HMGA2 Is a Driver of Tumor Metastasis

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Abstract

The non-histone chromatin-binding protein HMGA2 is expressed predominantly in the mesenchyme before its differentiation, but it is also expressed in tumors of epithelial origin. Ectopic expression of HMGA2 in epithelial cells induces epithelial–mesenchymal transition (EMT), which has been implicated in the acquisition of metastatic characters in tumor cells. However, little is known about in vivo modulation of HMGA2 and its effector functions in tumor metastasis. Here, we report that HMGA2 loss of function in a mouse model of cancer reduces tumor multiplicity. HMGA2-positive cells were identified at the invasive front of human and mouse tumors. In addition, in a mouse allograft model, HMGA2 overexpression converted nonmetastatic 4T07 breast cancer cells to metastatic tumors that homed specifically to liver. Interestingly, expression of HMGA2 enhanced TGFβ signaling by activating expression of the TGFβ type II receptor, which also localized to the invasive front of tumors. Together our results argued that HMGA2 plays a critical role in EMT by activating the TGFβ signaling pathway, thereby inducing invasion and metastasis of human epithelial cancers. Cancer Res; 73(14); 1–11. ©2013 AACR.

Introduction

HMGA2 is an architectural transcription factor predominantly expressed in the mesenchyme before its differentiation and a regulator of mesenchymal proliferation and differentiation (1, 2). Because of its endogenous expression pattern, HMGA2 is not detected in normal adult tissues (3) but is misexpressed and disrupted in several mesenchymal benign tumors, most predominantly lipomas (1). In mice, the Hmga2 null reveals a pygmy phenotype due to the decreased number of mesenchymal cells (3).

Despite its exquisite and specific expression pattern in the mesenchyme during normal development and its causal role in benign mesenchymal tumorigenesis, there have also been a number of intriguing observations with regard to HMGA2 in malignant epithelial tumors over the past decades. Interestingly, high HMGA2 expression is correlated to late stage of Dukes’ classification in patients with colorectal cancer (4) and associated with the high histologic grade of invasive ductal type of breast cancer (5). Misexpression of HMGA2 occurred in parallel with reduced survival rates of patients with breast cancer (6), colorectal cancer (CRC; ref. 7), lung cancer (8), and tumor recurrence in oral carcinoma (9).

The epithelial–mesenchymal transition (EMT) occurs during critical phases of embryonic development and tumor progression. Epithelial cells lose their characteristics and exhibit a molecular profile indicative of mesenchymal cells as well as a profound change in morphology. These include being loosely embedded in an extracellular matrix and an increased motile and invasive behavior. During this transition, mesenchymal cells acquire morphology appropriate for migration in an extracellular environment and settlement in areas that are involved in organ formation. This important concept is also required during tumor progression, and several signaling pathways have been uncovered that are common to EMT in both development and tumor progression (10). At the invasive front of the tumor, neoplastic cells are found that differ morphologically from other cells in the bulk of the tumor. The invasive front is defined as the one-cell-thick layer of the primary tumor juxtaposing, and small clusters of cancer cells that have advanced into the host stroma. These tumor cells are incohesive, lack polarity, and are dedifferentiated, acquiring a mesenchymal phenotype by the process of EMT (10). The dedifferentiation of epithelial cells to fibroblastoid, migratory, and more malignant cells also reveal a profoundly altered mesenchymal gene expression program (11). It is these mesenchymal-like capabilities of the cells that are said to be necessary for metastasis (10).

The TGFβ signaling pathway is a major inducer of the EMT during tumor development. In humans, the TGFβ superfamily represents a diverse set of growth factors (12). The TGFβ cytokines signal by bringing together 2 pairs of receptor serine/threonine kinases, the type I and type II receptors. On binding these ligands, active type II receptor contacts and phosphorylates the type I receptor (13), which leads to the activation of the canonical Smad pathway. Smad2 and Smad3,
which are known as the receptor-associated Smads (R-Smad), are then phosphorylated by the type I receptor and released to propagate the signal with Smad4 (12).

In the present study, to analyze the complexity of the process of metastasis, we have identified the architectural transcription factor, HMG2, as being essential for tumor progression, EMT, and metastasis in vitro and in vivo. Furthermore, we show for the first time that the molecular mechanism of HMG2 in tumor pathogenesis is mediated through the activation of the TGFβ signaling pathway in epithelial carcinomas.

Materials and Methods

Cell culture and invasion assay

The SW480, SW620, SW403, HCT116 and HT29 human colon cancer, MCF7, MDA-MB231, human breast cancer cell lines were purchased from American Type Culture Collection (ATCC) and propagated and maintained according to protocols supplied by ATCC. The 4T07, 4T1 mouse breast cancer cell lines were provided as a kind gift from Fred Miller (Wayne State University, Detroit, MI) and were grown in Dulbecco’s Modified Eagle Media (DMEM; Gibco) containing 10% FBS supplemented with 4 mmol/L L-glutamine, penicillin/streptomycin (Gibco). The transgene construct was made by cloning the human HMG2 cDNA into pcDNA3.1(+) plasmid (Invitrogen). Transfection of the pcDNA-HMG2 or the vector alone (mock) into the SW480 and MCF7 cells was conducted with the Lipofectamine 2000 (Invitrogen) method. The pCMV6-HMG2-GFP plasmid and pCMV6-AC-GFP (shuttle vector) plasmid were purchased from OriGene and transfected into 4T07 cells with electroporation. Twenty-four hours posttransduction, these cells were passaged into fresh particles in the presence of polybrene (4 μg/mL) and integrated into a SMARTvector 2.0 lentiviral shRNA (Thermo Scientific Dharmacon). 4T1 mouse breast cancer cells were transduced with the lentiviral Hmg2-shRNA particles and nontargeting control-shRNA (NTC-shRNA) particles in the presence of polybreine (4 μg/mL). Eighteen hours posttransduction, these cells were passaged into fresh growth medium containing puromycin (2 μg/mL) and maintained for a week so as to select stable transduced cells.

Immunocytochemistry

For immunocytochemistry, 10^5 cells were seeded onto CC2-coated chamber slides (Nalge Nunc) the day before staining. The primary antibodies for E-cadherin, β-catenin, vimentin (Sigma), and Smad3 (Cell Signaling Technology), ZEB1 (Cell Signaling Technology), and fibronectin (Abcam) were used at a dilution of 1:300, and appropriate rhodamine-tagged secondary antibodies were used at 1:500 dilution. The fluorescence was observed under an appropriately equipped Nikon microscope.

Immunohistochemistry and Western blotting

Paraffin-embedded murine and human samples were sectioned at 4 μm and stained with polyclonal anti-HMG2 antibody (3) and monoclonal anti-β-catenin (1:100 dilution, BD Biosciences), anti E-cadherin (1:50, Dako Cytomation), anti-Ki67 (1:200, Dako Cytomation), anti-TGFβ type II receptor (1:400, Abcam), and anti-insulin-like growth factor 2–binding protein (IGF2BP2; 1:400, Santa Cruz Biotechnology) antibodies using an immunoperoxidase technique (Vectastain ABC Elite kit, Vector Laboratories or Histo-mark SP kit, Zymed Laboratories). At least 3 sections from each tumor were stained for each antibody study conducted. Tissues from Hmga2-null mice were used as negative controls for the anti-Hmga2 antibody. Western blotting was conducted as described (9). Ten micrograms of total protein was run per lane and antibodies were used as described above.

Gene expression analysis

Total RNA was isolated from tissues and cells using RNAeasy Mini kit (Qiagen) according to the manufacturer’s instructions. All mRNA expression analyses were conducted with real-time quantitative reverse transcription (RT) PCR using a TaqMan Gene Expression Assay (Applied Biosystems) in an ABI Prism 7300HT Sequence Detection System (PE Biosystems). The RT-PCR reactions were carried out using TaqMan Reverse Transcriptase Reagents (Applied Biosystems). The relative expression data were calculated by the comparative Ct method as described elsewhere (15).

Mice

All mice were housed and handled according to the Institutional Animal Care and Use Committee guidelines. Hmga2-specific knockout mice have been described (3). Seventh-generation C57BL/6j-backcrossed Hmga2^−/− female mice were mated with C57BL/6j-Wnt1 male mice (The Jackson Laboratory). F1 Wnt1 transgenic, Hmga2^−/− male mice were then mated with F1 Wnt transgenic, Hmga2^−/− female littermates to obtain the F2 transgenic mice in the Hmga2^+/−, Hmga2^−/−, and Hmga2^−/− genetic backgrounds. PCR-based genotyping for the Wnt (16) and Hmga2 (2) loci have been described. For the mouse tumors from the Wnt mice, we examined 44 samples (3 sections each) for all antibody stains described, and for the in vivo metastasis study, we examined 10 mice with 3 sections for each tissue-type study.

Human tissue samples

De-identified human tissue samples were obtained from the Surgical Pathology archives of Columbia Presbyterian Hospital (New York, NY) from patients with breast and colorectal neoplasms who were staged by the Dukes’ classification (17). The study was conducted in compliance with HIPAA criteria. In the case of the colon cancer samples, the number of tumor examined is documented in Table 1 following the protocol as described in the Supplementary Materials and Methods using images as represented in Supplementary Fig. S1. For the human breast cancer studies, there were 100 samples with at least 3 sections from each tumor stained for HMG2 with a minimum of 19 samples (3 sections each) stained for TGFβRII and IGF2BP2.
HMGA2 and Metastasis

Table 1. Immunohistochemical expression indices for HMGA2 and Ki67 in different types of human colorectal tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>N</th>
<th>Entire neoplasm</th>
<th>Dysplasia or invasive front</th>
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<tr>
<td></td>
<td></td>
<td>HMGA2</td>
<td>Ki67</td>
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<td>Non-neoplastic</td>
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<td>8.5 ± 3.6</td>
<td>13.8 ± 5.1</td>
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<td>Adenoma</td>
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<td>3.6 ± 2.2</td>
<td>51.1 ± 7.4(^d)</td>
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<td>Carcinoma in situ(^a)</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Dukes’ A(^b)</td>
<td>9</td>
<td>28.7 ± 21.5(^e)</td>
<td>52.3 ± 16.8(^d)</td>
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<tr>
<td>Dukes’ B(^b)</td>
<td>12</td>
<td>32.8 ± 26.7(^d)</td>
<td>55.5 ± 16.5(^d)</td>
</tr>
<tr>
<td>Dukes’ C/D(^c)</td>
<td>9</td>
<td>65.7 ± 17.3(^d,a,h)</td>
<td>42.9 ± 18.0(^d)</td>
</tr>
<tr>
<td>Metastases(^c)</td>
<td>13</td>
<td>66.5 ± 20.8(^d,a,h)</td>
<td>37.4 ± 15.1(^d)</td>
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<td>30.8 ± 12.4(^e)</td>
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<td>Margin of tumor</td>
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<td>72.7 ± 26.3(^f)</td>
<td>54.0 ± 24.7</td>
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<td>P&lt;0.01 versus non-neoplastic</td>
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<td>78.3 ± 14.1(^f)</td>
<td>48.5 ± 20.8</td>
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<td>P&lt;0.01 versus adenoma</td>
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<td>69.3 ± 17.9</td>
<td>45.3 ± 13.5</td>
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<td>P&lt;0.01 versus entire neoplasm</td>
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<td>69.4 ± 18.4</td>
<td>44.6 ± 13.6</td>
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<td>P&lt;0.001 versus Dukes’ A</td>
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<tr>
<td>P&lt;0.01 versus Dukes’ B</td>
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NOTE: Indices for nuclear HMGA2 and Ki67 (i.e., proliferation) accumulation for human colorectal neoplasms are expressed as percentage of positive nuclei per total nuclei of colonic epithelial cells ± SD. \(n\), number of patient tumors examined. All \(P\) values are from the Student \(t\) test.

\(^a\)Dysplasia.
\(^b\)Invasive front.
\(^c\)Margin of tumor.
\(^d\)P<0.01 versus non-neoplastic.
\(^e\)P<0.01 versus adenoma.
\(^f\)P<0.01 versus entire neoplasm.
\(^g\)P<0.001 versus Dukes’ A.
\(^h\)P<0.01 versus Dukes’ B.

Tumor implantation

Twelve-week-old female BALB/cj mice (Taconic Farms) were implanted subcutaneously into the right fourth mammary gland with 2 \(\times\) 10\(^3\) of 4TO7, 4TO7-HMGA2-GFP, 4T1, and 4T1-Hmga2-shRNA mouse breast cancer cells (or a mixture of each 1 \(\times\) 10\(^5\) of 4TO7 cells and 4TO7-HMGA2-GFP cells) in 100 \(\mu\)L PBS using 28-gauge needle. Tumor size was measured 3 times a week by calipers, and tumor volumes were calculated as volume (mm\(^3\)) = \(L \times W^2 \times 0.5\). Mice were sacrificed when the tumor size exceeded 20 mm in diameter in either direction or at the end of the observation period (25 days).

Statistical analyses

All statistical analyses were conducted with PRISM v. 5.0d (Avenida De La Jolla). If statistically significant, comparisons between individual groups were conducted by the Student \(t\) test. The analysis of tumor-free percentage was conducted by log-rank test. \(P<0.05\) was considered significant.

Results

HMGA2 directly induces EMT and invasiveness in epithelial tumor cells

Our initial experiments to understand the function of HMGA2 in epithelial tumorigenesis examined the expression of HMGA2 in a number of human cancer cell lines. The selected cell lines were confirmed to exhibit anchorage-independent growth characteristics as previously defined (18), and interestingly, while the expression levels of HMGA1\(a\) and HMGA1\(b\) remained unchanged, the level of HMGA2 expression was found to be directly proportional to the in vitro anchorage-independent growth characteristics of the colon cancer cell lines (Fig. 1A, Supplementary Fig. S2; ref. 18). In addition, it was found that HMGA2 expression was inversely related to expression of E-cadherin (Fig. 1A). Although HMGA2 is identified in the SW480 line by the sensitive technique of quantitative RT-PCR (qRT-PCR), it is below the levels required for conversion to a mesenchymal and invasive phenotype (Fig. 1A and B).

To directly test whether HMGA2 was sufficient for the EMT in epithelial tumor cells, stable clones derived from the SW480 CRCs and MCF7 breast cancer epithelial cell lines were established that ectopically expressed HMGA2 under the control of the CMV promoter. Whereas mock-transfected SW480 (SW480-mock) cells maintained a regularly aligned pattern consistent with an epithelial morphology 3 days postconfluence, SW480 HMGA2-transfected (SW480-HMGA2) cells revealed a pronounced elongated, spindle-shaped mesenchymal phenotype (Fig. 1B). To confirm the morphologic appearance of the cells, molecular markers of the mesenchymal phenotype were examined using immunocytochemistry and Western blotting in the HMGA2- and mock-transfected cells. Consistent with the mesenchymal phenotype, the SW480-HMGA2 cells exhibited an enhanced expression of the mesenchymal marker vimentin (19), and a marked reduction in the expression of the epithelial marker E-cadherin (20), as compared with the mock-transfected controls (Fig. 1C and D). Similar results were obtained for cells of the MCF7 breast...
cancer cell line with the MCF7-HMGA2 cells (Fig. 1C and D) exhibiting enhanced relocalization of β-catenin expression predominantly in the nucleus (Fig. 1C), a characteristic of the EMT (10). Additional studies were conducted with other classic EMT-associated genes including ZEB1 and fibronectin with similar results (Supplementary Fig. S3).

Finally, the HMGA2-transfected SW480 and MCF7 clones exhibited invasion in the Matrigel assay (Fig. 1E) and closely matched the behavior of the control breast cancer cell line MDA-MB231, which is highly invasive. These results suggest that ectopic HMGA2 expression is sufficient as a “driver” for multiple cancer cell types to undergo the EMT, which leads to an increase in the invasive behavior of these cells.

**HMGA2 is expressed in tumor cells located at the invasive front of the primary tumor and in secondary metastatic lesions**

The direct relevance of the mechanistic studies to human tumors was investigated. In colorectal tumors, expression of HMGA2 was relatively absent in non-neoplastic (8.5 ± 3.6) and early adenomas (3.6 ± 2.2; Table 1, Fig. 2). In contrast, the carcinomas of increasing severity (Dukes’ A through D) showed a progressively increased proportion of HMGA2-positive neoplastic cells (Table 1, Fig. 2) in the entire neoplasm. However, the number of HMGA2-positive cells at the invasive front remained constant showing a preferential distribution of HMGA2-positive cells at the invasive front. Indeed, there was no statistical difference between the number of cells expressing HMGA2 in the entire neoplasm and invasive front in the most severe grade of tumors (Dukes C/D), as these tumors are mostly of a dedifferentiated phenotype (17, 21) and also contain well-differentiated areas (Supplementary Fig. S1; ref. 22). In addition, HMGA2 staining can clearly be seen in the metastatic lesion with increased β-catenin staining and loss of E-cadherin (Fig. 2).

Also, the proliferation index (PI) for colon epithelial cells significantly increased in adenomas as compared with non-neoplastic tissue, whereas at the same time, the fraction of HMGA2-positive cells decreased (Table 1). The fact that proliferation remained constant and independent of tumor location in all tumor stages indicates that HMGA2 expression does not occur concomitantly with expansion of noninvasive neoplastic colonic cells but correlates with the invasive cancer cells that exhibit EMT.

In addition, cells at the invasive front in CRCs have undergone EMT (21), where the overwhelming majority of cells are HMGA2-positive. Two well-established markers for EMT in CRCs are membrane-to-nucleus relocalization of β-catenin (21) and loss of E-cadherin expression along the cell membrane (10). Importantly, the mesenchymal-like cells at the invasive front, which exhibited the strongest nuclear HMGA2 accumulation, also showed nuclear relocalization of β-catenin and loss of membrane E-cadherin expression (Fig. 2).

**HMGA2 activates the TGFβRII and enhances TGFβ signaling pathway**

To define the molecular mechanism of HMGA2-related EMT, we decided to investigate the possible relationship between the HMGA2 and TGFβ signaling pathway, which is well-established to play a role in the EMT (20) and invasion (23). Consistent with the expression of HMGA2 and its bona fide downstream target gene, IGF2BP2 (24, 25), the TGFβ/RII
was highly expressed in MCF7-HMGA2 and MDA-MB231 cells compared with MCF7-mock cells (Fig. 3A and B). TGFβRI expression level was unchanged (data not shown). Depletion of HMGA2 on HMGA2 siRNA-treated MDA-MB231 cells down-regulated TGFβRII mRNA expression (Fig. 3C) and protein (Fig. 3D).

Next, the activation of the TGFβRII by HMGA2 was examined through the phosphorylation status of Smad3. Whereas Smad3 is typically localized in the cytoplasm, activation of the TGFβ pathway causes Smad3 to be phosphorylated and migrate to the nucleus (26). Although Smad3 expression levels were unchanged in MCF7 or MCF7-HMGA2 cells even after TGFβ1 was added (Fig. 3E), in the MCF7-HMGA2 cells, the levels of phospho-Smad3 increased as compared with the MCF7-mock cells with a dramatic enhancement observed in phospho-Smad3 expression levels in the presence of exogenous ligand, TGFβ1. Just as importantly, Smad3 is observed exclusively in the cytoplasm of MCF7-mock cells but in the MCF7-HMGA2 cells, expression is most prominent in the nucleus (Fig. 3F). Taken together, these results suggest that HMGA2 increases the susceptibility to the TGFβ ligand through the activation of TGFβRII and strongly suggests that HMGA2 is upstream of the TGFβ pathway.

To elucidate the direct relevance of the in vitro mechanistic studies, human breast and CRC tissues were investigated. In breast cancer tumors, a coincident expression pattern was observed for HMGA2 and TGFβRII. Expression was coincident for the 2 proteins within the cells of CRC tumor tissue at the invasive front (Fig. 4, bottom, left 2 lines), but not in the well-differentiated area of the tumor (Fig. 4, middle, left 2 lines). IGF2BP2 was also strongly expressed at the invasive front of human advanced breast cancer tumors (Fig. 4, top, right 2 lines) and severe grade of human CRC tumors (Fig. 4, bottom, right 2 lines), but not in the well-differentiated area of CRC tumors (Fig. 4, middle, right lines). In conclusion, the expression of the TGFβ type II receptor was coincident with HMGA2 and IGF2BP2, which is expressed in the cells that undergo EMT at the invasive front.

**HMGA2 and TGFβRII expression and loss of Hmga2 in the MMTV-Wnt1 transgenic mouse breast cancer model**

In the MMTV-Wnt1 transgenic mouse model, the expression of Wnt1 induces hyperplasia of breast at 2 weeks of age, and by 1 year, 90% develop mammary adenocarcinomas (16, 27). Therefore, the MMTV-Wnt1 transgenic mouse was used as an animal model of epithelial tumors. Interestingly, Hmga2 was expressed in the hyperplastic mammary glands and mammary adenocarcinomas of the Wnt1 transgenic mice (Fig. 5A) but not in the normal breast tissue of the wild-type littermates.

Mammary tumors in the MMTV-Wnt1 mouse develop as multiple small tumors that are expansive and infiltrate the stroma. The edge of each of the multiple small tumors was defined as the invasive front. The majority of HMGA2-
expressing cells are detected at the invasive front of individual tumors on the Hmga2+/+ genotypic background (Fig. 5B, top). Interestingly, the TGFβRII and IGF2BP2 expression were observed at the same invasive front on the Hmga2+/+ genotypic background (Fig. 5B, top). In addition, the loss of E-cadherin (Fig. 5B, top) and relocalization of β-catenin (Supplementary Fig. S4) were also detected at the same invasive front of Wnt1-mediated tumors on the Hmga2+/+ genetic background.

Having obtained the encouraging result that Hmga2 was expressed in the MMTV-Wnt1 tumors, the next step was to determine whether this correlative result could be extended. Therefore, to examine the in vivo effect of Hmga2 on the initiation and progression of Wnt1-mediated tumorigenesis, the Hmga2 mutation (3) was bred onto the background of the MMTV-Wnt1 transgenic mouse for 2 generations (the Hmga2fl/fl null mice are sterile) to obtain MMTV-Wnt1 transgenic mice with Hmga2+/+ (n = 17), Hmga2−/− (n = 18), and Hmga2−/− (n = 9) genetic backgrounds. There was no difference in the timing and size of the tumors on the different Hmga2 backgrounds. Remarkably, there were 70% fewer mice with tumors on the Hmga2−/− background as compared with the Hmga2+/+ background (Fig. 5C). Interestingly, although Wnt1-mediated tumor formation typically results in a heterogeneous glandular structures with frequent blood- or secretion-filled cysts (27), no obvious glandular and cystic structures were observed on the Hmga2−/− genotypic background (Fig. 5B, bottom).

Interestingly, a small number of tumors did develop on the null background, which was further analyzed by immunohistochemistry. Unlike in the wild-type tumors, TGFβRII and IGF2BP2 expression were absent at the edge of tumor on the Hmga2−/− genetic background (Fig. 5B, bottom). In addition, β-catenin did not relocalize to the nucleus and was detected both at the edge and throughout the tumor (Supplementary Fig. S4). Interestingly, E-cadherin was present both...
throughout the tumor and at the edge of the tumor (Fig. 5B, bottom). These results suggest that HMGA2 is required in mammary tumorigenesis for tumor development and progression that is initiated through the Wnt1 pathway, via TGFβ signaling pathway.

HMGA2 and metastasis

The next series of studies took advantage of 2 well-characterized mouse mammary tumor cell lines derived from a single spontaneously arising, mammary tumor in a BALB/cJ mouse (28) to further investigate direct role of HMGA2 in metastasis in vivo. 4TO7 cells disseminate from the primary tumor and can enter the bloodstream but cannot proliferate at their secondary site in the distant organs (28, 29). On the other hand, 4T1 cells have full metastatic properties where the cells from the primary tumor disseminate into the bloodstream and form macrometastasis in the liver and lung (29). A third cell line was generated, which was a derivative of the 4TO7 cell line but expressed HMGA2-GFP (4TO7-HMGA2-GFP; see Materials and Methods). As was the case for the cell line described in Fig. 1B, the 4TO7 cell attained a mesenchymal phenotype upon transfection of HMGA2 exhibiting spindle cell metaplasia and loss of E-cadherin. Interestingly, in our study, TGFβRII was upregulated in 4T1 cells as compared with 4TO7 cells (Supplementary Fig. S5).

Mice were inoculated with 2 different cell lines and formed palpable primary tumors within 6 days. HMGA2 was enhanced in the primary tumors injected with the 4T1 cells (Fig. 6A). Cells have full metastatic properties where the cells from the primary tumor disseminate into the bloodstream and form macrometastasis in the liver and lung (29). A third cell line was generated, which was a derivative of the 4TO7 cell line but expressed HMGA2-GFP (4TO7-HMGA2-GFP; see Materials and Methods). As was the case for the cell line described in Fig. 1B, the 4TO7 cell attained a mesenchymal phenotype upon transfection of HMGA2 exhibiting spindle cell metaplasia and loss of E-cadherin. Interestingly, in our study, TGFβRII was upregulated in 4T1 cells as compared with 4TO7 cells (Supplementary Fig. S5).

Mice were inoculated with 2 different cell lines and formed palpable primary tumors within 6 days. HMGA2 was enhanced in the primary tumors injected with the 4T1 cells (Fig. 6A).
Remarkably, 4T07-HMGA2-GFP cells formed primary tumors more rapidly than the 4T07-mock cells after 9 days (Fig. 6B) and grew at the same rate as the 4T1 cells. In the 4T1 metastatic tumors, HMGA2-expressing cells were detected at the edge of the lung tumors and liver stroma (Glisson’s capsule) into the parenchyma (Supplementary Fig. S6A).

To determine whether the loss of Hmga2 reduced the invasive properties of the 4T1 mouse invasive breast cancer cells and subsequent tumor development, we generated 4T1 cells stably expressing Hmga2 shRNA (4T1-Hmga2-shRNA cells; see Materials and Methods). Hmga2 mRNA levels were remarkably reduced in the 4T1-Hmga2-shRNA cells (Fig. 6C) and these 4T1-Hmga2-shRNA cells exhibited marked reduction of invasion in a Matrigel invasion assay (Fig. 6D). Most remarkably, 7 days after injection the 4T1-Hmga2-shRNA cells formed primary tumors more slowly than the 4T07-NTC-shRNA cells (Fig. 6E). The number of invaded cells was counted per field (magnification, ×100) in at least 10 different fields per filter. The numbers are shown as mean ± SD from 2 experiments carried out in triplicate. 

A, Hmga2 mRNA expression in the primary tumor of 4T1- and 4T07-derived cells. B, primary tumors derived from 4T07-HMGA2 cells rapidly developed similar to 4T1 cells until 16 days after implantation when compared with 4T07-mock cells, *p < 0.05 (4T07-mock vs. 4T07-HMGA2 cells); †, p < 0.05 (4T07-mock vs. 4T07-HMGA2 cells and 4T07-mock vs. 4T1 cells). C, Migration assay (4T1-NTC-shRNA and 4T1-Hmga2-shRNA cells). D, Matrigel invasion assay (4T1-NTC-shRNA and 4T1-Hmga2-shRNA cells). The number of invaded cells was counted per field (magnification, ×100) in at least 10 different fields per filter. The numbers are shown as mean ± SD from 2 experiments carried out in triplicate. E, primary tumors derived from 4T1-Hmga2-shRNA cells slowly developed 7 days after implantation when compared with 4T1-NTC-shRNA cells, *p < 0.0001. F, liver metastases (arrows) were induced in 4T07 cells stably overexpressing HMGA2 (4T07-HMGA2) but not in 4T07-mock–transfected (4T07-mock) cells, †, p < 0.001. G, 4T07-HMGA2 cells were localized to the invasive front (arrows) but not at the noninvasive front of the primary tumor developed from the mixture between 4T07-mock and 4T07-HMGA2 cells. 4T07-HMGA2 cells were detected in the metastatic liver lesion (liver parenchyma, arrows). Scale bars, 100 μm. H, schematic of HMGA2-induced metastasis. HMGA2 induces expression of the TGFβRII. These cells then undergo EMT, migrate, and localize to the invasive front of the tumor where they interact with TGFβ from the stroma facilitating invasion and metastasis.
HMGA2 and Metastasis

Finally, the HMGA2-GFP fusion protein was traced after implantation of a mixture of equal numbers of 4T07-mock and 4T07-HMGA2-GFP cells. At the primary site, 4T07-HMGA2-GFP cells were localized at the invasive front and were not detected in the centre of the primary tumors (Fig. 6G, top). Similarly, in the liver metastases, the majority of the 4T07-HMGA2-GFP cells were detected especially in the liver parenchyma invaded by cancer cells emanating from the blood vessel into the stroma (Fig. 6G, bottom; Supplementary Fig. S7).

These results suggest that when HMGA2 is expressed in cells during tumor development, the cells have the ability to localize to the invasive front of the primary tumor and HMGA2 can directly induce liver metastasis in the mouse allograft model.

Discussion

The described studies define a fundamental pathway in the EMT and tumor metastasis. Our initial in vitro experiments established that HMGA2 converts noninvasive cell types into their invasive counterparts through the induction of the EMT. Importantly, the cells at the invasive front of human tumors preferentially express HMGA2 where the tumor cells exhibit the EMT, and it is these cells that are said to be destined to become metastatic and progress further onto malignancy (10). The genetic studies with the mouse model of tumorigenesis reveal that the HMGA2 pathway is essential for tumor pathogenesis for the majority of tumors. Furthermore, the molecular mechanism by which HMGA2 mediates the induction of EMT was determined to go through the TGFβ canonical pathway and specifically through the activation of the TGFβRII. Most decisively, the expression of TGFβRII was absent in the HMGA2-independent tumors leading to the conclusion that HMGA2-dependent tumor pathogenesis is mediated via TGFβRII. Consistent with the distribution of the expression pattern of HMGA2 and its activation of the TGFβRII, we showed that the TGFβRII is also expressed exclusively at the invasive front of human tumors.

The hypothesis for a relationship between genes that are involved in mesenchymal proliferation and differentiation and the EMT and hence invasion and metastasis is further strengthened by studies of these genes in human tumors. For HMGA2, the present study showed that the protein is localized to the invasive front. Consistent with these findings, there were a number of studies that suggested a positive correlation between HMGA2 expression and severity of tumor grade for a variety of tumor cell types (5, 9). Furthermore, the presence of HMGA2 in the blood (presumably due to metastasizing circulating tumor cells) correlates with either tumor recurrence or mortality (6). Where analyzed, there are a number of similar observations as for HMGA2 on the expression of Snail, Slug, and Twist in human tumors. Both Snail and Twist are maximally expressed in multiple tumor types of the most severe grade (30, 31), and expression in breast cancer is related to patients survival (32). Pertinently, Snail was mainly expressed at the invasive front in human esophageal cancer (33), and both Snail and Twist are also found at the invasive front of the tumor in malignant parathyroid neoplasia (34). In addition, Slug is predominantly expressed at the invasive front of pancreatic tumor (35). We would predict that future cellular localization studies would reveal that these genes are also expressed at the invasive front in cells that have undergone EMT. The correlative human studies in conjunction with the in vitro and in vivo studies described in the Results clearly define the functional significance of HMGA2 expression in the EMT and metastasis. Although all 3 of these mesenchyme-specific genes induce EMT, this does not imply that they are in the same molecular pathway. For example, Twist does not induce Snail expression in carcinoma cells (36), and our studies show there was not a statistically significant difference in Twist or Snail expression by HMGA2 in MCF7 cells (Supplementary Fig. S8). It should be noted that HMGA2 was said to induce the expression of Snail and Twist in normal murine mammary gland epithelial (NMuMG) cells (see below; ref. 37). Cumulatively, these results suggest that in various cell types, mesenchyme-specific molecular pathways are activated in tumor cells for cells to ultimately metastasize and potentially the particular EMT driver directs metastasis to a specific tissue.

The in vitro studies strongly indicate a role for HMGA2 in the EMT, and the clinical studies suggest a role for HMGA2 in metastasis. Therefore, to understand the function of HMGA2 in metastasis directly in vivo, the 2 closely related mouse breast cancer cell lines, 4T07 and 4T1 were used. The 4T07-HMGA2-GFP cells localized to the invasive front of the primary tumor. Therefore, expression of the HMGA2 pathway causes the cells to migrate to the exterior of the tumor within a relatively short amount of time in tumor development (20 days). This would suggest that HMGA2 expression in tumor cells requires few, if any, additional genetic events to attain the capability to migrate to the invasive front of a tumor. Compared with the parental 4T07 cells that are detected only within the blood vessels of Glisson’s capsule, the 4T07-HMGA2-GFP cells are converted to a metastatic form in their ability to invade and colonize the liver parenchyma, similar to the 4T1 cells. Whether these 4T07-HMGA2-GFP cells at the exterior of the primary tumor require additional genetic events to metastasize to distant organs remains to be elucidated. Interestingly, the 4T07-HMGA2-GFP cells did not metastasize to the lung 25 days after implantation, as do the 4T1 cells. This tissue specificity of colonization could be a function of HMGA2 or due to the loss of the 4T07 cells’ capability to metastasize to the lung. Such a finding warrants further investigation and is an area of future study.

In the present study, ectopic HMGA2 expression mediates EMT through an interaction via the TGFβ signaling pathway that has been strongly implicated in EMT induction in vitro for a number of cell types (20, 38). Specifically, ectopic HMGA2 expression induced EMT and invasiveness through upregulation of the TGFβRII in MCF7 breast cancer cells (Figs. 1 and 3) without the activation of TGFβ1 expression (Supplementary Fig. S9). Also, in our study, the endogenous HMGA2 expression was not increased by TGFβ1 treatment in the MCF7 breast cancer cells (Supplementary Fig. S10). It should be noted that with NMuMG cells, although Thuault and colleagues (38) did observe EMT induced by the ectopic expression of HMGA2, treatment of these cells by TGFβ1 was found to increase
HMGA2 expression from only the endogenous locus. This apparent discrepancy may lie in the difference between the 2 breast cancer cell types that were used, which may represent tumor cells at variant competencies to the ligand. Most importantly, the function of HMGA2 in the EMT via the TGFβ pathway and, more specifically, TGFβRII, derived from the in vitro studies were directly showed to be relevant to in vivo tumors from both human and mouse studies. TGFβRII was highly expressed at the invasive front of the human breast (Fig. 4, top) and colon cancer (Fig. 4, bottom) tissue consistent with the expression of HMGA2 in vivo as well as in the mammary carcinomas of the MMTV-Wnt1 mouse model. In conclusion, HMGA2 activates the expression of the TGFβRII, directly or indirectly, in tumor cells and allows these tumor cells to respond to TGFβ, which may be secreted from the tumor microenvironment (39, 40) during invasion and metastasis (Fig. 6H).

In addition, IGF2BP2 identified as a target gene of HMGA2 both during development (24) and in tumorigenesis (25) was detected in the cells at the invasive front of the MMTV-Wnt1 tumor. This suggests that HMGA2 could regulate metastasis not only through the TGFβ pathway but also by recruitment through the activation of the downstream pathway. Therefore, HMGA2 can be considered a “driver” of the EMT and metastasis, and delineation of the HMGA2 pathway will potentially define a series of genes specific for invasion and metastasis of epithelial cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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