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HMGA2 and Bach-1 cooperate to promote breast cancer cell malignancy

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Author contribution statement

Behzad Mansoori has provided constructing an idea or hypothesis for research.

Behzad Baradaran and Pascal H.G. Duijf have provided planning methodology to reach the conclusion.

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Behzad Baradaran and Behzad Mansoori have provided organizing and supervising the course of the project or the article and taking the responsibility provided personnel, environmental and financial support, tools, and instruments that are vital for the project have been provided by Behzad Baradaran.

Behzad Mansoori and Ali Mohammadi have provided biological materials, reagents and referred patients.

Taking responsibility in execution of the experiments, patient follow-up, data management and reporting have been provided by Behzad Mansoori, Zahra asadzadeh, Solmaz Shirjang, Mahsa minuee and Freydoon Abedi Gaballu.

Taking responsibility in logical interpretation and presentation of the results have been provided by Behzad Mansoori, Pascal H.G. Duijf, Morten F. Gjerstorff.

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Abstract

During breast cancer progression, tumor cells acquire multiple malignant features. The transcription factors and cell cycle regulators HMGA2 and Bach-1 are overexpressed in several cancers, but the mechanistic understanding of how HMGA2 and Bach-1 promote cancer development has been limited. We found that HMGA2 and Bach-1 are overexpressed in breast cancer tissues and their expression correlates positively in tumors but not in normal tissues. Individual HMGA2 or Bach-1 knockdown downregulates expression of both proteins, suggesting a mutual stabilizing effect between the two proteins. Importantly, combined HMGA2 and Bach-1 knockdown additively decreases cell proliferation, migration, EMT and colony formation, while promoting apoptotic cell death via upregulation of caspase-3 and caspase-9. First the first time, we show that HMGA2 and Bach-1 overexpression in tumors correlate positively and that the proteins cooperatively suppresses a broad range of malignant cellular properties, such as proliferation, migration, clonogenicity and evasion of apoptotic cell death. Thus, our observations suggest that combined targeting of HMGA2 and Bach1 may be an effective therapeutic strategy to treat breast cancer.

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Introduction

Cancer has long been among the severest threats to human health. This condition not only reduces the quality of life, but also increases the mortality rate (Al-Hajj et al., 2003). Breast cancer is a highly heterogeneous disease and the most common malignant tumor type among women. The incidence and mortality of breast cancer have steadily increased in recent years (Hillion et al., 2016). Breast cancer progression involves multiple genetic events, which may activate dominant-acting oncogenes or disrupt the function of specific tumor suppressor genes. Various breast cancer susceptibility genes have been identified and it is well established that germline variants or mutations in oncogenes or tumor suppressor genes are important risk factors for breast cancer development (Bertucci et al., 2005).

High mobility group A2 (HMGA2) is a small non-histone nuclear protein that belongs to the family of HMG proteins. These proteins are architectural transcription factors, which not only bind to AT-rich DNA sequences to alter chromatin structure and then directly affect downstream transcription, but also interact with other transcription factors and indirectly...
regulate downstream transcription (Hammond and Sharpless, 2008; Reeves, 2001). HMGA2 has been reported to function as an oncogene and to be overexpressed in almost all human malignancies, including gastric cancer, oral squamous cell carcinoma, colon cancer, pancreatic cancer, ovarian cancer, and breast cancer (Hristov et al., 2009; Meyer et al., 2007; Miyazawa et al., 2004; Rogalla et al., 1997). HMGA2 influences a variety of biological processes, including DNA repair (Singh et al., 2015), apoptosis (Natarajan et al., 2013), cell proliferation (Li et al., 2015), senescence (Narita et al., 2006), epithelial-to-mesenchymal transition (EMT) (Tan et al., 2014; Wu et al., 2011) and telomere maintenance (Li et al., 2011). The expression of HMGA2 has been linked to epithelial-to-mesenchymal transition (EMT), a process in which epithelial cells adopt a mesenchymal phenotype and increases their ability to migrate and invade (Bai et al., 2015). HMGA2 also regulates apoptosis and proliferation in various cancers (Morishita et al., 2013). For example, it promotes proliferation, migration, invasion, and EMT in epithelial ovarian carcinomas (EOC) and bladder cancer (Shi et al., 2016a; Stigbrand, 2017). HMGA2 also disrupts the apoptosis process through interfering with the expression of pro-apoptotic genes, including those encoding Bax, Bcl-xl and caspase 9 (Shi et al., 2016b).

Bach-1 (BTB and CNC homology 1) is a MAF-related transcription factor, which forms a heterodimer with members of the small Maf family in gene promoter regions (Davudian et al., 2016a). Bach-1 regulates of vital cellular process during cancer development, including hemoglobin cell balance, oxidative stress response, cell cycle progression, transcription suppression, apoptosis, and EMT (Davudian et al., 2016a; Wang et al., 2014; Warnatz et al., 2011). Ectopic expression of Bach-1 was shown to enhance the malignancy of breast cancer cells, while Bach-1 knockdown considerably suppresses breast cancer development (Liang et al., 2012). Our previous study on breast cancer cells showed that the Bach-1 promotes breast cancer cell proliferation, migration and apoptosis (Aletaha et al., 2017). Recently, it was reported that Bach-1 is a master regulator of metastasis by regulation of metastasis-related genes including MMP1, MMP9 and CXCR4 in prostate cancer, colorectal cancer and breast cancer (Shajari et al., 2017). It is also known as a master regulator of breast cancer to bone metastasis (Davudian et al., 2016b).

This study aims to investigate the correlation between HMGA2 and Bach-1 expression in clinical breast cancer samples. In addition, it seeks to study the effects of individual and combined silencing of corresponding target genes.
on the survival, apoptosis, migration, and clonogenicity of breast cancer cells.

Materials and Methods

Ethics statement and patient samples

To analyze the tissue specimens, all patients gave informed consent to use excess pathological specimens for research purposes. The employed protocols in this study were approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1396.982). Twenty-four samples of pathologically diagnosed breast cancer tissue at stage 2 or 3 and marginal normal tissue samples were acquired from patients with breast cancer. Four patients were diagnosed with distant metastases. The breast cancer tissues were confirmed by the Mamotype breast cancer assay kit (NGBI, Tabriz, Iran). The supplied tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until further use. The cancer tissue samples were obtained from the Noureh Nejat Hospital affiliated with Tabriz University of Medical Sciences.

Cell culture and reagents

Human invasive breast cancer cells (MDA-MB-468) and human non-invasive breast cancer cells (MCF-7) were obtained from the Pasteur Institute (Tehran, Iran). The cells were maintained in RPMI-1640 with 10% FBS (GIBCO, Carlsbad, CA, USA) and cultured at 37°C with 5% CO2. The cells were used in the logarithmic growth phase in all experiments.

siRNA transfection

2×10^5 cells were seeded into 6-well cell culture plates in RPMI-1640 medium free of serum and antibiotics. Eighty pmol of the siRNA (Table 1) were transfected into the cells by using jetPRIME reagent according to the manufacturer’s instructions. Briefly, the siRNAs and jetPRIME reagent were diluted in jetPRIME dilution buffer (Poly plus, France) and incubated for 30 min at room temperature. Subsequently, the mixtures, containing the siRNA, were added to each well containing cells in opti-MEM medium. The cell culture plates were then incubated for 5–7 h at 37°C in a CO2 incubator. After that, RPMI-1640 medium with 20% FBS was added into each well with transfected cells.
Western blotting

After transfection of siRNAs into MCF-7 and MDA-MB-468 cell lines, the cells were harvested and washed once with PBS buffer. The total proteins of the cells were extracted with RIPA lysis buffer (Santa Cruz Biotechnology, CA, USA) according to the manufacturer’s instructions. Briefly, the cell pellet was resuspended in lysis buffer, which contained protease inhibitors, phosphatase inhibitors and PMSF. The suspension was mixed and vortexed for 10 sec and then incubated on ice for 10 min. Afterwards, the lysates were centrifuged at 12,000 rpm for 10 min at 4°C. Subsequently, 50 mg/ml of each protein sample was separated on 5% stacking and 10% running gel on SDS-PAGE electrophoresis. Then, proteins were blotted onto PVDF membrane (Roche Diagnostics GmbH, Mannheim, Germany) using a semi-dry immunoblotting system. The membranes were blocked with 0.5% Tween-20 in PBS for 2 h on a shaker at RT and then incubated with monoclonal antibody against HMGA2, Bach-1, caspase 3, caspase 9, MMP-9, CXCR4, VEGFA or beta-actin as a reference protein overnight at 4°C (1:1000, Santa Cruz Biotechnology, California, USA). After washing the membranes, they were incubated with secondary antibodies conjugated with horseradish peroxidase (1:5000, diluted in PBS), rabbit anti-goat secondary antibody for Bach-1 and rabbit anti-mouse antibody for caspase 3, caspase 9, MMP9, CXCR4 and VEGFR, then incubated for 1 h at RT while rocking. The protein bands were visualized using the enhanced chemiluminescence kit (Roche Diagnostics GmbH, Mannheim, Germany) and Western blot imaging system (Sabz.co, Iran). Finally, the intensity of bands was analyzed by ImageJ software (National Institutes of Health, Maryland, USA) and the different protein band intensities normalized to the respective beta-actin bands.

Cell proliferation assay

The MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5 diphenyltetrazolium bromide) assay was employed to probe the effects of HMGA2 and Bach-1 silencing, individually and in combination, on the viability of breast cancer cells. Briefly, 15×10^3 MCF-7 and MDA-MB-468 cells were seeded into each well of 96-well plate. Then, the cells were transfected with optimal concentrations of the specific siRNA, individually and in a combination.
After 48 h, 50 µl of MTT solution (2mg/ml PBS) was added to each well. After incubation for 2-4 hours at 37 °C, the supernatant was replaced with 200 µl of DMSO. After shaking of the plate at 1000 RPM for 1 min, the absorption of each well was read at 570nm using an ELISA microplate Reader (Sunrise RC, Tecan, Switzerland).

**Apoptosis Annexin V/PI assay**

To measure the rate of apoptosis and necrosis of the cells, MCF-7 and MDA-MB-468 cells were seeded into six-well plates at a density of 2×10^5 cells per well. After 48 hours of the treatment, the cells were harvested and stained using the Annexin V/PI apoptosis detection kit (Exbio, Czech Republic) according to the manufacturer's instruction. Then, the rate of apoptosis was analyzed using flow cytometry (MACS Quant 10, Miltenyi Biotech GmbH). The achieved data were analyzed using the FlowJo software package (Treestar, Inc., San Carlos, CA).

**DAPI staining assay**

To assess the effect of gene silencing on chromatin fragmentation, DAPI staining was performed. To this purpose, 15×10^3 cells were seeded into 96-well plates. The cultivated cells were silenced individually or in a combination for 48 h. Then, the cells were fixed with 5% of formaldehyde for 4 h and permeabilized with Triton X-100 (0.1%) for 5 min. Following that, the cells were stained with DAPI (0.1%) for 10 min. Finally, the cells were monitored in the DAPI channel of a fluorescence imaging system (Cytation 5, Biotek, USA).

**Wound healing assay**

To evaluate the effect of HMGA2 and Bach-1 on the cellular mobility of MCF-7 and MDA-MB-468 cells, 5x10^5 cells transfected with specific siRNAs were seeded in 24-well cell culture plates. After the cells were attached to the bottom of the wells, scratches were applied to each well using sterile yellow sampler tips. After that, images were taken of the wells by an inverted light microscope (Optika, XDS-3, Italy) at 0 and 48 h after transfection. Mobility of the cells from the edge of the gap area was determined in siRNAs transfected group compared to the negative controls (NC).
Clonogenicity Assays

Colony formation assays were performed to investigate the effect of individual and combined silencing of HMGA2 and Bach-1 on the ability of the breast cancer cells to form colonies. To this purpose, $5 \times 10^3$ cells were seeded in 6-well plates after transfection and the cells were maintained for 2 weeks. The colonies were fixed, stained with crystal violet, and then photographed. Finally, they were counted manually, and the numbers of the colonies for each condition were plotted.

Quantitative real-time PCR

Total tissue and cellular RNA were isolated using RiboEX reagent (GeneAll, GeneAll biotechnology, Seoul, Korea). cDNA was synthesized from 1 µg of total RNA via thermal cycler system (Bio-Rad, Hercules, CA). qRT-PCR was performed with the LightCycler 96 instrument (Roche Diagnostics, Mannheim, Germany) using 2X SYBR green master mix.

The primer sequences are listed in Table 2. The evaluation of Bach-1, HMGA2, ALDH, SOX2, CD133 and Nanog expression was performed by an initial denaturation step at 94°C for 10 min, followed by 45 cycles at 94°C for 10 sec, 59°C for 35 sec, and finally 72°C for 20 sec. Also, beta actin expression was used as a reference gene.

Bioinformatics

HMGA2 and BACH1 expression levels in normal breast, primary and metastatic breast carcinomas were compared using mRNA expression data from The Cancer Genome Atlas (TCGA) breast cancer RNAseq V2 dataset (Network, 2012). These data were downloaded, normalized, processed and analyzed as previously described (Vaidyanathan et al., 2016a; Vaidyanathan et al., 2016b).

Statistical analysis

All experiments were repeated in triplicate and analyzed via GraphPad Prism software version 7.0 (GraphPad Prism; San Diego, CA, USA). All data were expressed as means ± standard deviation (SD). The correlation assay was analyzed by the Pearson test. Student's t-test and one-way analysis of variance (ANOVA) were used for comparing of the groups with this article is protected by copyright. All rights reserved.
parametric data. A p-value less than 0.05 was considered to indicate a statistically significant difference.

Results

Expression of HMGA2 and Bach-1 is increased and positively correlates in breast cancer samples

qRT-PCR was used to evaluate the expression levels of HMGA2 and Bach-1 in breast cancer tissues. The clinicopathological characters of breast cancer tissues represent in table 3. Among all 24 paired breast cancer tissues and adjacent normal breast tissues, all of the cases showed overexpression of HMGA2 (Figure 1A) and (22/24) of the cases displayed the overexpression of Bach-1 (Figure 1B). The relative expression levels of HMGA2 were 0.6±0.15 and 1.5±0.37 in the adjacent normal tissues and breast cancer tissues, respectively (p-value <0.0001). Furthermore, the relative expression levels of Bach-1 in the adjacent normal tissues and breast cancer tissues were 0.79±0.25 and 1.44±0.39, respectively (p-value <0.0001). To investigate the association between HMGA2 and Bach-1 expression levels in human breast cancers, Pearson correlation analyses were performed. Figure 1D shows that the Pearson correlation coefficient is 0.65, which identified a positive correlation (p value=0.003). RNAseq data from The Cancer Genome Atlas (TCGA) breast carcinoma dataset also showed that there was a positive correlation with Pearson correlation coefficient of 0.23 (p value=2.4×10^{-14}) in primary tumor samples (Figure 1F) and 0.82 (p value=0.04) in metastatic tumor samples (Figure 1G). Thus, HMGA2 and Bach-1 are overexpressed in breast cancer and their expression levels correlate positively.

Combined HMGA2 and Bach-1 knockdown additively suppresses the levels of these proteins in breast cancer cells

We next knocked down HMGA2 and Bach-1 expression in the MCF-7 and MDA-MB-468 breast cancer cell lines. To do so, we transfected HMGA2 and Bach-1 siRNAs separately and in combination. Western blot analysis showed that the silencing of HMGA2 in MCF-7 and MDA-MB-468 cells resulted in relative HMGA2 protein expression levels of 0.65±0.02 and 0.61±0.06, respectively, and 0.38±0.01 and 0.33±0.006 in combined HMGA2 and Bach-1 silencing (Figure 2A-D). Silencing of Bach-1 resulted in relative Bach-1 protein expression levels of 0.59±0.01 and 0.79±0.04, respectively, and in 0.42±0.02 and 0.47±0.01 in dual silencing relative to...
negative control (Figure 2A, B, E and F). Interestingly, the silencing of HMGA2 reduced the expression level of Bach-1 to 0.77±0.01 and 0.71±0.001 and, conversely, Bach-1 suppression decreased the expression level of HMGA2 to 0.79±0.04 and 0.8±0.04 in MCF-7 and MDA-MB-468 cells, respectively. In addition, the dual silencing suppressed the corresponding proteins more than individual silencing.

**HMGA2 and Bach-1 cooperate to promote breast cancer cell proliferation**

We next assessed whether individual and combined knockdown of HMGA2 and Bach-1 expression have an anti-proliferative effect on breast cancer cells. To that end, we performed MTT assays. Individual knockdown of HMGA2 or Bach-1 decrease the proliferation rates of MCF-7 (Figure 3A) and MDA-MB468 (Figure 3B) cells by 20 to 40% (p < 0.001). However, the combined silencing reduced the proliferation rates by more than 40%. These results indicate that the dual knockdown reduced the proliferation of these breast cancer cells more efficiently than individual knockdown (p < 0.05).

**HMGA2 and Bach-1 cooperate to evade apoptosis in breast cancer cells**

To determine if single or combined HMGA2 and Bach-1 knockdown promotes apoptosis, corresponding siRNAs were transfected into MCF-7 and MDA-MB-468 cells. We found that dual HMGA2 and Bach-1 knockdown more readily induced apoptosis than individual knockdown. The apoptosis rates for individual HMGA2 and Bach-1 silencing were 35.3±0.02 and 35.5±0.02 in MCF-7 cells (Figure 4A and B) and 22.19±1.02 and 21.37±1.22 in MDA-MB-468 cells, respectively (Figure 4C and D). Interestingly, the rates of apoptosis following dual knockdown were higher: 37.3±1.42 and 40.4±1.82 in MCF-7 and MDA-MB-468 cells, respectively (Figure 4B and D). DAPI staining confirmed that the chromatin fragmentation following dual knockdown was higher than after individual knockdown in both breast cancer cell lines (Figure 4E, F). We also determined the expression levels of caspase-3 and caspase-9 in both MCF-7 (Figure 4G, I and J) and MDA-MB-468 cells (4H, K, and L). This similarly showed additional increases after dual knockdown. Thus, we observed cooperative increases in apoptosis and in both caspase-3 and caspase-9 expression following combined knockdown of HMGA2 and Bach-1 expression in breast cancer cell lines.
HMGA2 and Bach-1 cooperate to promote breast cancer cell migration and EMT-related protein expression

To study the role of HMGA2 and Bach-1 in cell migration, we performed wound healing assays. Single and dual siRNA-mediated knockdown of HMGA2 and/or Bach-1 block the migration of MCF-7 and MDA-MB-468 breast cancer cells (Figure 5A-D). Individual HMGA2 and Bach-1 gene silencing led to reduced migration rates of MCF-7 cells to $331 \pm 11$ and $446.3 \pm 33.3$ and of MDA-MB-468 cells to $531 \pm 11$, and $613 \pm 25.9$, respectively. Again, double knockdown decreased the migration rates even more, specifically to $269.7 \pm 27$ and $369.7 \pm 26.3$ in MCF-7 and MDA-MB-468 cells compared to $613 \pm 25.94$ and $813 \pm 26$ in the respective control cells.

To assess if HMGA2 and Bach-1 affect the expression of EMT-related proteins, we determined the protein levels of CXCR4, MMP9 and VEGFR by Western blotting. This revealed that the relative expression levels of these proteins were consistently reduced in individual knockdown experiments and were lowest following dual knockdown (Figure 5E-J). Thus, knockdown of HMGA2 and/or Bach-1 reduces breast cancer cell migration and EMT-related protein expression and combined knockdown enhances these effects.

HMGA2 and Bach-1 cooperate to promote breast cancer cell stemness

qRT-PCR was used to evaluate the expression levels of ALDH, Sox2, CD133 and Nanog in breast cancer tissues. The relative expression levels of ALDH were $1.01 \pm 0.23$ and $1.11 \pm 0.15$ in the adjacent normal tissues and breast cancer tissues, respectively (p-value = 0.073) (Figure 6A). Furthermore, the relative expression levels of Sox2 in the adjacent normal tissues and breast cancer tissues were $0.63 \pm 0.11$ and $0.78 \pm 0.24$, respectively (p-value = 0.0093) (Figure 6B). Moreover, the relative expression of CD133 in the adjacent normal tissues and breast cancer tissues were $1.96 \pm 0.26$ and $2.05 \pm 0.24$, respectively (p-value = 0.237) (Figure 6C). In addition, the relative expression of Nanog in the adjacent normal tissues and breast cancer tissues were $0.51 \pm 0.15$ and $0.53 \pm 0.11$, respectively (p-value = 0.237) (Figure 6D). In order to understand if individual and/or combined knockdown of HMGA2 and Bach-1 could decrease breast cancer cell clonogenicity, we performed colony formation assays. With $526.5 \pm 21$ and $651.5 \pm 35.23$ in MCF-7 cells and $226.5 \pm 21$ and $283.5 \pm 15.8$ in MDA-MB-468 cells, respectively, the colony numbers following HMGA2 and Bach-1 single knockdown were significantly reduced compared to control cells (Figure 6E-H). Interestingly, the colony numbers in MCF-7 and MDA-MB-
468 were 351.5±32 and 102.3±13.45 in the dual knockdown groups. In comparison to the respective control cells, whose numbers were 851.5±38 and 351.5±35, respectively, these were considerably reduced. In addition, using qRT-PCR, we found that combined knockdown of HMGA2 and Bach-1 markedly reduced the mRNA expression of the stem cell markers ALDH, Sox2, CD133 and Nanog (Figure 6I-L). In conclusion, HMGA2 and Bach-1 cooperate to reduce breast cancer cell stemness.

Discussion

Breast cancer is the leading cause of cancer-related death in women worldwide (Jemal et al., 2011; Siegel et al., 2015). In recent decades, increasing efforts have been made to elucidate the molecular mechanisms underlying breast cancer development. The overexpression or activation through mutations of some oncogenes, or, conversely, reduced expression or mutational inactivation of tumor suppressor genes promote breast cancer progression. (Mansoori et al., 2016a; Mansoori et al., 2016b). A number of genes that play a role in breast cancer progression have been identified. Accumulating evidence demonstrated that the deregulation of HMGA2 is associated with cancer development. HMGA2 displayed high-level expression in some malignant tumors, such as gastric cancer, oral squamous cell carcinoma, colon cancer, pancreatic cancer and ovarian cancer (Ding et al., 2014; Lee et al., 2015; Shi et al., 2015). However, limited information is available to date regarding to the function of HMGA2 in breast cancer. Additionally, Bach-1, a basic leucine zipper transcription factor, has been shown to transcriptionally regulate the expression of a range of genes that are associated with breast cancer metastasis, and more significantly, it promotes breast cancer cell invasion and metastasis (Shajari et al., 2017). Therefore, Bach-1 may be a significant target for effective therapeutic intervention in tumor metastasis (Nie et al., 2016). The oncogenic properties of HMGA2 and Bach-1 are shown to be involved in aggressive tumor growth, cell differentiation, DNA damage response, participation of the EMT and apoptosis (Shajari et al., 2017; Shi et al., 2016a). Here, we showed that the expressions of HMGA2 and Bach-1 mRNA in breast cancer tissues and cells were significantly higher than the normal breast tissues. Moreover, we found a positive correlation between HMGA2 and Bach-1 expression in primary and metastatic breast cancer tissue, but not in normal breast.

It has been reported that HMGA2 and Bach-1 can regulate apoptosis and proliferation in various cancers (Li et al., 2014; Shajari et al., 2017). Our study supports those observations, as we found that HMGA2 and Bach-1
knockdown increased apoptosis and decreased proliferation of breast cancer cells. More importantly, however, we here show that HMGA2 and Bach-1 directly affect each other’s expression and cooperate to promote malignancy, because combined knockdown more potently promotes apoptosis and restrains cell proliferation compared to knockdown of either one alone.

Our previous studies indicated that HMGA2 silencing induces apoptosis and inhibits cell migration in human colorectal cancer (HCT-116) and breast cancer through caspase 9 (Esmailzadeh et al., 2017). However, Shi et al. identified a novel anti-proliferative role of HMGA2 in the induction of apoptosis through caspase 2 in primary human fibroblast cells (WI38). They showed that WI38 cells express a high level of HMGA2 and exhibit apoptotic nuclear phenotypes (Shi et al., 2015).

We found that downregulation of HMGA2 inhibits the proliferation of breast cancer cells and induces apoptosis in vitro. To investigate whether the reduced cell proliferation was due to the increase of apoptosis, we performed flow cytometry analysis. This showed that HMGA2 knockdown led to a higher rate of apoptosis in the breast cancer cells. Kaur et al. found that loss of HMGA2 led to decreased cell growth, proliferation, and colony formation. However, this was associated with an increase in apoptosis (Kaur et al., 2015). It is thus clear that HMGA2 plays an important role in apoptosis. In addition, downregulation of Bach-1 induces apoptosis. Our results show that Bach-1 has a positive effect on breast cancer cell proliferation and migration, while it inhibits apoptosis. In the present study, we show that the combined downregulation of HMGA2 and Bach-1 using siRNAs additively inhibits proliferation and induces apoptosis in breast cancer cells.

Our previous study showed that in breast cancer cells HMGA2 knockdown alone increases caspase activity, especially of caspases 3 and 9, indicating induction of apoptosis through the intrinsic mitochondrial pathway (Mansoori et al., 2016b). Here, our Western blot analyses showed upregulation of caspase-3 and caspase-9. Therefore, we demonstrate that the additive effect of combined targeting of HMGA2 and Bach-1 similarly causes apoptosis by invoking the intrinsic mitochondrial pathway.

In the past several decades, HMGA2 and Bach-1 have been shown to be associated with EMT (Aletaha et al., 2017; Davudian et al., 2016b; Morishita et al., 2013; Watanabe et al., 2009; Wu et al., 2011). HMGA2 plays a critical role in EMT by activating the TGFβ signaling pathway,
thereby inducing invasion and metastasis of human epithelial cancer cells (Morishita et al., 2013). In addition, HMGA2 overexpression was shown to induce protein changes consistent with EMT and enhanced epithelial cell invasion and migration both in vitro and in vivo via regulation of EMT-related genes, including vimentin, E-cadherin and MMP-9 in gastric cancer, bladder cancer and ovarian cancer (Shi et al., 2016a; Wu et al., 2011). The importance of Bach-1 in the regulation of EMT has been shown in several studies. Our recent studies demonstrated that suppression of Bach-1 using siRNAs markedly inhibits colon and prostate cancer cell migration and reduces the expression of metastasis-related genes, including CXCR4, MMP-9 and HMGA2 in vitro (Davudian et al., 2016b; Shajari et al., 2017). In this work, we also find that the individual knockdown of HMGA2 and Bach-1 reduces EMT and significantly decreases MMP9, CXCR4 and VEGFR protein levels in breast cancer cells. However, our observations that the combined suppression of HMGA2 and Bach-1 cooperatively inhibits EMT and MMP9, CXCR4 and VEGFR protein expression constitutes a major novel observation.

Recent studies demonstrated that HMGA2 plays an oncogenic role in glioblastoma and gastric cancer, promoting cell clonogenicity and tumorigenicity. Targeting HMGA2 decreases stemness, tumorigenicity and self-renewal capabilities (Kaur et al., 2016; Li et al., 2017). Here, we find that individual and combined suppression of HMGA2 and Bach-1 inhibits the clonogenicity and stemness of breast cancer cells. One of our previous studies indicated that knockdown of Bach-1 in prostate cancer cells reduces the expression of genes that promote migration and metastasis and mesenchymal markers of stemness (Davudian et al., 2016b). The results of cancer stem cell-related genes showed Sox2 significantly increase in breast cancer tissues compared to adjacent normal. Moreover, the expression level of ALDH, CD133, and Nanog increased in tumor samples but there are not significant, it’s better to mention the most of tumor tissues involved in this study were in the primary tumor and a few numbers was in advance stage. Our results consistent with Finicelli et al. which they demonstrated Sox2 and Nanog overexpressed in breast cancer tissues (Finicelli et al., 2014). Qiu et al showed ALDH expression in a different type of breast cancer tissue, and they demonstrated significant overexpression was seen in the invasive or metastatic type of breast cancer tissues(Qiu et al., 2014). Xia showed CD133 as cancer stem cell marker overexpressed in breast cancer stem cell and invasive and metastatic breast cancer.(Xia, 2017). Sun et al. demonstrated that HMGA2 induces tumorsphere formation, clonogenicity and
tumorigenicity in vitro and in vivo by increasing the expression of the stem cell markers CD44, ALDH1, Sox2, and Oct4 (Sun et al., 2017). Consistent with this, in the present study, we find that the expressions of the stem cell markers CD133, ALDH, SOX2, and NANOG decrease after knockdown HMGA2 and Bach-1 alone and more significantly in combination. Thus, overexpression of HMGA2 and Bach-1 jointly promotes clonogenicity and stemness in breast cancer cells.

Consistent with our observations, knockdown of Bach-1 was previously reported to significantly reduce breast cancer cell proliferation. However, in this study, we demonstrate that the knockdown of HMGA2 and Bach-1 individually, as well as in a combination reduces the proliferation rate of breast cancer cells. In fact, combined knockdown inhibits proliferation more significantly than individual knockdown.

In conclusion, this study shows that the expression of HMGA2 and Bach-1 is elevated and positively correlates in breast cancer. In addition, HMGA2 and Bach-1 individual knockdown, but even more so combined knockdown suppress cell proliferation, migration, EMT, stemness and clonogenicity and induces apoptosis. Thus, our study provides a rationale for combined targeting of HMGA2 and Bach-1 in a cancer therapeutic setting.

Conflict of Interest

All the authors declare no conflict of interest.

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Figures

Figure 1. HMGA2 and Bach-1 are overexpressed in breast cancer and their expression correlates positively. The expression levels of both HMGA2 (A) and Bach1 (B) are increased in the breast cancer tissues compared to the margin tissues. The results are expressed as mean ± SD; Ω, p < 0.001. Correlation analysis between HMGA2 and Bach-1 in the margin and tumor tissues (C and D). (E-G) Scatter plots comparing HMGA2 expression to Bach-1 expression in normal breast (E), primary breast cancer (F) and metastatic breast cancer (G) in samples from The Cancer Genome Atlas (TCGA). R and p values: Pearson correlations.

Figure 2. Additive knockdown of HMGA2 and Bach-1 expression in breast cancer cell lines. HMGA2 and Bach-1 siRNAs reduce the expression of corresponding proteins, individually and together in breast cancer cells. The combination silencing of HMGA2 and Bach-1 enhanced the knockdown efficiency compared to the individual knockdown. (A, B) Western blots. (C-F) Quantification of normalized protein levels. The data are represented as mean±SD (n = 3); Ω P < 0.001 versus the negative control cells.
Figure 3. Effect of dual HMGA2 and Bach-1 silencing on MCF-7 and MDA-MB-468 cell proliferation. Forty-eight hours after HMGA2 and/or Bach-1 individual or combined knockdown, the proliferation rates of the indicated breast cancer cell lines was determined by MTT assays. The results are expressed as mean±SD (n = 3); *P < 0.05, Ω P < 0.001 versus the control group.

Figure 4. Dual siRNA-mediated knockdown of HMGA2 and Bach-1 in breast cancer cells strongly promotes apoptosis. AnnexinV/PI apoptosis analyzed on MCF-7 (A, B) and MDA-MB-468 breast cancer cells (C, D). Chromatin fragmentation was evaluated using DAPI staining in MCF-7 (E) and MDA-MB-468 cells (F) in which HMGA2 and Bach-1 were knocked down individually or in combination. Relative Caspase-3 and Caspase-9 (4G-L) protein levels were evaluated by immunoblotting and densitometry (ImageJ software), respectively. The results are expressed as mean±SD (n = 3); Ω P < 0.001 versus the control group.
Figure 5. Dual HMGA2 and Bach-1 knockdown additively reduces breast cancer cell migration and EMT-related protein expression. Images and the numbers of cells in the scratched areas at 0 and 48 h after individual and combined knockdown of HMGA2 and/or Bach-1 in MCF-7 (A, B) and MDA-MB-468 cells (C, D). Relative CXCR4, MMP9, and VEGFR protein expression levels after individual and combined knockdown in MCF-7 (E-G) and MDA-MB-468 cells (5H-J). CXCR4, MMP9, and VEGFR protein expression were evaluated by Western blotting. Data are presented as mean±SD (n=3); Ω p<0.001 versus the control groups.
Figure 6. Combined HMGA2 and Bach-1 knockdown additively suppresses breast cancer cell clonogenicity. The expression levels of ALDH (A) and Sox2 (B), CD133 (C) and Nanog (D) represented in the breast cancer tissues compared to the margin tissues. MCF-7 and MDA-MB-468 cells in which HMGA2 and Bach-1 were knocked down individually or in combination were allowed to form colonies in 6-well plates. The colony numbers for MCF-7 cells (E, G) and MDA-MB-468 cells (F and H) were quantified. Relative ALDH, SOX2, CD133 and Nanog mRNA expression levels after individual and combined knockdown in MCF-7 and MDA-MB-468 breast cell lines is shown (I-L). mRNA expression was evaluated by qRT-PCR. Data are presented as mean±SD (n=3); * p<0.05, θ p<0.001, Ω p<0.001 versus control.
Table 1. the HMGA2 and Bach-1 siRNA sequences.

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*Table 3.* Association between BACH1 and HMGA2 mRNA expressions with clinicopathological features of breast cancer patients
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