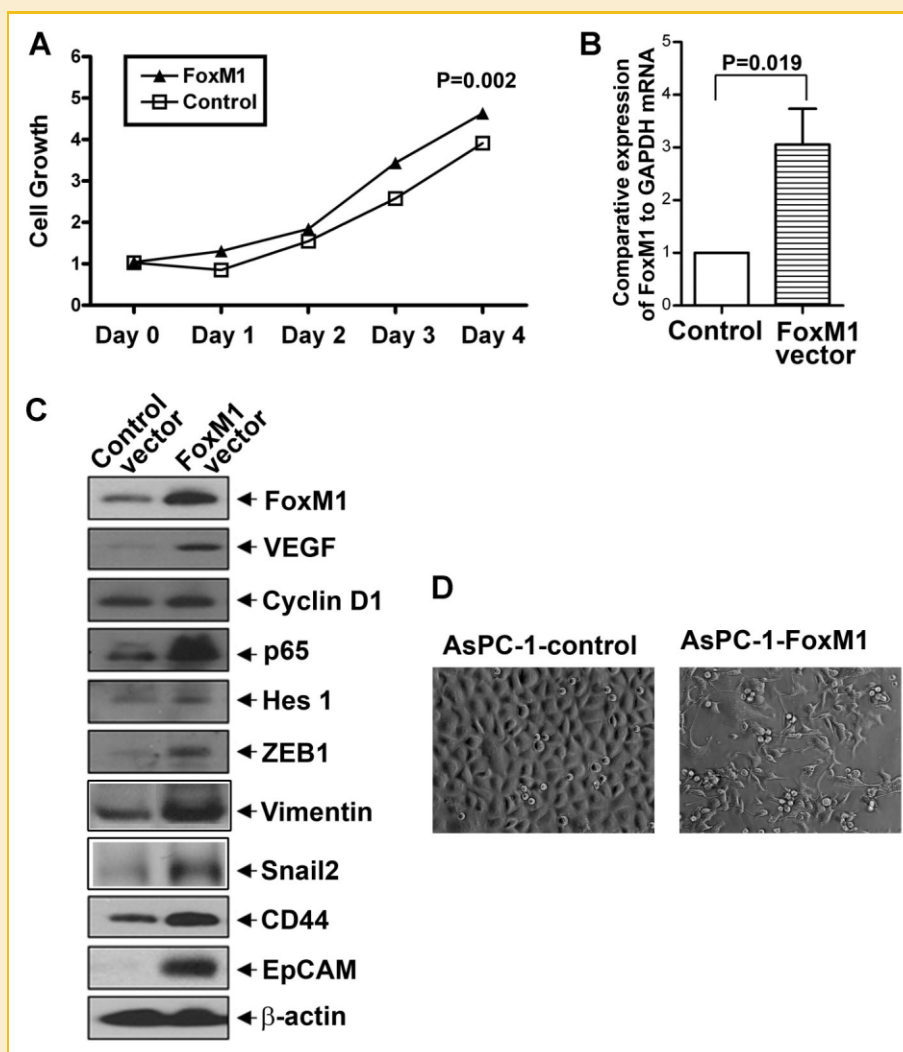


Fig. 1. FoxM1 expression vector increased cell growth (A), the comparative expression of FoxM1 mRNA (B), the relative expression of FoxM1, cyclin D1, VEGF, p65, Hes-1, ZEB1, CD44, and EpCAM at the protein levels (C), and the acquisition of EMT phenotype (D; magnification $\times 20$) in human pancreatic cancer AsPC-1 cells. FoxM1 over-expressing AsPC-1 cells were established by stable gene transfection technique, as described under the Methods section.

EpCAM in AsPC-1 cells (Fig. 1C). These results suggest that FoxM1 is involved in the regulation of the expression of these proteins. The morphological examination showed that the acquisition of mesenchymal phenotype in FoxM1 over-expressed AsPC-1 cells, compared to those of AsPC-1-control cells (Fig. 1D), suggesting that forced over-expression of FoxM1 leads to the acquisition of EMT phenotype in AsPC-1 cells.

OVER-EXPRESSION OF FOXM1 DIFFERENTIALLY MEDIATED THE EXPRESSION OF LET-7 AND MIR-200 IN ASPC-1 CELLS

To investigate whether or not over-expression of FoxM1 could alter the expression of miRNAs, we examined the relative miRNA levels of miR-21, let-7a, b, c, and miR-200b, c in AsPC-1-control and AsPC-1-FoxM1 cells. The results demonstrate that AsPC-1-FoxM1 cells had decreased expression of let-7b, c and miR-200b, c, compared to AsPC-1-control cells (Fig. 2). These results suggest that over-expression of FoxM1 down-regulates the expression of

miRNAs, let7-b, c and miR-200b, c in AsPC-1 cells, further suggesting that FoxM1 could be involved in the regulation miRNA expression in AsPC-1 cells.

RE-EXPRESSION OF MIR-200B CAUSED REVERSAL OF EMT PHENOTYPE IN FOXM1-OVER-EXPRESSED ASPC-1 CELLS

A large number of studies have reported that miR-200 is involved in the regulation of the acquisition of EMT phenotype by targeting the expression of ZEB1 and ZEB2. We investigated whether or not miR-200 family could regulate FoxM1-mediated EMT phenotype in AsPC-1 cells, we transfected miR-200b precursor into AsPC-1-FoxM1 cells in this study. We confirmed that the transfection of miR-200b precursor significantly increased the level of miR-200b in AsPC-1-FoxM1 cells (Fig. 3A). The results also showed that re-expression of miR-200b decreased the relative mRNA levels of ZEB1 and vimentin (mesenchymal phenotype cell biomarkers), and increased the relative mRNA level of E-cadherin, an epithelial cell biomarker, in FoxM1-over-expressed AsPC-1 cells as assessed by real time RT-PCR assay (Fig. 3A). The data from Western blot

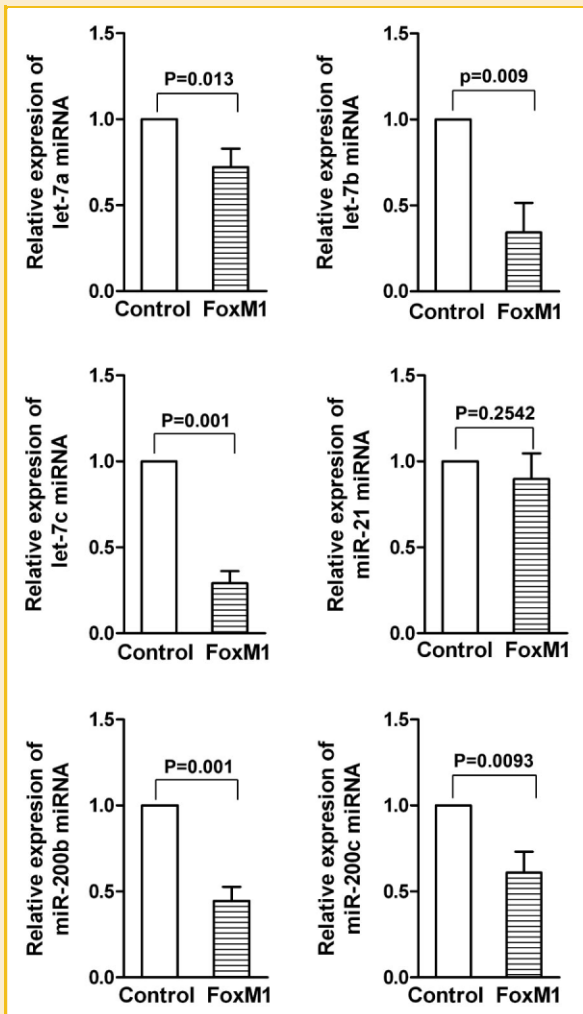


Fig. 2. FoxM1 over-expression differentially regulated the miRNA expression of let-7a, b, c and miR-200b, c in AsPC-1 cells. The comparative expressions of miRNAs against control RNU6B miRNA were measured by real-time RT-PCR technique.

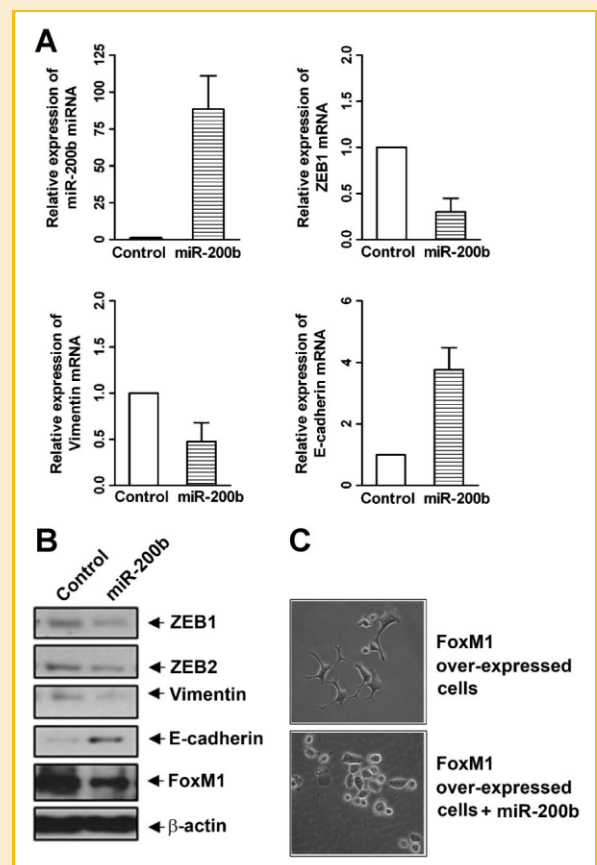


Fig. 3. Re-expression of miR-200b differentially regulated the expression of EMT phenotype marker mRNAs (A) and proteins (B), and reversed EMT phenotype (C; magnification $\times 20$) in FoxM1 over-expressing AsPC-1 cells. Over-expression of miR-200b was established in FoxM1 over-expressing AsPC-1 cells by transient transfection with its precursor. Real-time RT-PCR and Western blotting analysis were performed to measure the relative levels of mRNAs and proteins, respectively.

analysis also demonstrated that re-expression of miR-200b could diminish the relative protein levels of ZEB1, ZEB2, vimentin, and FoxM1, and increase the relative protein level of E-cadherin in FoxM1-over-expressed AsPC-1 cells (Fig. 3B). Morphological study showed reversal of EMT phenotype by re-expression of miR-200b in FoxM1-over-expressed AsPC-1 cells. However, control miRNA-transfected AsPC-1-FoxM1 cells showed mesenchymal phenotype (Fig. 3C). These results clearly suggest that re-expression of miR-200b could reverse the EMT phenotype consistent with down-regulation of FoxM1 expression and EMT phenotypic biomarkers in FoxM1-over-expressed AsPC-1 cells.

GENISTEIN TREATMENT INHIBITED CELL SURVIVAL AND CLONOGENICITY IN FOXM1 ASPC-1 CELLS

To investigate whether or not genistein, a known natural chemopreventive agent predominantly found in soybeans, could inhibit cell growth, we examined the effect of genistein treatment on cell survival and growth by MTT and clonogenic assays. The results indicate that AsPC-1-FoxM1 cells had 5% more cell survival compared to AsPC-1-control cells after 3 days of incubation and genistein treatment decreased cell survival in both AsPC-1-control and AsPC-1-FoxM1 cells in a dose-dependent manner after 3 days

of treatment (Fig. 4A). AsPC-1-FoxM1 cells show 30% more numbers of colonies, compared to AsPC-1-control cells (Fig. 4B). These results suggest that forced over-expression of FoxM1 leads to increased cell growth and clonogenicity, which could be easily attenuated by genistein treatment (Fig. 4B).

GENISTEIN INHIBITED THE PROTEIN EXPRESSION OF EMT PHENOTYPE MARKERS IN FOXM1-OVER-EXPRESSED ASPC-1 CELLS

To examine the effect of genistein on the protein expression of cyclin D1, NF- κ B, FoxM1, EMT markers, and CSC surface markers in AsPC-1-control and AsPC-1-FoxM1 cells, Western blot analysis was performed. We found increased protein levels of cyclin D1, NF- κ B p65 subunit, FoxM1, ZEB1, ZEB2, Snail2, and vimentin, and CSC surface markers (CD44 and EpCAM) in AsPC-1-FoxM1 cells, and decreased level of E-cadherin protein, compared to those of the control cells (Fig. 4C), which suggest that FoxM1 is involved in the regulation of these proteins. Interestingly, genistein treatment showed decreased expression of cyclin D1, NF- κ B, FoxM1, EMT markers and increased the protein level of E-cadherin in both the cell lines (Fig. 4C), suggesting that genistein could reverse EMT phenotype of AsPC-1-FoxM1 cells.

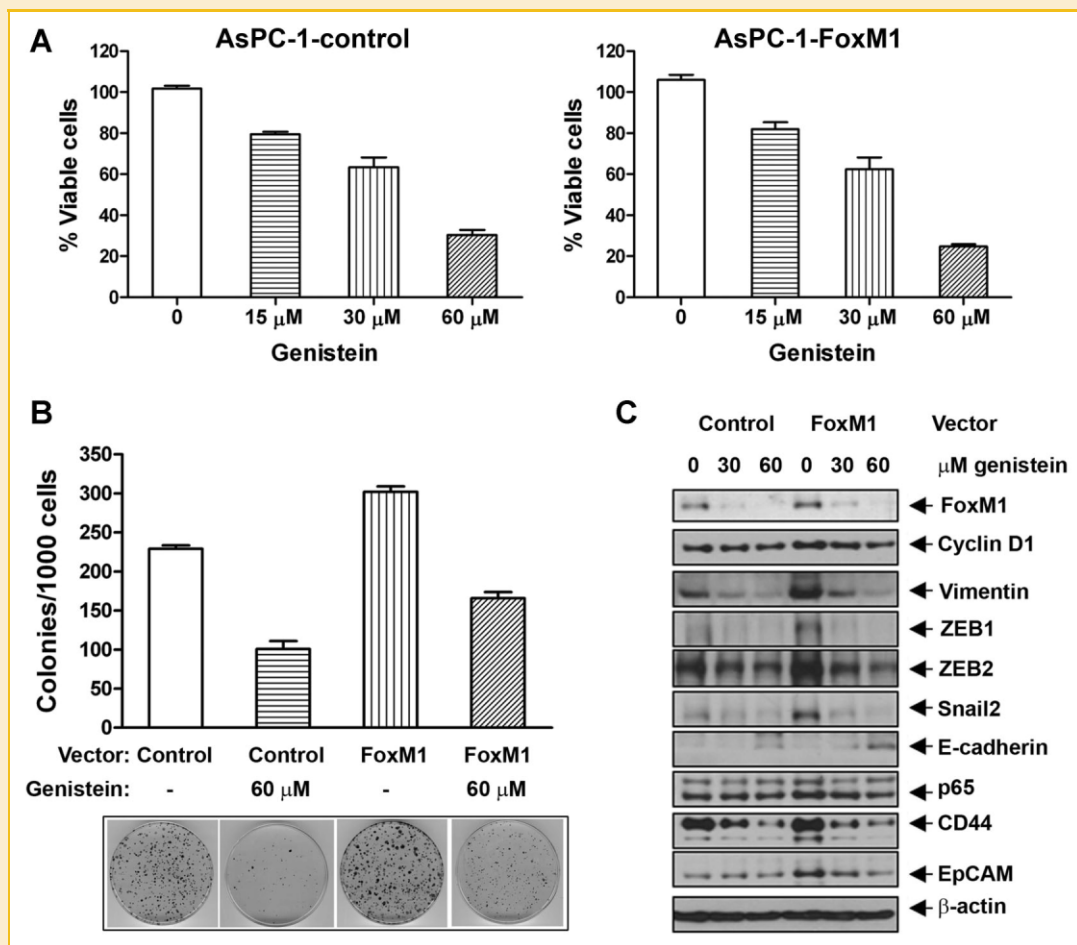


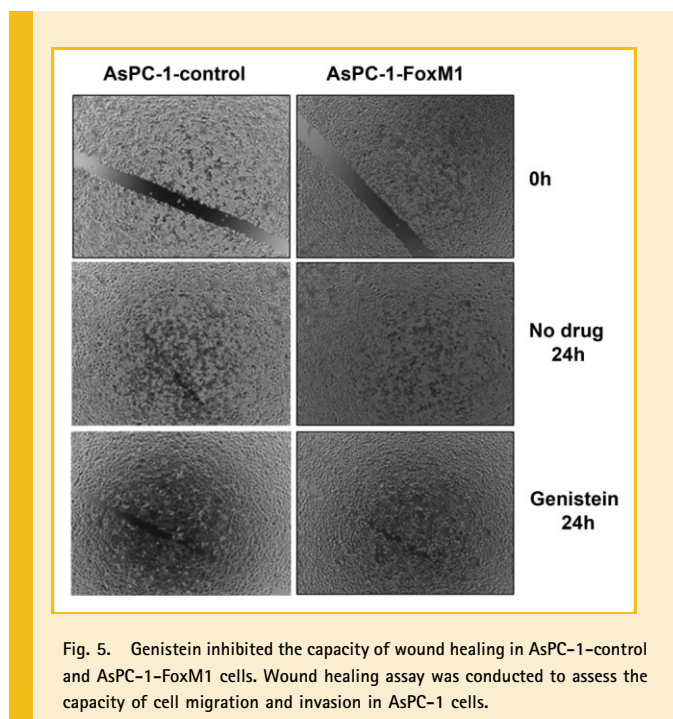
Fig. 4. Genistein treatment inhibited cell survival (A), clonogenicity (B), and the relative levels of expression of proteins (C) in AsPC-1-control and AsPC-1-FoxM1 cells. Different concentrations of genistein were used for both cell lines for 3 days followed by MTT assay, clonogenic assay, and Western blotting analysis, respectively.

GENISTEIN INHIBITED CELL MIGRATION AND INVASION IN FOXM1-OVER-EXPRESSED ASPC-1 CELLS

To examine the effect of genistein on cell migration and invasion in FoxM1-over-expressed AsPC-1 cells, we conducted wound-healing assay. The results show that AsPC-1-FoxM1 cells had more capacity of wound healing, compared to AsPC-1-control cells (Fig. 5), suggesting that FoxM1-over-expression lead to increased cancer cell migration and invasion capacity, which was found to be inhibited by genistein treatment.

GENISTEIN INHIBITED SPHERE-FORMING (PANCREATOSPHERE) CAPACITY BY ALTERING THE EXPRESSION OF CSC CELL SURFACE MARKERS IN FOXM1-OVER-EXPRESSED ASPC-1 CELLS

Sphere-forming assay was performed to assess the CSC or CSC-like cell self-renewal capacity of AsPC-1-FoxM1 cells. We found increased formation (35% more) of pancreatospheres consistent with increased protein levels of CSC surface markers, CD44 and EpCAM, in the pancreatospheres, compared to AsPC-1-control cells (Figs. 6A and 7), which indicate that forced over-expression of FoxM1 led to increased capacity of pancreatospheres and CD44 and EpCAM in the pancreatospheres, further suggesting that FoxM1 is involved in the regulation of CSC phenotype. Interestingly, we found that genistein treatment showed decreased formation of pancreatospheres consistent with decreased expression of CD44 and EpCAM in the pancreatospheres (Figs. 6A and 7). We further investigated whether genistein could inhibit the formation of secondary pancreatospheres of AsPC-1-FoxM1 cells. The results show that genistein could significantly decrease the formation of secondary pancreatospheres in AsPC-1-FoxM1 cells after 1 and 3 weeks of treatment (Fig. 6B and C). These results suggest that genistein can inhibit the capacity of CSC self-renewal in FoxM1-over-expressed AsPC-1 cells.



DISCUSSION

A large body of evidences suggests that EMT plays an important role during embryonic development, and in the formation of fibroblasts during inflammation and wound healing [Desmouliere, 1995; Yang and Weinberg, 2008; Peter, 2009]. Emerging evidences suggest that EMT also plays a key role in carcinogenesis. For examples, many of the EMT-inducing transcription factors such as Snail1, Snail2, ZEB1, ZEB2, Twist1, FoxC2 have been found to be associated with tumor aggressiveness, invasion and metastasis [Yang and Weinberg, 2008], which leads to tumor recurrence. EMT phenotype has been observed in drug-resistant human pancreatic cancer cells as documented in our previous report [Ali et al., 2010], and recently reviewed by our laboratory, suggesting that the acquisition of EMT phenotype is associated with drug resistance and cancer cell metastasis [Wang et al., 2010b].

In the current study we found, for the first time, that forced over-expression of FoxM1 leads to the acquisition of EMT phenotype by up-regulation in the protein expression of mesenchymal cell markers, ZEB1, ZEB2, Snail2, and vimentin, and by down-regulation of epithelial cell marker, E-cadherin in AsPC-1 cells. These results strongly suggest the importance of FoxM1 signaling in tumor cell aggressiveness through the acquisition of EMT phenotype in pancreatic cancer cells. Therefore, targeting FoxM1 signaling by novel approaches would be useful for reversing the EMT phenotype, which would likely results in the reversal of drug resistance and elimination of cancer cells. To that end we document for the first time that forced over-expression of FoxM1 led to decreased expression of critical miRNAs (let-7b, c and miR-200b, c) that are associated with the acquisition of EMT phenotype and that the re-expression of miR-200b lead to the reversal of EMT phenotype, which could also be achieved by treating EMT phenotypic cells with genistein. Our results are consistent with reports documenting the importance of miR-200 family in the regulation of EMT phenotype [Gregory et al., 2008b; Park et al., 2008b; Peter, 2009].

The expression of miR-200 family members has been found to be downregulated in TGF- β -induced EMT phenotype of Madin-Darby canine kidney (MDCK) cells. However, the expression of miR-200 is highly enriched in the epithelial such as NCI60 cells panel. Most importantly, the deregulation of their expression in cancer cells was found to be mechanistically associated with EMT mediated through the regulation of ZEB1 and ZEB2 expression [Gregory et al., 2008b; Korpál et al., 2008; Park et al., 2008b; Peter, 2009]. In our previous study, we showed that over-expression of PDGF-D in prostate cancer cells led to the acquisition of EMT phenotype, consistent with down-regulation in the expression of miR-200 family, and forced re-expression of miR-200b in PDGF-D-over-expressing PC3 cells led to the reversal of EMT phenotype by deregulation in the expression of ZEB1, ZEB2, and Snail2 [Kong et al., 2009; Ali et al., 2010; Kong et al., 2010]. We have also demonstrated that the down-regulation of miR-200 expression in human pancreatic cancer cells is consistent with gemcitabine resistant EMT phenotypic MiaPaCa-2 cells [Ali et al., 2010]. The previously published data are consistent with our results in FoxM1 over-expressing pancreatic cancer cells, further suggesting that re-expression of miR-200b could be a novel therapeutic approach for the reversal of EMT phenotype, which

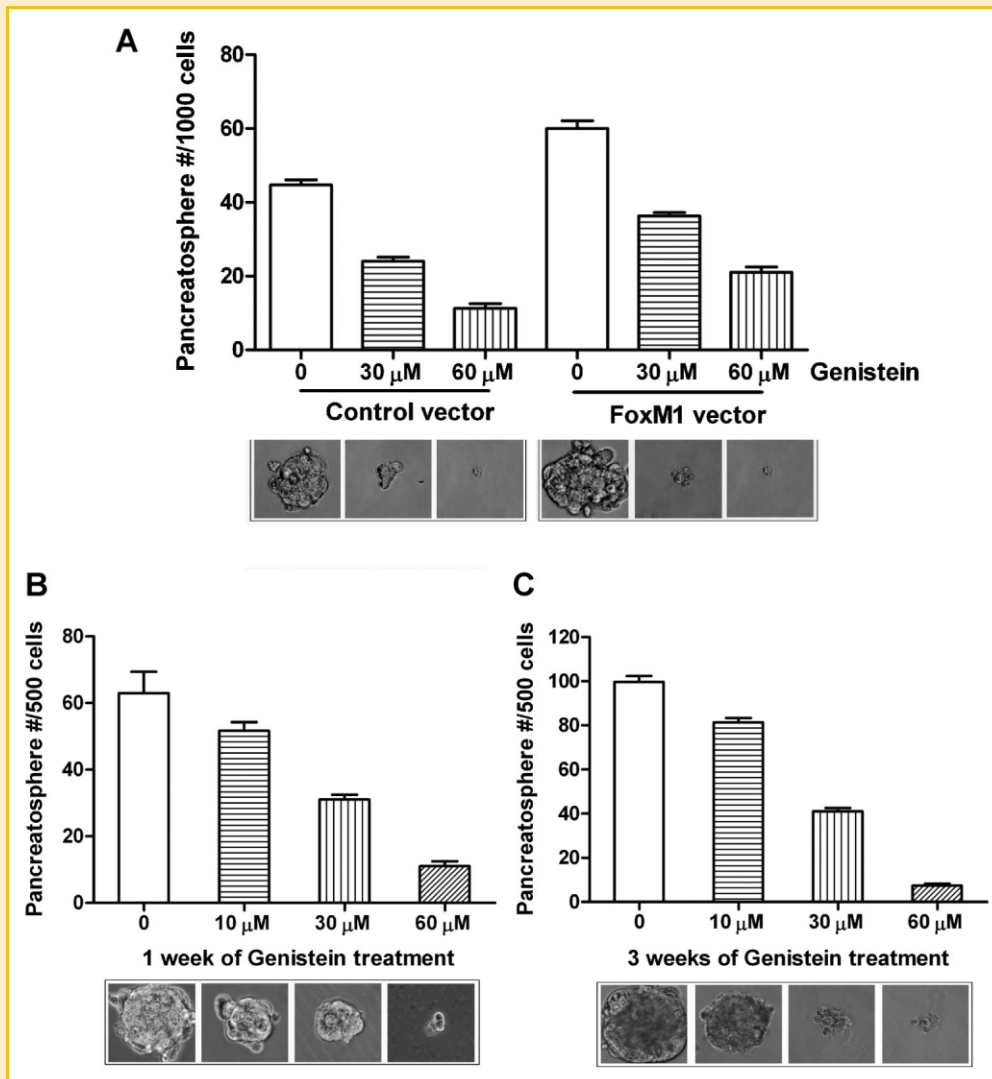


Fig. 6. Genistein inhibited the capacity of CSC-self-renewal in primary pancreatospheres of AsPC-1-control and AsPC-1-FoxM1 cells after 1 week of treatment (A) and secondary pancreatospheres of FoxM1 over-expressing AsPC-1 cells after 1 and 3 weeks of treatment (B, C). Secondary pancreatospheres were generated by desegregations of primary pancreatospheres using accutase solution, as described in the Materials and Method section.

would likely contribute to improve the overall survival of patients diagnosed with pancreatic cancer. Interestingly, we also report, for the first time, that re-expression of miR-200b resulted in decreased expression of FoxM1 in FoxM1 over-expressing AsPC-1 cells, suggesting that miR-200b might participate in the regulation of FoxM1 expression. The miR-200 family including miR-200b has been recently reported to down-regulate the expression of Notch-1 in prostate and pancreatic cancer cells [Kong et al., 2010; Brabletz et al., 2011]. Moreover, Notch-1 signaling plays an important role in tumorigenesis, in part, through activation of FoxM1 signaling [Wang et al., 2008; Wang et al., 2010c; Wang et al., 2011]. Our unpublished data show that re-expression of miR-200b resulted in the down-regulation of Notch-1 in pancreatic cancer cells, which could be responsible for the down-regulation of FoxM1 as observed in our current study.

A number of studies have suggested that the processes of EMT phenotype are also associated with the characteristics of cancer stem

cell (CSC) or CSC-like cells. CSC constitute only a very small proportion of malignant cells in a tumor mass and possess the ability to self-renew giving rise to differentiated tumor cells [Lee et al., 2008; Sarkar et al., 2009; Hermann et al., 2010; Ischenko et al., 2010]. The CSC theory has fundamental clinical implications especially because CSC has been identified in many malignant tumor tissues including pancreatic cancers and CSCs are considered to be highly resistant to chemo-radiation therapy [Lee et al., 2008; Creighton et al., 2010; Hermann et al., 2010; Ischenko et al., 2010]. Thus, CSCs have been believed to play critical roles in drug resistance and cancer metastasis, suggesting that targeting CSC self-renewal capacity would likely eliminate CSCs that are the root cause of tumor recurrence as reviewed by us recently [Wang et al., 2010b]. In our current study we showed, for the first time, that forced over-expression of FoxM1 led to increased self-renewal capacity of AsPC-1 cells consistent with increased expression of CSC cell surface markers such as CD44 and EpCAM, and these processes could be

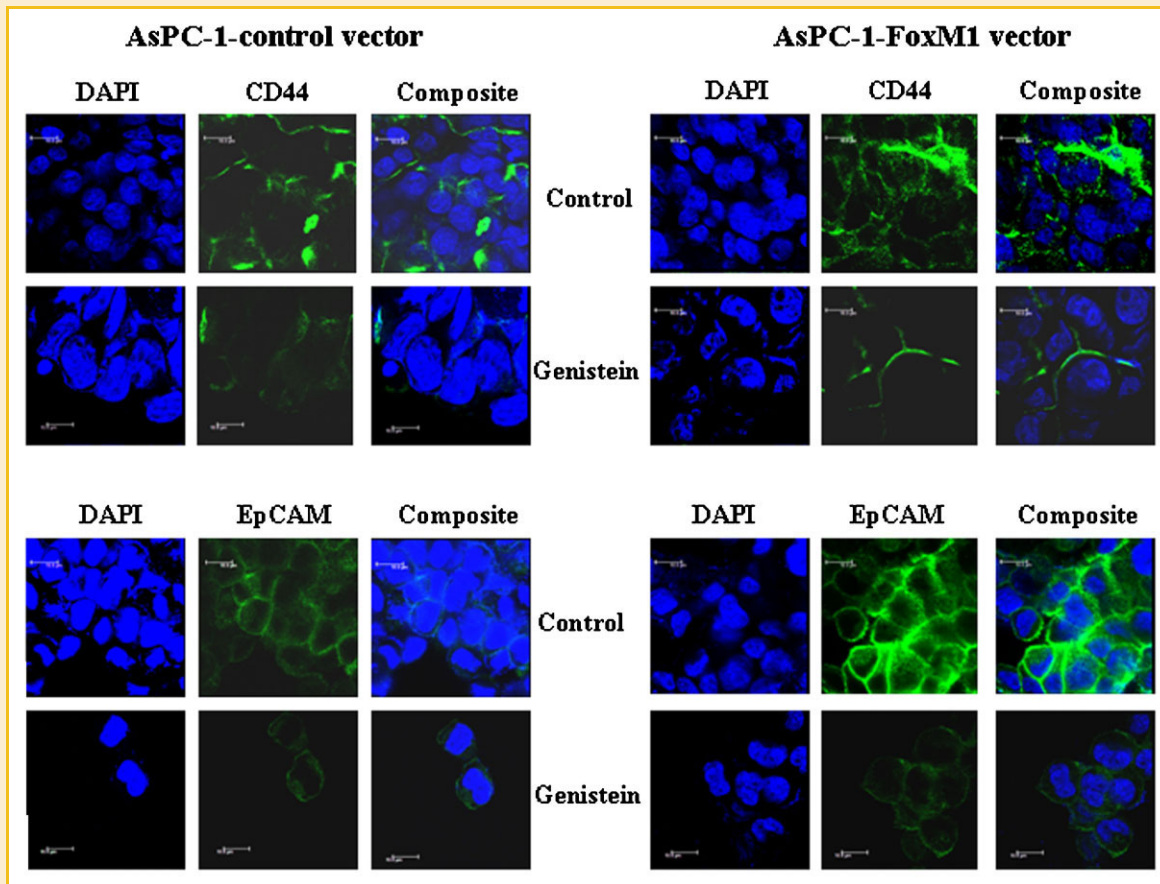


Fig. 7. Genistein inactivated the expression of CSC surface markers such as CD44 and EpCAM in pancreatospheres of AsPC-1-control and AsPC-1-FoxM1 cells. Immunofluorescent staining and confocal microscopy image analysis (Magnification $\times 250$) was conducted using pancreatospheres of AsPC-1-control and AsPC-1-FoxM1 cells after 1 week of $60 \mu\text{M}$ of genistein treatment.

reversed by either re-expression of miR-200b or the treatment of cells with genistein.

A large body of literature suggests that genistein can inhibit the growth of various cancer cells such as breast, prostate, and pancreatic cancer cells both in vitro and in vivo. From gene expression profiles, genistein has been found to differentially regulate genes that are critical for the control of cell proliferation, cell cycle, apoptosis, oncogenesis, transcription regulation, and cell signal transduction pathways in our previous study, which was consistent with apoptosis inducing effects of genistein mediated through inactivation of NF- κ B and Akt signaling pathways [Sarkar et al., 2006; Wang et al., 2011]. In our previous reports, we also showed that genistein could inhibit cell growth and invasion by the down-regulation of FoxM1 expression in prostate and pancreatic cancer cells [Wang et al., 2011], which further suggest that genistein could be a novel anti-tumor agent for the treatment of pancreatic cancer by targeting multiple oncogenic pathways and by targeted elimination of EMT and CSC phenotypic cells. Our results are supported by a recent report showing that genistein could decrease the protein expression of CSC genes such as Sox2, Oct4, and Nanog in human embryonic carcinoma NCCIT cells [Regenbrecht et al., 2008]. From these results, we conclude that forced over-expression of FoxM1 leads to increased cell growth, clonogenicity, acquisition

of EMT, CSC self-renewal capacity, cell migration and invasion, and increased capacity to form pancreatospheres, all of which were consistent with increased expression of EMT and CSC biomarkers, consistent with decreased expression of miR-200 whose re-expression led to the reversal of EMT phenotype, which was also achieved by genistein treatment. Therefore, genistein could be useful for the reversal of EMT and CSC characteristics, and thus genistein could become an important agent for the prevention of tumor recurrence and/or treatment of pancreatic cancer with better treatment outcome in the future by targeting multiple oncogenic pathways including EMT and CSC phenotypes.

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