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Report

HMG-I/Y in human breast cancer cell lines

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Summary

The *HMG-I/Y* gene encodes the HMG-I and -Y architectural, chromatin binding proteins originally identified based on their association with chromosomal DNA. HMG-I/Y proteins bind to AT-rich regions in chromosomal DNA and alter gene expression. Increased HMG-I/Y protein expression also correlates with neoplastic transformation. Previous work from our laboratory has shown that *HMG-I/Y* is a direct c-Myc target gene involved in neoplastic transformation in Burkitt's lymphoma. We also observed that HMG-I/Y proteins have several oncogenic properties. In this report, we show that HMG-I/Y proteins are increased in several human breast cancer cell lines compared to a human breast cell line derived from normal breast cells. Decreasing HMG-I/Y proteins using an antisense ribozyme approach inhibits transformation in human breast cancer cells, suggesting that HMG-I/Y is important for the transformed phenotype observed in these cells. In addition, increased expression of the HMG-I isoform in normal human breast cells leads to transformation. These results suggest that *HMG-I/Y* is an oncogene important in the pathogenesis of human breast cancer. Although additional studies with animal models are needed, the antisense experiments, which result in blocking transformation suggest that this approach may have therapeutic potential in patients with breast cancer characterized by increased *HMG-I/Y* expression.

Introduction

The *HMG-I/Y* gene encodes the HMG-I and -Y architectural, chromatin binding proteins originally identified based on their association with chromosomal DNA [1–8]. These proteins differ by eleven internal amino acids that are present in the HMG-I isoform and result from alternatively spliced mRNA [1–8]. A difference in the biologic activities of these isoforms has not been established. Both HMG-I and -Y proteins contain AT hook DNA binding domains that mediate specific binding to AT-rich regions in the minor groove of chromosomal DNA [9–12]. HMG-I/Y proteins have been shown to function in transcriptional regulation [9–32]. Specifically, HMG-I/Y relieves histone H1-mediated repression of transcription [18–20]. Moreover, HMG-I/Y is essential for viral induction of the *interferon-β* gene [13–17]. Other genes have been implicated as potential gene targets of HMG-I/Y, including *E-selectin* [21–22], *nitric oxide synthase* [23], *tumor necrosis factor beta* [24], *interleukin 2 receptor alpha* [25–26], *interleukin 4* [27], *human gp91-phox* [28–29], *epsilon-immunoglobulin G* [30], *T-cell receptor alpha* [31], *c-fos* [32], and the smooth muscle specific gene *SM22* [32]. How these transcriptional activities contribute to the biologic function of HMG-I/Y proteins is not yet clear.

HMG-I/Y proteins are also overexpressed in several mammalian malignancies [7], including thyroid [33–39], cervical [40], prostate [41–43], lymphoid [44], and mammary [45–47]. We have previously demonstrated that *HMG-I/Y* is a c-Myc target gene involved in Myc-mediated transformation [44, 48–49]. We also showed that overpression of HMG-I or -Y leads to neoplastic transformation in several experimental cell lines, including Rat 1a fibroblasts and CB33 human lymphoid cells [44, 49]. Moreover, decreasing HMG-I/Y in Burkitt's lymphoma cells blocks transformation [44], suggesting that HMG-I/Y is critical to the transformed phenotype observed in these cells. Other investigators have also observed that decreasing HMG-I/Y proteins interferes with cell growth

and transformation [47, 50–51], again suggesting that HMG-I/Y proteins are involved in malignant transformation.

HMG-I/Y protein expression is correlated with progressive transformation of murine mammary cells [45]. Increased *HMG-I/Y* expression was also identified in primary breast cancers compared to normal breast cells using serial analysis of gene expression [46]. Moreover, *HMG-I/Y* gene expression is stimulated by epidermal growth factor (EGF) or estrogen in the aggressive, metastatic Hs578T breast cancer cell line, but not the less aggressive MCF7 human breast cancer cells [52]. These findings suggest that *HMG-I/Y* may contribute to the pathogenesis of the malignant phenotype observed in these human breast cancer cells [45–47, 52].

In this report, we show that HMG-I/Y proteins are increased in several human breast cancer cell lines, compared to a cell line derived from normal human breast cells. We also demonstrate that decreasing HMG-I/Y proteins in the Hs578T breast cancer cells interferes with transformation in the soft agar assay. In addition, we show that increased expression of the HMG-I isoform in normal human breast cells leads to a transformed phenotype in tissue culture. Our findings indicate that increased expression of the HMG-I protein is oncogenic in normal breast cells and HMG-I/Y proteins are critical to the malignant phenotype observed in the human Hs578T breast cancer cells. Although additional work with animal models is needed, the antisense experiments, which result in blocking transformation suggest that this approach could have therapeutic implications.

Materials and methods

Cell culture and transfection

Hs578T, MCF7, and MDA-MB-231 cells were maintained in Dulbecco's minimal essential media supplemented with 10% fetal bovine serum as previously described [52–54]. Hs578Bst cells were grown in Hybri-Care (ATCC) supplemented with epidermal growth factor (30 ng/ml) and 10% fetal bovine serum as described before [53].

To determine if HMG-I/Y is required for transformation in breast cancer cells, HMG-I/Y proteins were decreased in the Hs578T cell lines using an antisense ribozyme approach [55]. The Hs578T breast cells were transfected with each antisense construct in separate transfection experiments, using Lipofectin according to manufacturer's instructions (GIBCO/BRL). Single cell clones were isolated from each transfection with cloning cylinders according to the manufacturer's instruction (Scienceware Cloning Cylinders).

Plasmids

The HMG-I/Y antisense construct (CT-HMG-I/Y AS) [44] directed at the carboxyl-terminus of the HMG-I/Y protein was made using the vector pU1/RIBOZYME [55], which incorporates an autocatalytic hammerhead ribozyme structure within the complementary sequence. The regions of complementarity are predicted to align the autocatalytic ribozyme structure with the consensus sequence (GUC) for ribozyme cleavage within the targeted *HMG-I/Y* message. The *HMG-I/Y* oligonucleotide sequence 5′ and 3′ to the ribozyme structure has been described [44]. A second HMG-I/Y ribozyme antisense construct with antisense sequence directed at the amino-terminus (NT-HMG-I/Y AS) was also made using the vector pU1/RIBOZYME [55]. The *HMG-I/Y* sequence 5′ to the ribozyme structure was: TGCTCCTCCTCCGAG; the *HMG-I/Y* sense sequence 3′ to the ribozyme structure was: TCCTGCGAGATGCCC. The parent vector pU1/RIBOZYME was used as a control vector for both antisense vectors.

The adeno-associated virus vector pTRUF5 [56] was used to overexpress HMG-I in the Hs578Bst cells. The HMG-I cDNA was cleaved from pBS-HMG-I [9] using *Hinc* II and *Not* I. This was shuttled into the pTRUF5 after restriction with *Xba*, *Klenow* treatment, and further restriction with *Not* I, thus replacing the green fluorescent protein (GFP) sequence with HMG-I, and creating pTR-HMG-I. The pTRUF5 vector expressing GFP was used as a control [56].

Western analysis

For western blot analysis of HMG-I/Y, total cell lysates collected from plates of exponentially growing cells were boiled in 2 × Laemmli buffer and analyzed by SDS/4–20% gradient polyacrylamide gel electrophoresis (PAGE) and subjected to western analysis [57–58] using a chicken polyclonal antibody raised against the amino-terminus of HMG-I/Y diluted 1 : 200–1 : 500 [44, 49]. For analysis of HMG-C, a rabbit polyclonal antibody raised against the amino-terminus of HMG-C was diluted 1 : 500 [44, 49]. The actin monoclonal antibody AC15 (Sigma Immunochemicals) was diluted 1 : 2,500–1 : 5,000 and used to

control for sample loading. Reactive proteins were detected by enhanced chemiluminescence (ECL, Amersham). Autoradiograms from the gels were scanned and all bands were quantified using the N I H Image program. β-actin was used as a loading control.

Cellular growth rate determinations

The growth rates of the Hs578T cells were determined as previously described [44, 49], with the following exceptions. Cells were seeded at 5×10^4 into six separate 5 cm tissue culture dishes. Duplicate dishes were harvested every 24 h for 3 days and the cells were counted.

Soft agar assays

The soft agar assay was performed as previously described [44, 49] except that 1.5×10^5 Hs 578T cells were suspended in 3 ml of 0.4% agarose and poured onto a 5 ml 0.7% agarose bed in 100 mm tissue culture dishes. Colonies greater than 0.2 mm were counted after 3–4 weeks.

Hs578Bst transformation assay

After infection of 5×10^4 Hs578Bst cells with pTR-HMG-I (2×10^{10} pfu) or pTRUF5 control (2×10^{10} pfu) in 5 cm dishes, cells were observed for foci formation. Transformed foci were counted 2 weeks after infection.

Results

HMG-I/Y proteins are increased in human breast cancer cells

HMG-I/Y proteins are increased in several human cancers [7], including thyroid [33–39], cervical [40], prostate [41–43], and lymphoid [44]. To determine if HMG-I/Y proteins are also increased in human breast cancer compared to normal breast tissue, we performed western analysis of HMG-I/Y protein expression in breast cancer cell lines as well as a breast cell line derived from normal breast tissue. We observed that HMG-I/Y proteins are increased in the Hs578T (12-fold), MCF-7 (3-fold), and MDA-MB-231 (19-fold) breast cancer cells, compared to the Hs578Bst cells from normal breast tissue (Figure 1(A)). The Hs578Bst cells and Hs578T cells are derived from a patient with an infiltrating ductal carcinoma of epithelial origin; Hs578T cells are derived from the ductal carcinoma; the Hs578Bst cells are from normal myoepithelial tissue distant to the ductal carcinoma [53]. The Hs578T cells grow aggressively in soft agar, exhibit invasive characteristics in matrigel assays, and metastasize after injection in nude mice [59]. The MDA-MB-231 breast cancer cells are derived from metastatic mammary adenocarcinoma and are invasive in matrigel assays [54] and tumorigenic in nude mice. In contrast, the MCF-7 cells were derived from the pleural fluid of a metastatic mammary adenocarcinoma (ATCC HTB-22), although they retain many characteristics of normal mammary epithelial cells [60]. In addition, these cells grow to a limited extent in soft agar [47, 60], are noninvasive in matrigel assays, and nontumorigenic in nude mice [47, 60–61]. Interestingly, HMG-I/Y proteins are highest in the metastatic Hs578T and MDA-MB-231 cell lines compared to the less aggressive MCF-7 cells, indicating a possible correlation with HMG-I/Y protein expression and the more aggressive phenotype in these breast cancer cell lines. Of note, other investigators have recently confirmed the increased expression of HMG-I/Y in the Hs578T cells compared to Hs578Bst cells [47].

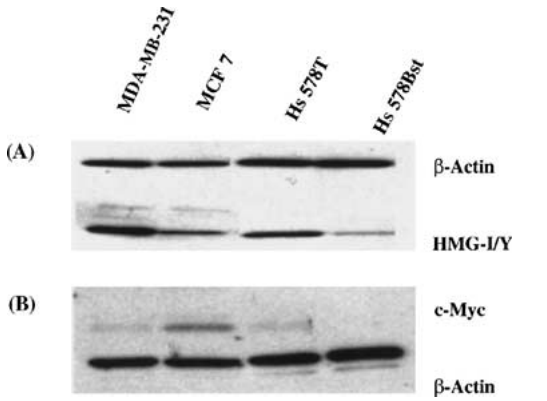

Figure 1. HMG-I/Y proteins are increased in human breast cancer cells. (A) HMG-I/Y proteins are increased in three human breast cancer cell lines [MDA-MB-231 (12-fold), MCF-7 (3-fold), and Hs578T (19-fold) and compared to a human breast cell line derived from normal breast cells (Hs578Bst). Note that HMG-I/Y is highest in the metastatic cell lines, MDA-MB-231 and Hs578T. (B) c-Myc is also increased in the breast cancer cell lines, with highest levels in the MCF-7 cells.

Because we have previously shown that *HMG-I/Y* is a c-Myc target gene, we also evaluated c-Myc protein expression in these cell lines to determine if HMG-I/Y proteins were increased as a result of increased c-Myc oncoprotein. We observed that the c-Myc protein is increased in all of the breast cancer cell lines (Figure 1(B)). It is, therefore, possible

that c-Myc is contributing, at least in part, to the increased HMG-I/Y expression. Of note, the c-Myc levels do not correlate precisely with HMG-I/Y protein expression. For example, c-Myc protein is highest in the MCF7 cells. In contrast, HMG-I/Y proteins are highest in the Hs578T and MDA-MB-231 cells. The higher levels of HMG-I/Y in the Hs578T and MDA-MB-231 cells may be related to another protein(s) that enhances HMG-I/Y expression independent of c-Myc. The lower levels of HMG-I/Y in the MCF-7 cells may be secondary to a relative decrease in this other factor(s). This is consistent with our previous promoter studies showing that *HMG-I/Y* is regulated by other factors in addition to c-Myc [44]. These additional factors may be increased in the more aggressive breast cancer cell lines.

Decreasing HMG-I/Y proteins inhibits transformation in breast cancer cells

To determine if HMG-I/Y is required for transformation in breast cancer cells, HMG-I/Y proteins were decreased in the aggressive Hs578T cell lines using an antisense ribozyme approach [55]. Five single cell clones were isolated from the Hs578Tcells transfected with the carboxyl-terminal antisense vector, CT-HMG-I/Y AS, and of these five single cell clones, two were found to have significantly reduced HMG-I/Y proteins. Seven single cell clones were isolated from the cells transfected with the amino-terminal antisense vector, NT-HMG-I/Y AS. Of these seven single cell clones, one had markedly reduced HMG-I/Y proteins. In the cells with decreased HMG-I/Y protein, the related protein, HMG-C, was unaffected (Figure 2(A)), indicating that the antisense vectors caused a specific, significant decrease in HMG-I/Y proteins. Thus, from the 12 antisense single cell clones, three or 25% showed decreased HMG-I/Y proteins.

Two clones with decreased HMG-I/Y were subsequently analyzed in the soft agar assay to determine if transformation was affected by decreasing HMG-I/Y proteins in these cells (Figure 2(A) and data not shown). Note that HMG-I/Y proteins were decreased by 75% in the NT-HMG-I/Y AS clone and 83% in the CT-HMG-I/Y AS clone compared to control cells transfected with the vector without antisense sequence. We observed that transformation was significantly inhibited in both clonal cell lines with decreased HMG-I/Y proteins compared to a single cell line transfected with control ribozyme vector (Figure 2(B–C)). These results suggest that HMG-I/Y is important for transformation in these cells. Cellular growth rates were determined in the antisense and control cell lines and found to be similar (Figure 2(D)), indicating that transformation was not blocked simply by decreasing cell growth. These findings suggest that colony formation was inhibited through a transformation-specific mechanism independent of decreased growth rate.

Several single cell control cell lines transfected with the ribozyme vector without antisense sequence were also analyzed further for HMG-I/Y protein expression, transforming activity in soft agar, and cellular growth rates. Of 21 single cell control clones from Hs578T breast cancer cells transfected with control ribozyme vector alone, only one (5%) had decreased HMG-I/Y protein expression, denoted clone #5 (Figure 2(E)). Western analysis of clones #1–5 are shown in Figure 2(E); the remaining clones without decreased HMG-I/Y proteins are not shown. The basis for the decrease in HMG-I/Y protein expression in the clone #5 is not clear. This cell line may have had a spontaneous decrease in HMG-I protein independent of the transfection. Alternatively, the control ribozyme vector may have integrated such that *HMG-I/Y* expression was adversely affected.

Single cell control isolates were analyzed further in soft agar, including the clone #5 with decreased HMG-I/Y proteins. The control clone #1, used in the preceding experiments with the antisense clones was included and arbitrarily assigned a value of 100% transformation capability. We observed that all control cell lines without decreased HMG-I/Y had similar transforming activity in soft agar (Figure 2(F)). Of note, clone #5, which had decreased HMG-I/Y proteins, also exhibited decreased colony formation in the soft agar assay. These results further support the previous finding that levels of HMG-I/Y proteins correlate with the transformation phenotype in these breast cancer cells. Cellular growth rates were similar in all the control cell lines (Figure 2(G)), suggesting that the decreased transforming activity observed in clone #5 with decreased HMG-I/Y proteins did not result from decreased proliferative capacity in these cells.

Increased expression of HMG-I in Hs578Bst normal breast cells leads to a transformed phenotype

Because our antisense experiments showed that HMG-I/Y proteins are important for the transformed phenotype in breast cancer cells, we next determined if overexpression of HMG-I in normal breast cells results in transformation using an adeno-associated

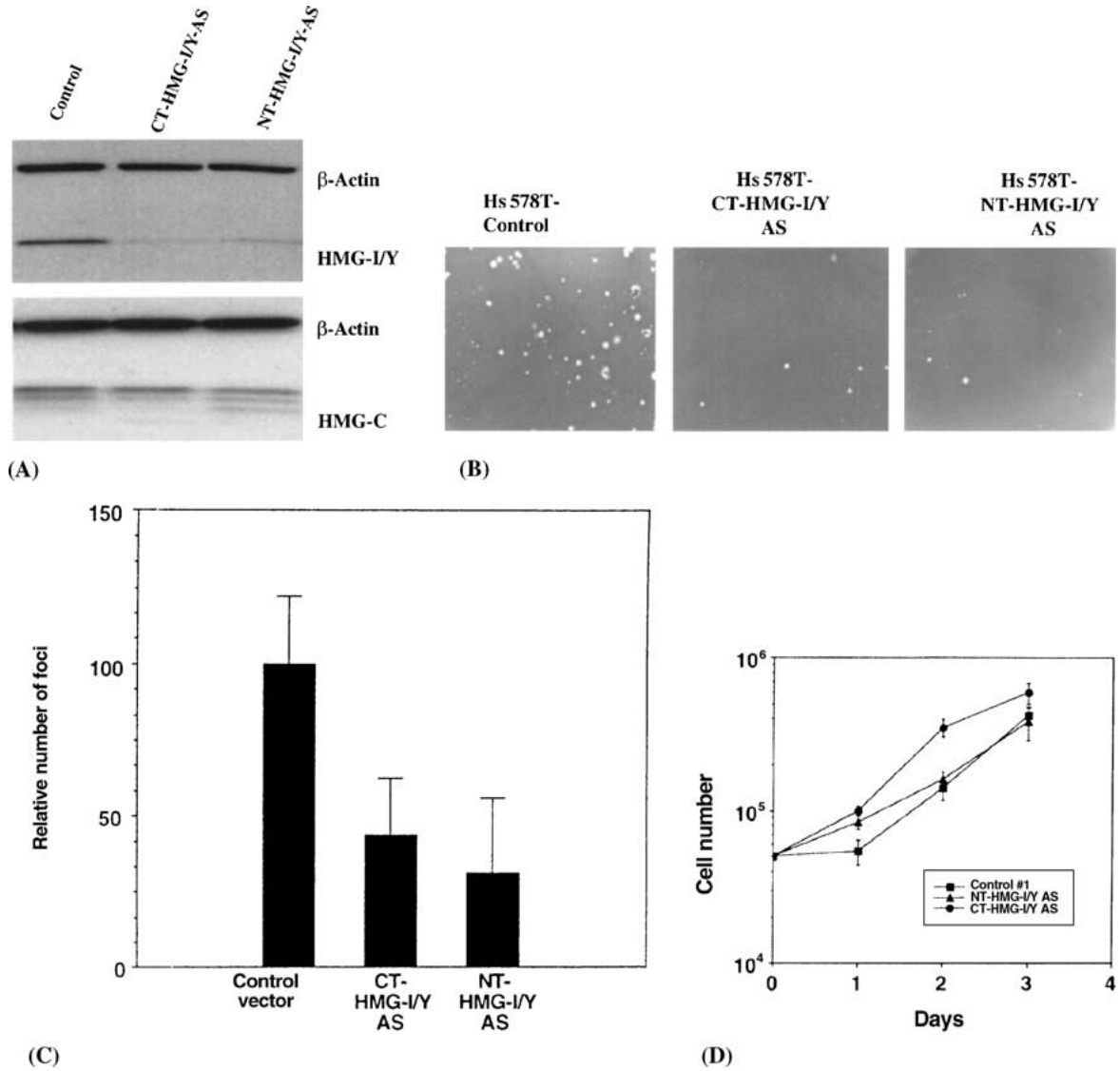

Figure 2. Transformation was decreased in the antisense cell lines with decreased HMG-I/Y proteins. (A) Western analysis showing decreased HMG-I/Y proteins in two monoclonal antisense cells lines as follows: Control Hs578T monoclonal cells transfected with vector alone, Hs578T monoclonal cells transfected with the carboxyl-terminal antisense construct and designated CT-HMG-I/Y AS, and Hs578T monoclonal cells transfected with the amino-terminal antisense construct and designated NT-HMG-I/Y AS. The blot was probed with the HMG-I/Y antibody as well as an antibody to β-actin to control for protein loading. Note the decreased in HMG-I/Y proteins in the cells transfected with the antisense constructs. Western analysis of HMG-C protein in the control and antisense cell lines was also performed. Note that HMG-C is not decreased in the HMG-I antisense cell lines. (B) The antisense Hs578T breast cancer cells have decreased transformation in soft agar as shown. Note that anchorage-independent cell growth was inhibited similarly in cells transfected with the carboxyl- or amino-terminal antisense constructs. (C) The number of colonies in the antisense cell lines was decreased compared to the control breast cancer cell line transfected with vector control. This experiment was repeated 2–4 times and results are expressed as the average (bar) +/− the standard deviation. (D) The cell growth rates of the control and antisense cell lines were similar, suggesting that transformation was not inhibited simply by decreasing cell growth rates in these cells. These findings suggest that HMG-I/Y protein expression is correlated with transformation in the Hs578T breast cancer cells. (E) Western analysis showing HMG-I/Y proteins in additional, monoclonal, control cell lines transfected with vector alone. Note that HMG-I/Y protein expression is similar in all control cell lines, excluding clone #5, in which it was decreased. Western analysis of HMG-C protein in the monoclonal control cell lines transfected with control vector show that HMG-C is similar in the control cell lines. (F) The number of colonies in the control cell lines were similar, except for clone #5, which had decreased HMG-I protein and decreased transformation in soft agar. This experiment was repeated 2–4 times and results are expressed as the average (bar) +/− the standard deviation. (G) The cell growth rates of the control cell lines were similar. These findings further suggest that HMG-I/Y protein is required for transforming activity in these cells.

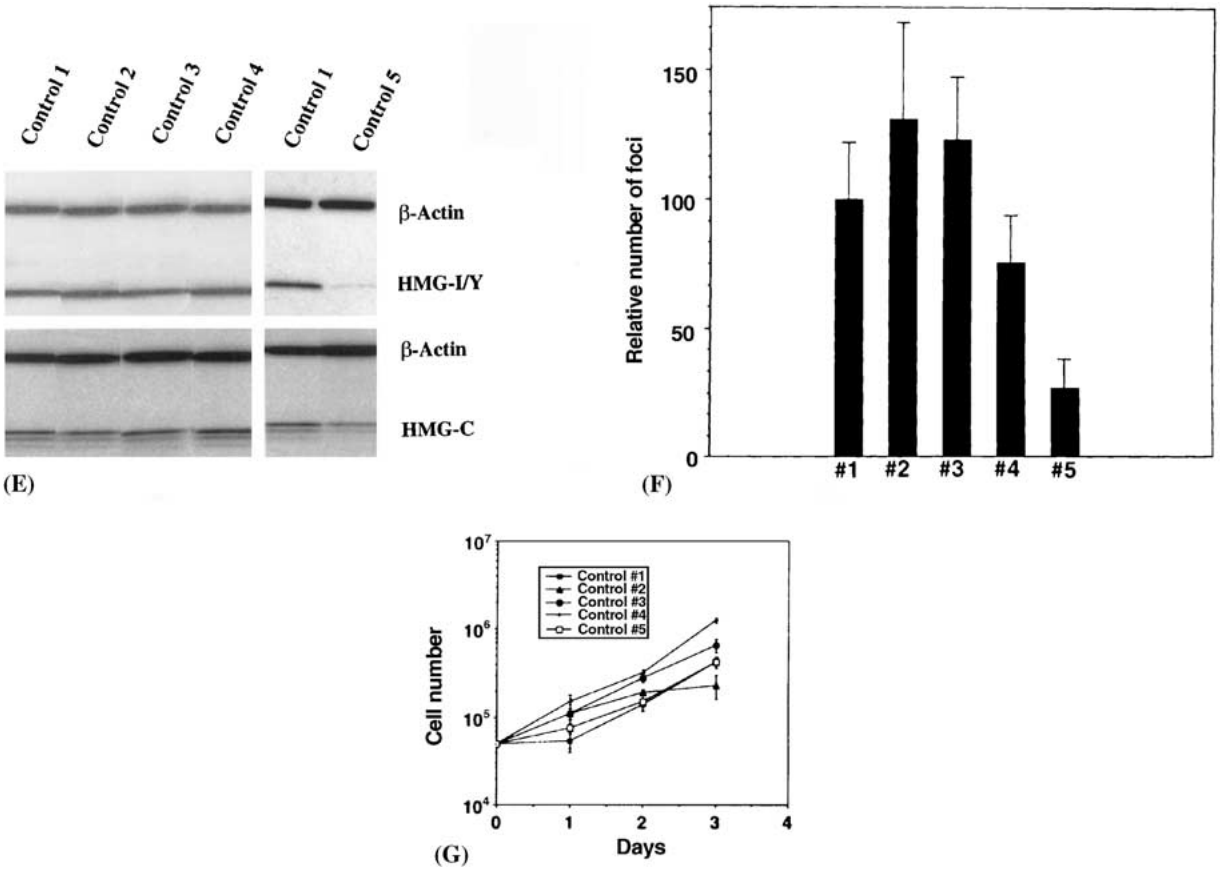

Figure 2. (*continued*)

viral (AAV) vector-mediated gene transfer approach [56]. We observed that Hs578Bst cells overexpressing HMG-I protein formed transformed foci after 2 weeks. Transformed foci were not observed in cells infected with the control pTRUF5 vector expressing GFP (Figure 3(A)). Western analysis shows that the HMG-I protein is overexpressed in the cells transduced with the pTR-HMG-I vector by 3-fold (Figure 3(B)). Thus, our results show that increased expression of HMG-I leads to transformation in normal breast cells.

Discussion

Breast cancer is one of the most common malignancies affecting women today, striking almost one in every eight women [62]. A number of genetic abnormalities have been associated with the disease, including gene amplification and overexpression [62], both of which can result in abnormal increases in proteins that are important for cell growth. Increases in proteins that regulate cell growth may lead to the development of breast and other cancers by causing the cells to grow in an uncontrolled fashion. Determining the genes and proteins that give rise to breast cancer will provide invaluable insight into the causes for this disease and should lead to new treatment strategies.

The *HMG-I/Y* gene encodes the HMG-I and -Y architectural, chromatin binding proteins [1–8]. These proteins have been shown to function in transcriptional regulation [9–32]. Moreover, increased expression of HMG-I/Y proteins has been observed in a number of human cancers [7, 33–47]. In addition, increased expression of HMG-I/Y proteins correlates with transformation in murine mammary cells [45]. To determine if HMG-I/Y proteins may play a role in the pathogenesis of human breast cancer, we evaluated the expression of HMG-I/Y proteins in several human breast cancer cell lines compared to a human breast cell line derived from normal breast cells. In this report, we show that HMG-I/Y proteins are increased in the three breast cancer cell lines studied,

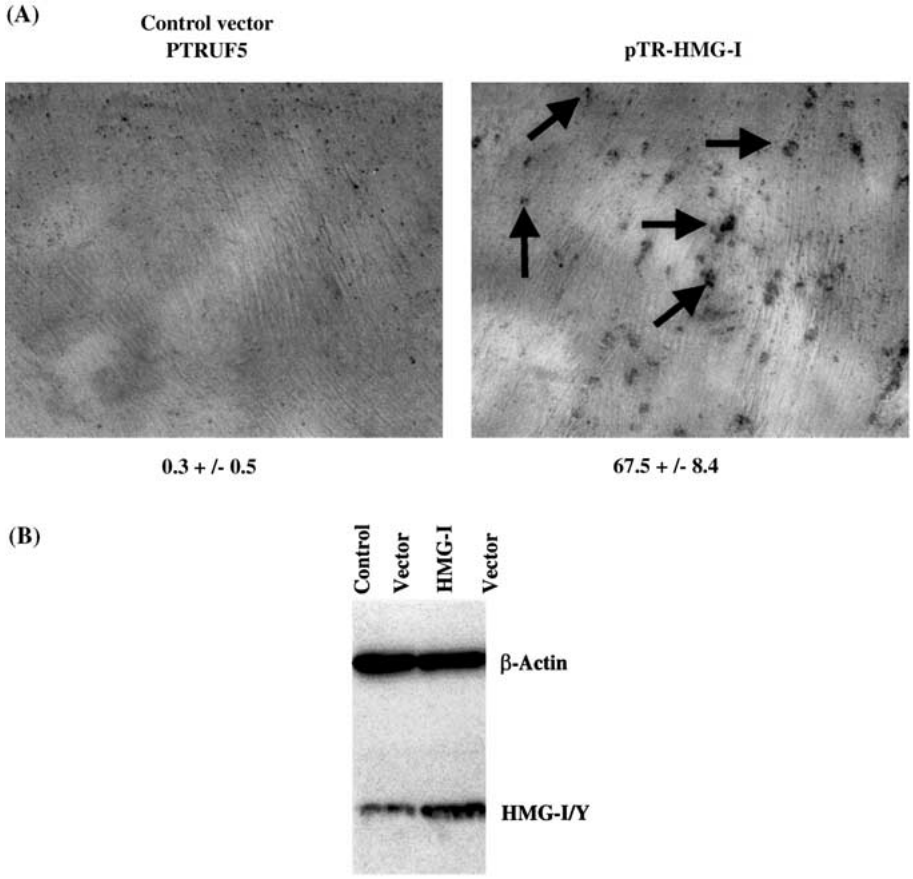

Figure 3. Overexpression of HMG-I in normal human Hs578Bst cells leads to transformation. (A) The transformed foci in the Hs578Bst cell line transduced with 2×10^{10} pfu pTR-HMG-I compared to 2×10^{10} pfu pTRUF5 control vector. Note that only cells transduced with the pTR-HMG-I vector form foci. The number of transformed foci formed in the cells overexpressing HMG-I or control GFP vector are indicated. The transduction experiments were performed in triplicate and repeated twice; results are expressed as the mean (bar) +/− the standard deviation from the different experiments. (B) Western analysis shows that the Hs578Bst cells transduced with pTR-HMG-I vector overexpress the HMG-I protein by 3-fold relative to the Hs578Bst cells transduced with the control pTRUF5 vector alone. All lanes were blotted with the HMG-I antibody as well as the β-actin antibody to control for sample loading.

including the less aggressive MCF-7 cells, as well as the metastatic Hs578T and MDA-MB-231 cell lines. Interestingly, HMG-I/Y proteins are highest in the aggressive, metastatic cell lines, indicating that HMG-I/Y expression may correlate with a more aggressive phenotype. Future studies are needed to determine if HMG-I/Y proteins are increased in additional breast cancer cell lines as well as tissue specimens, and whether there is a correlation between the phenotype of the cancer and HMG-I/Y expression.

To determine if HMG-I/Y proteins are required for the transformed phenotype observed in these breast cancer cells, we specifically decreased HMG-I/Y protein using an antisense approach [44, 55]. We observed that cells with decreased HMG-I/Y proteins have decreased colony formation in the soft agar assay. Other investigators have also observed that transforming capability correlates with HMG-I/Y protein expression [47, 51]. Interestingly, we did not observe a decrease in cell growth rates in the breast cancer cells with decreased HMG-I/Y proteins, suggesting that transformation in soft agar was inhibited through a transformation-specific mechanism, and not simply by decreasing cell proliferation. In contrast, when we used this approach in Burkitt's lymphoma cells, we observed a more complete inhibition of transformation in soft agar as well as a decrease in cell growth rates in the Burkitt's cells [44]. Of note, there appeared to be a greater decrease in the HMG-I/Y proteins on western analysis using the antisense ribozyme approach in the Burkitt's cells [44]. Other investigators have also observed a decrease in cellular growth rates in

cells with decreased HMG-I/Y proteins using antisense strategies [47, 50–51]. The decreased cellular growth rates observed in other cells could reflect a toxic effect of the antisense vector or a consequence of decreased HMG-I/Y proteins. It is possible that greater decreases in HMG-I/Y proteins will lead to decreased cellular proliferation in all cells. Alternately, proliferative capacities in some cell lines may be more sensitive to decreases in HMG-I/Y proteins. Regardless of the mechanisms involved, decreasing HMG-I/Y proteins leads to decreased transformation in the soft agar assay. These findings suggest that this approach could be used therapeutically for patients with breast cancer and other malignancies associated with increased HMG-I/Y proteins. Further study is needed to determine if antisense HMG-I/Y strategies decrease malignant cell growth in animal models of malignancy associated with increased HMG-I/Y expression.

We have previously shown that HMG-I/Y proteins have several oncogenic properties [44, 49]. Specifically, we observed that increased expression of HMG-I/Y proteins in rat fibroblasts and CB33 human lymphoid cells leads to a transformed phenotype in soft agar [44, 49]. Moreover, fibroblasts with increased expression of HMG-I/Y proteins are tumorigenic in nude mice [44, 49]. Here, we show that increased expression of HMG-I protein in Hs578Bst breast cells derived from normal breast tissue likewise become transformed in tissue culture. These studies demonstrate that HMG-I/Y proteins are oncogenic in a variety of cell and tissue types.

How the HMG-I/Y proteins contribute to neoplastic transformation is not yet clear. A variety of mechanisms are possible. For example, HMG-I/Y proteins could function by binding specifically to DNA upstream of genes involved in regulating cell growth. After binding to DNA, the HMG-I/Y proteins could recruit additional transcription factors and bend DNA, forming a structure similar to the enhanceosome observed for the *interferon-β* promoter [13–17]. Alternatively, the HMG-I/Y proteins could function more generally by binding to chromatin at several sites and altering chromatin structure, thereby increasing transcription less specifically [19–20]. Finally, as reported for the related HMG-C protein, HMG-I/Y proteins may form a chimeric protein that alters transcription of genes critical to cell growth and neoplastic transformation [63–76].

In summary, we have shown that HMG-I/Y proteins are increased in several human breast cancer cell lines. We have also observed that decreasing HMG-I/Y proteins interferes with transformation in breast cancer cells. In addition, overexpression of HMG-I in breast cells leads to a transformed phenotype. Our findings suggest that *HMG-I/Y* is an oncogene important in the pathogenesis of human breast cancer. Moreover, although further work in animal models is warranted, the antisense approach may have therapeutic implications. Additional studies are also needed to ascertain the mechanisms involved in transformation by HMG-I/Y, as well as the cellular processes that are affected by decreasing HMG-I/Y proteins.

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