A Requirement for Dimerization of HP1Hs\(\alpha\) in Suppression of Breast Cancer Invasion*

Laura E. Norwood\(‡\), Timothy J. Moss\(‡\), Naira V. Margaryan\(§\), Sara L. Cook\(\dagger\), Lindsay Wright\(\dagger\), Elisabeth A. Seftor\(\dagger\), Mary J. C. Hendrix\(\dagger\), Dawn A. Kirchmann\(\dagger\), and Lori L. Wallrath\(\dagger,\‡,\§\)

From the \(\dagger\)Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242 and the \(\dagger\)Children’s Memorial Research Center, Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine at Northwestern University, Chicago, Illinois 60611

The development and progression of cancer is controlled by gene expression, often regulated through chromatin packaging. Heterochromatin protein 1Hs (HP1Hs), one of three human HP1 family members, participates in heterochromatin formation and gene regulation. HP1Hs possesses an amino-terminal chromodomains, which binds methylated lysine 9 of histone H3 (meK9 H3), and a carboxy-terminal chromoshadow domain (CSD) that is required for dimerization and interaction with partner proteins. HP1Hs is down-regulated in invasive metastatic breast cancer cell compared with poorly invasive nonmetastatic breast cancer cell. Expression of EGFP-HP1Hs in highly invasive MDA-MB-231 cells causes a reduction in in vitro invasion, without affecting cell growth. Conversely, knock-down of HP1Hs levels in the poorly invasive breast cancer cell line MCF-7 increased invasion, without affecting cell growth. To determine whether functions of the CSD were required for the regulation of invasion, mutant forms of HP1Hs were expressed in MDA-MB-231 cells. A W174A mutation that disrupts interactions between HP1Hs and PXVXL-containing partner proteins reduced invasion similar to that of the wild type protein. In contrast, an I165E mutation that disruptions dimerization of HP1Hs did not decrease invasion. No gross changes in localization and abundance of HP1Hs\(\beta\), HP1Hs\(\gamma\), and meK9 H3 were observed upon expression of wild type and mutant forms of HP1Hs in MDA-MB-231 cells. Taken together, these data demonstrate that modulation of HP1Hs alters the invasive potential of breast cancer cells through mechanisms requiring HP1 dimerization, but not interactions with PXVXL-containing proteins.

Mortality from breast cancer occurs by the spread of cancer cells to secondary sites within the body, a process called metastasis (1). Breast cancer cells must acquire several properties in order to disseminate from the primary tumor, including the ability to degrade and migrate through the extracellular matrix, a process called invasion (2). Invasion is one of the first steps in the metastatic cascade and is a strong indicator of tumor progression. Two classes of proteins have been identified that regulate metastasis progression: activators and suppressors (3). Metastasis suppressors are genes that promote metastasis, whereas metastasis suppressors inhibit metastasis. In contrast to tumor suppressors, metastasis suppressors do not affect growth of the primary tumor (3). Although several genes have been identified that regulate metastasis, clinically valuable predictive or prognostic molecular markers for metastasis have yet to be determined (4–6). Currently, the best indicator for metastasis is lymph node micrometastases (4). However, microarray-based studies to identify transcription profiles that predict metastasis are in development (5, 7).

In an attempt to identify genes with altered expression in breast cancer metastasis, a differential display analysis was performed comparing gene expression between poorly invasive nonmetastatic and highly invasive metastatic breast cancer cell lines (8). The mRNA encoding heterochromatin protein 1Hs (HP1Hs)\(^3\) was found to be down-regulated (1.5-fold) in highly invasive metastatic breast cancer cell lines compared with poorly invasive nonmetastatic breast cancer cell lines (8, 9). Consistent with this reduction in mRNA, HP1Hs protein levels showed an even larger disparity (7.1-fold) between the two types of breast cancer cell lines (9). HP1Hs protein was also less abundant in metastatic tissues than in primary breast cancer tissues, suggesting that the down-regulation in highly invasive metastatic breast cancer cell lines recapitulate trends that were observed in clinical samples (9).

HP1 is a highly conserved protein originally identified in Drosophila melanogaster as a component of chromatin near centromeres (10, 11). HP1 proteins are classically known to silence genes that have been juxtaposed to centric chromatin, and the degree of silencing is dependent on HP1 dosage (12, 13). Three HP1 family members have been identified in humans: HP1Hs, HP1H\(\beta\), and HP1H\(\gamma\). All HP1 family members consist of two conserved domains, the chromodomab (CD) and the chromoshadow domain (CSD), that are separated by a less conserved hinge region. The CD, within the amino-terminal region, folds into a structure containing three β-sheets and an α-helix that form a hydrophobic protein interaction pocket (14, 15). The HP1 CD has been shown to specifically interact with di- and trimethylated lysine 9 of histone H3 (meK9 H3), an epigenetic mark generated by SET (suppressor of variegation, enhancer of zest and Trithorax) domain-containing histone methyltransferases (15–18). This interaction targets HP1 proteins to specific regions of the genome that are enriched in meK9 H3, such as those near centromeres (16, 19).

The CSD, at the carboxy-terminal region, has an amino acid sequence and structure similar to that of the CD (20–22). A major distinction between the CD and CSD is that the CSD forms homo- and heterodimers with other HP1 proteins through an α-helix (20, 21, 23).

---

*This work was supported by Grant DAMD17-02-1-0424 from the Dept. of Defense Breast Cancer Research Program (to L. L. W. and D. A. K.), Susan G. Komen Dissertation Research Award DIS50403121 (to L. E. N.), and the Order of the Eastern Star Breast Cancer Research Fund (to M. J. C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore indicate this fact.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Biochemistry, The University of Iowa, 3136 Medical Education Research Facility (MERF), Iowa City, IA 52242. Tel.: 319-335-7920; Fax: 319-384-4770; E-mail: lori-wallrath@uiowa.edu.

3 The abbreviations used are: HP1, heterochromatin protein 1; CD, chromodomain; CSD, chromoshadow domain; meK9 H3, methylated lysine 9 of histone H3; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; shRNA, short hairpin RNA; shHP1Hs\(\alpha\), short hairpin RNA targeted to HP1Hs\(\alpha\); shGFP, short hairpin RNA targeted to EGFP; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; NLS, nuclear localization signal; RNAi, RNA interference; BrdUrd, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; RSV, Rous sarcoma virus.
The dimerization of CSDs forms an interaction platform for proteins containing the amino acid sequence motif PXXVL (21, 22). Many different types of nuclear proteins contain PXXVL motifs, including nuclear architecture proteins such as the lamin B receptor, transcriptional regulators such as KRAB-associated protein 1 (KAP1), and chromatin assembly and modifying proteins such as chromatin assembly factor 1 (CAF1p150) (24). However, there have been proteins identified that bind to HP1 but do not utilize the PXXVL interaction platform, such as BRM-related gene 1 (BRG1) and suppressor of variegation 3–9 homolog 1 (SU(V39h)1) (25, 26).

In this report, the function of HP1HsX has been investigated by assaying invasion and growth of breast cancer cells upon modulation of HP1HsX levels. Structure/function analysis has revealed that HP1HsX dimerization is essential for regulating invasion. Data presented here are consistent with the hypothesis that HP1HsX functions as a breast cancer metastasis suppressor.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—MCF-7 cells were kindly supplied by Dr. F. Miller (Karmanos Cancer Institute, Detroit, MI), and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37 °C with 5% CO2 in complete medium (RPMI 1640, Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 10 μg/ml gentamycin (Cellgro).

**Adenoviral Constructs**—The I165E and W174A mutants of HP1HsX were generated by site-directed mutagenesis (QuikChange, Invitrogen) using the pEGFP-HP1HsX as a template (9). The primers used to generate the I165E mutation are: 5′-CCACAATTTGTTGACATATTGCAT-3′ and 5′-CATAAATGGTTCCCAATTTTGG-3′. The primers used to generate the W174A mutation are: 5′-GAGACTGAGAACGGGCATGATCCGCA-3′ and 5′-GGATATGCATGCGCTGTGGTCCA-3′. The mutant and wild-type EGF-P-HP1HsX were cloned into the Ad5 RSV K-NpA shuttle vector (University of Iowa Gene Transfer Vector Core). As a control, the enhanced green fluorescent protein (EGFP) gene was fused to a nuclear localization signal (isolated from pShooter pCMV/myc/nuc plasmid, Invitrogen) and cloned into the Ad5 RSV K-NpA shuttle vector. The adenoviral constructs were packaged into virus and used for infection of breast cancer cells (University of Iowa Gene Transfer Vector Core).

**In Vitro Invasion Assays**—MCF-7 cells were plated in 24-well plates on top of glass coverslips. Twenty-four hours post-plating, the cells were either examined for EGFP fluorescence (unfixed cells) or fixed with 100% methanol for 5 min and allowed to dry for 1 h. Fixed cells were rehydrated with 1× phosphate-buffered saline (PBS) and immunostained with α-HP1HsX, α-HP1HsY, and α-HP1HsZ (MAB145.3 from Dr. Frank Rauscher III; MAB3448 and MAB3450 from Chemicon), di-meK9 H3 (07–441, Upstate Biotechnology), tri-meK9 H3 (07–442, Upstate Biotechnology), and β-tubulin (Developmental Studies Hybridoma Bank, University of Iowa). Goat α-rabbit horseradish peroxidase (Upstate Biotechnology) and goat α-mouse horseradish peroxidase (Tissue Culture/Hybridoma Core, University of Iowa) were used as secondary antibodies.

**Localization of Proteins**—Twenty-four hours post-infection, cells (1 × 105 MDA-MB-231; 5 × 104 MCF-7) were collected and plated on glass coverslips in 24-well plates. Twenty-four hours after plating, the cells were either examined for EGFP fluorescence (unfixed cells) or fixed with 100% methanol for 5 min and allowed to dry for 1 h. Fixed cells were rehydrated with 1× phosphate-buffered saline (PBS) and immunostained with α-HP1HsX, α-HP1HsY, and α-HP1HsZ (MAB145.3 from Dr. Frank Rauscher III; MAB3448 and MAB3450 from Chemicon), di-meK9 H3, or α-tri-mK9 H3 (07–441 and 07–442, respectively; Upstate Biotechnology). A rhodamine red conjugated goat α-mouse antibody was used for detection (Molecular Probes). Cells were visualized under a fluorescent microscope (Leica DMLB) for EGFP and rhodamine fluorescence using the ×100 objective.

**Western Analysis**—Protein expression was determined by Western analysis using antibodies against GFP (Molecular Probes), HP1HsX (clones MAB145.3 and MAB15.19 (2), kind gifts of Frank Rauscher III, Wistar Institute), HP1HsY (MAB3448, Chemicon), HP1HsZ (MAB3450, Chemicon), di-meK9 H3 (07–441, Upstate Biotechnology), tri-meK9 H3 (07–442, Upstate Biotechnology), and β-tubulin (Developmental Studies Hybridoma Bank, University of Iowa).

**Cell Cycle Assays**—Forty-eight hours post-infection, cells were collected and plated (5 × 105 cells MDA-MB-231; 2 × 105 MCF-7) in each well of a 6-well plate. Cells were collected for each sample from one well at specific time points, and the cell number was counted using a hemocytometer.

**BrdUrd Incorporation Assays**—Forty-eight hours post-infection, 5 × 105 cells were plated in 24-well plates on top of glass coverslips, incubated for 24 h (37 °C with 5% CO2), and then treated with 10 μM BrdUrd.
Three lines of evidence demonstrate that EGFP does not alter HP1Hs localization between low levels of HP1Hs localization pattern (Fig. 3 and data not shown). Expression of EGFP-and data not shown), the pattern anticipated from previous localization studies (9). MDA-MB-231 cells infected with a virus expressing EGFP-NLS did not have a significantly different invasive potential than uninfected cells (106 versus 100%, respectively; \( p = 0.455 \)) (Fig. 2B). MDA-MB-231 cells infected with EGFP-HP1Hs have a reduced invasive potential compared with uninfected cells (76 versus 100%, respectively; \( p = 0.023 \)) or compared with EGFP-NLS (72 versus 100%, respectively; \( p = 0.015 \)) (Fig. 2, B and C). These results provide evidence that HP1Hs is involved in the suppression of breast cancer cell invasion, consistent with previously published data (9).

**Results**

Expression of HP1Hs in MDA-MB-231 Cells—Because of the correlation between low levels of HP1Hs and breast cancer invasion/metastasis, our objective was to modulate the levels of HP1Hs in breast cancer cell lines and assay for effects on cell growth and invasion. Adenoviral constructs expressing either enhanced green fluorescent protein (EGFP) tagged with a nuclear localization signal (NLS) as a negative control or EGFP fused to HP1Hs were generated. To determine whether GFP altered the function of HP1Hs, the EGFP-HP1Hs fusion protein was expressed in *Drosophila*, for which functional assays exist (30). Three lines of evidence demonstrate that EGFP does not alter function of HP1Hs: 1) EGFP-HP1Hs localized to sites of endogenous HP1 on *Drosophila* salivary gland polytene chromosomes; 2) EGFP-HP1Hs enhanced silencing of genes near heterochromatin in a dosage-dependent manner; and 3) EGFP-HP1Hs rescued lethality associated with mutations in the gene encoding HP1 (30).

To determine the effects of EGFP-HP1Hs expression in breast cancer cells, MDA-MB-231 cells were infected with adenovirus encoding EGFP-HP1Hs at levels similar to that of endogenous HP1Hs in MCF-7 cells, recapitulating physiological levels in noninvasive breast cancer cells (Fig. 1). As a negative control, EGFP-NLS was expressed in MDA-MB-231 cells at levels comparable with that of EGFP-HP1Hs (Fig. 2A). The localization of EGFP-HP1Hs was examined in both fixed and unfixed cells. EGFP-HP1Hs localized to small discrete foci within the nucleus and co-localized with endogenous HP1Hs in fixed cells (Fig. 3 and data not shown), the pattern anticipated from previous localization studies (9, 19, 31, 32). In contrast, EGFP-NLS exhibited a diffuse nuclear localization pattern (Fig. 3 and data not shown). Expression of EGFP-NLS did not alter endogenous HP1Hs expression as assayed by reverse transcriptase PCR, nor did EGFP-NLS cause a change in endogenous HP1Hs localization (data not shown). These data demonstrate that the EGFP-tagged proteins properly localize in the nucleus of MDA-MB-231 cells.
PXVXL-containing proteins are not required to suppress invasion in MDA-MB-231 breast cancer cells.

Localization and Expression of HP1\(^{\alpha}\), HP1\(^{\alpha}\), and meK9 H3 Are Not Dramatically Altered by Expression of HP1\(^{\alpha}\) in MDA-MB-231 Cells—HP1\(^{\alpha}\) is one of three HP1 family members in human cells. To investigate the effect of expression of EGFP-HP1\(^{\alpha}\) on HP1\(^{\alpha}\) and HP1\(^{\alpha}\) localization and abundance, immunolocalization studies and Western analyses were performed. Expression of EGFP-NLS, EGFP-HP1\(^{\alpha}\), EGFP-I165E, or EGFP-W174A had no obvious effect on HP1\(^{\alpha}\) localization or expression (Fig. 3 and data not shown). Therefore, alterations in the level or localization of HP1\(^{\alpha}\) do not appear to influence the expression of other HP1 family members in MDA-MB-231 cells.

Current models for heterochromatin formation and spreading include binding of HP1 to meK9 H3, recruitment of SUV39h histone methyltransferases through direct interactions with HP1, and subsequent methylation of adjacent histones (17, 20). Thus, there is a potential that the decrease in invasion observed after expression of EGFP-HP1\(^{\alpha}\) could result in changes in meK9 H3. Western analysis and immunolocalization were performed to determine whether the localization and abundance of di- or tri-meK9 H3 changed after infection of the adenoviral constructs in MDA-MB-231 cells. Neither gross localization (Fig. 4A) nor expression levels (Fig. 4B) of di- or tri-meK9 H3 were observed to change after expression of EGFP-NLS, EGFP-HP1\(^{\alpha}\), EGFP-I165E, or EGFP-W174A. These observations lead to the conclusion that global changes in di- and tri-meK9 H3 are unlikely to be involved in the suppression of the invasive potential of the metastatic breast cancer cell line, MDA-MB-231, by EGFP-HP1\(^{\alpha}\).

HP1\(^{\alpha}\) Does Not Affect Growth of MDA-MB-231 Cells—Because HP1\(^{\alpha}\) is able to suppress invasion, a key step in metastasis, and is
down-regulated in metastatic tissue from breast cancer patients, HP1\textsuperscript{Hs\alpha} is hypothesized to be a metastasis suppressor protein (9). Metastasis suppressor proteins are defined as being able to suppress metastasis without affecting the growth of tumor cells (3). To test for the effect of HP1\textsuperscript{Hs\alpha} expression on the growth rate of MDA-MB-231 cells, growth curves of uninfected cells and of cells infected with one of the adenoviral constructs (EGFP-NLS, EGFP-HP1\textsuperscript{Hs\alpha}, EGFP-W174A, and EGFP-I165E) were compared (Fig. 5A). During the time period when the in vitro invasion assays were performed (48–72 h post-infection), there was no difference in growth between the samples. Therefore, growth rate differences do not explain the decrease in invasion of MDA-MB-231 cells infected with EGFP-HP1\textsuperscript{Hs\alpha}. After 72 h, all samples infected with adenovirus showed a slower growth rate than uninfected cells. No statistical difference was observed among the EGFP-NLS-, EGFP-HP1\textsuperscript{Hs\alpha}-, EGFP-I165E-, and EGFP-W174A-infected cells through 144 h post-infection (Fig. 5A). The slower growth rate is most likely because of adenoviral effects.

To further determine the effects of HP1\textsuperscript{Hs\alpha} expression in MDA-MB-231 cells, cell cycle progression was investigated using propidium iodide fluorescence-activated cell sorting. No differences were observed between uninfected cells and those infected with EGFP-HP1\textsuperscript{Hs\alpha} during G\textsubscript{0}-G\textsubscript{1}, S, or G\textsubscript{2}-M phases of the cell cycle 48 h post-infection (Fig. 5B). Although there were no changes during G\textsubscript{0}-G\textsubscript{1}, S, or G\textsubscript{2}-M phases, there might be differences in the ability of the cells to undergo DNA replication. BrdUrd incorporation studies were performed using uninfected MDA-MB-231 cells, cells expressing EGFP-NLS, and cells expressing EGFP-HP1\textsuperscript{Hs\alpha}. No differences in BrdUrd staining were observed between the samples after 12, 6, 3, or 1 h of BrdUrd treatment (84 h post-infection) (Fig. 5C and data not shown). Taken together, expression of EGFP-HP1\textsuperscript{Hs\alpha} in MDA-MB-231 cells does not result in altered DNA replication, cell cycle progression, or cellular growth rate. These data are consistent with the hypothesis that HP1\textsuperscript{Hs\alpha} is a metastasis suppressor in breast cancer cells.

Knock-down of HP1\textsuperscript{Hs\alpha} in MCF-7 Cells—If expression of HP1\textsuperscript{Hs\alpha} is sufficient to suppress invasive potential in breast cancer cells, a reduction of HP1\textsuperscript{Hs\alpha} is predicted to increase the invasive ability of poorly invasive breast cancer cells. To test this prediction, the poorly invasive nonmetastatic breast cancer cell line, MCF-7, previously shown to have high levels of HP1\textsuperscript{Hs\alpha} (9), was investigated for phenotypic differences after knock-down of HP1\textsuperscript{Hs\alpha} by RNAi.

MCF-7 cells were infected with adenovirus expressing short hairpin RNAi molecules targeted to either HP1\textsuperscript{Hs\alpha} (shHP1\textsuperscript{Hs\alpha}) or GFP (shGFP) as a negative control, and expression levels of HP1\textsuperscript{Hs\alpha} were subsequently compared. Levels of HP1\textsuperscript{Hs\alpha} decreased to less than 5% of that present in uninfected cells 48 h post-infection and remained at that level for ~7 days (Fig. 6A and data not shown). As there are three mammalian HP1...
family members, the effects of HP1Hs knock-down on the expression of HP1Hs and HP1Hs were investigated. No dramatic changes in protein levels of HP1Hs or HP1Hs were detected upon knock-down of HP1Hs (Fig. 6A). These data show that HP1Hs knock-down can be achieved through adenoviral shRNA infection of MCF-7 cells and that the loss of HP1Hs does not dramatically alter the levels of HP1Hs or HP1Hs.

Previous immunofluorescent data have shown that HP1Hs is organized in multiple discrete foci in MCF-7 cell nuclei (9). Uninfected cells showed similar immunofluorescent signal intensity and punctate distribution of HP1Hs in the nucleus, as reported previously (9). Cells infected with shGFP as a negative control showed an intensity of signal and localization of HP1Hs similar to uninfected cells. The localization of HP1Hs was investigated to determine the effects of knock-down in MCF-7 cells using an EGFP marker in the shHP1Hs viral backbone to discriminate between infected and uninfected cells (Fig. 6B). Infection efficiency based on visualization of EGFP fluorescence was 90%. Cells infected with shHP1Hs were evaluated based on expression of GFP. Those cells not expressing EGFP showed a pattern of HP1Hs expression similar to uninfected cells (Fig. 6B). In contrast, cells with high EGFP levels showed an almost complete loss of HP1Hs immunofluorescent signal, although the signal that remained was present in a punctate pattern (Fig. 6B). Therefore, the levels of HP1Hs were reduced, but the localization pattern of remaining HP1Hs continued to be in discrete foci.

Knock-down of HP1Hs in MCF-7 Cells Results in Increased Invasive Potential—To test for whether reduction of HP1Hs in a poorly invasive nonmetastatic cell line increases invasion, an in vitro assay was used to evaluate the invasive ability of MCF-7 cells with or without knock-down. Cells infected with a virus expressing shGFP as a negative control did not have a significantly altered invasive potential compared with uninfected cells (87 versus 100%, respectively; p = 0.338) (Fig. 7A).
HP1\(^{H\alpha}\) in Breast Cancer Invasion

**FIGURE 6.** Knock-down of HP1\(^{H\alpha}\) in MCF-7 cells. MCF-7 breast cancer cells were infected with adenovirus expressing shHP1\(^{H\alpha}\) or shGFP as a negative control. After 48 h, cells were harvested and subjected to Western analysis (A) or fixed with methanol and subjected to immunofluorescence (B). A, Western analysis of HP1\(^{H\alpha}\), HP1\(^{H\beta}\), and HP1\(^{H\alpha}\) levels following HP1\(^{H\alpha}\) knock-down. B, immunofluorescence of MCF-7 cells following HP1\(^{H\alpha}\) knock-down that were immunostained with antibodies recognizing HP1\(^{H\alpha}\) and GFP. Arrows indicate infected cells (green) with knock-down of HP1\(^{H\alpha}\). Arrowheads indicate uninfected cells.

**FIGURE 7.** Knock-down of HP1\(^{H\alpha}\) in MCF-7 cells increases invasion without affecting growth. A, MCF-7 cells either uninfected or infected with adenovirus expressing shHP1\(^{H\alpha}\) or shGFP were assayed for invasion through a laminin/collagen IV/gelatin membrane for 24 h, and the relative percent of invasion was calculated. Three independent cultures were tested in triplicate. The \(p\) values were calculated using Excel t test. B, line graph showing the growth rate of MCF-7 cells. MCF-7 cells were either uninfected (solid line) or infected with an adenovirus expressing shGFP (dashed line) or shHP1\(^{H\alpha}\) (dotted line) and assayed for growth. The black outlined box represents the time frame of the invasion assays (48–72 h post-infection).

contrast, cells expressing shHP1\(^{H\alpha}\) increased the invasiveness of MCF-7 cells compared with shGFP (136 versus 87%, respectively; \(p = 0.028\)) or compared with uninfected cells (136 versus 100%, respectively; \(p = 0.017\)) (Fig. 7A). These results provide evidence that a reduction of HP1\(^{H\alpha}\) is sufficient to increase the invasive potential of MCF-7 breast cancer cells.

**Knock-down of HP1\(^{H\alpha}\) Does Not Alter Growth of MCF-7 Cells**—To further explore the possibility that HP1\(^{H\alpha}\) is a metastasis suppressor, the effects of HP1\(^{H\alpha}\) knock-down on growth in MCF-7 cells was investigated. The growth rates of MCF-7 cells uninfected and infected with either shHP1\(^{H\alpha}\) or shGFP were compared. No changes in growth between the different samples were noted during the time points when invasion was assayed (Fig. 7B). Therefore, growth rate differences do not explain the increase in invasion of MCF-7 cells. However, the adenovirus-infected cells showed a reduced growth rate compared with uninfected cells after 72 h post-infection, most likely because of viral effects (Fig. 7B). Thus, studies in both highly invasive metastatic and poorly invasive nonmetastatic breast cancer cells show that modulation of HP1\(^{H\alpha}\) levels changes the invasive properties of the cells without affecting cellular growth rates.

**DISCUSSION**

Cancer progression is accompanied by cumulative genetic alterations, with transformation and immortalization of cells being well studied (35). However, the molecular mechanisms that result in dissemination of cells from the primary tumor to distant sites in the body are poorly understood (3, 36–40). Although progress is being made in understanding stromal interactions, motility/chemotactic migration, and the epithelial to mesenchymal transition that accompanies metastasis, there is still a deficiency in mechanistic knowledge and predictive power in treating breast cancer metastasis (41–43). Because of this lack of understanding and an inability to accurately anticipate which patients will develop metastatic lesions, many patients who will never develop metastatic disease are treated unnecessarily with cytotoxic therapeutic agents (5, 44, 45). In other cases, those who will acquire metastases are often either undertreated or given therapies to which the metastases are resistant (5, 44, 45). This lack of diagnostic power may account for the large difference in 5-year relative survival rates between localized and metastatic breast cancer (98 versus 26%, respectively) (46). Better prognostic and predictive markers are needed to correctly assess and treat patients.

There is precedent for regulating cancer progression through chromatin organization (47). Alterations in global chromatin structure could simultaneously affect disparate pathways involved in cancer progression. The HP1 family of proteins, which regulates chromatin organization, has been shown to control chromosomal stability and transcriptional regulation (24, 48–51). Improper chromatin organization, including abnormal expression and localization of HP1 family members, is linked to leukemia cell proliferation (52). Additionally, HP1\(^{H\alpha}\) has been associated with tumor progression in multiple human cancers; HP1\(^{H\alpha}\) mRNA levels are reduced in advanced forms of papillary thyroid carcinoma, medulloblastoma, and breast cancer (9, 53, 54).

The cause of HP1\(^{H\alpha}\) mRNA down-regulation in MDA-MB-231 cells relative to MCF-7 cells has been investigated, with evidence showing differential regulation by an E-box element in the 5′-promoter region (30). Yet, how HP1\(^{H\alpha}\) mediates a change in invasive potential has not
been elucidated. We hypothesize that HP1\(^{\text{Hsc}}\) regulates genes involved in invasion/metastasis in breast cancer cells. This could be dependent upon the ability of HP1\(^{\text{Hsc}}\) to dimerize and/or interact with partner proteins to regulate gene expression.

In breast cancer, decreased levels of HP1\(^{\text{Hsc}}\) have previously been shown to correlate with metastasis to distant sites (9). We have demonstrated that the level of HP1\(^{\text{Hsc}}\) regulates \textit{in vitro} invasion; increased expression of HP1\(^{\text{Hsc}}\) reduces invasive potential, whereas decreased expression enhances invasive potential. Altering levels of HP1\(^{\text{Hsc}}\) does not completely reverse the invasive phenotype. This incomplete effect is likely due to cell-by-cell variation in expression of HP1\(^{\text{Hsc}}\) likely due to cell-by-cell variation in HP1\(^{\text{Hsc}}\) expression of HP1\(^{\text{Hsc}}\) in H3 K9 methylation status might occur by variation in HP1\(^{\text{Hsc}}\) levels (9).

To provide further evidence that HP1\(^{\text{Hsc}}\) is a metastasis suppressor, limiting invasion while not altering growth rate. Clinical studies are consistent with these in \textit{vitro} findings, where down-regulation of HP1\(^{\text{Hsc}}\) in breast cancer cell invasion is a metastasis suppressor, \textit{in vivo} metastasis assays using a mouse model are necessary (58).

To dissect the mechanism of how HP1\(^{\text{Hsc}}\) regulates invasion of breast cancer cells, two functional properties of the HP1\(^{\text{Hsc}}\) CSD were analyzed: dimerization of HP1 and interactions with PXVXL motif proteins (21, 22). Here, two CSD mutations were investigated for effects on breast cancer cell invasion. The HP1\(^{\text{Hsc}}\) I165E mutation disrupts CSD dimerization and, consequently, interactions with partner proteins that require dimerization (20, 33, 34). The analogous mutation in mouse HP1\(^{\text{Hsc}}\) (W170A) does not result in mislocalization, showing anticipated nuclear foci (22). The analogous mutation in \textit{Drosophila} HP1 (W200A) retains binding at euchromatic sites. Consistent with these findings, the I165E mutation shows diffuse nuclear staining, whereas the W174A mutation shows punctate nuclear foci in human breast cancer cells (Fig. 3). Collectively, these studies demonstrate that the majority of HP1 chromosomal associations require dimerization rather than interactions with PXVXL-containing partner proteins.

The I165E and W174A point mutations also differentially affect \textit{in vitro} invasion in MDA-MB-231 cells. The I165E mutant protein is unable to suppress invasion, whereas the W174A can suppress \textit{in vitro} invasion. This suggests that dimerization is also required for suppression of breast cancer \textit{in vitro} invasion, whereas interactions with PXVXL partner proteins are not required.

The necessity of having dimerization, but not the PXVXL binding platform, for suppressing breast cancer cell invasion does not eliminate the possibility of partner protein involvement. Although many proteins interact with the HP1 family members through the PXVXL motif, others do not use this mechanism. For example, interaction between BRG1 and HP1\(^{\text{Hsc}}\) does not require the PXVXL platform (25). In addition, the interaction of HP1 family members with SUV39H1 does not involve a PXVXL motif but does require HP1 dimerization (26). Thus, HP1 partner proteins that do not interact through the PXVXL platform might also play a role in HP1\(^{\text{Hsc}}\)-mediated regulation of breast cancer invasion.

Another possible mechanism for regulating breast cancer invasion is HP1\(^{\text{Hsc}}\) dimerization itself. Chromosomal association of HP1 generates a compact chromatin structure, possibly because of interactions between HP1 proteins. The loss of this compact structure could lead to inappropriate activation of genes that are responsible for the invasive phenotype of breast cancer cells (24, 48, 59). In addition, dimerization of HP1 molecules at distant chromosome sites is proposed to form loop structures that bring enhancers into proximity with promoters (24, 60). In such a case, reduction in HP1 levels could lead to repression of genes that prevent invasion. In mammalian cells, HP1 dimerization is complex and might involve both homo- and heterodimerization (61). The composition of a heterodimer could specify chromatin localization and/or partner interactions. This idea is consistent with data showing partial overlapping localization of HP1 family members (62–64). Further studies that would distinguish the functions of HP1 homo- and heterodimers would help to clarify this issue.

We propose a model in which HP1\(^{\text{Hsc}}\) regulates genes involved in breast cancer cell invasion. Evidence that HP1\(^{\text{Hsc}}\) regulates genes comes from microarray studies in \textit{Drosophila} and mammalian cells, where hundreds of genes both increase and decrease in expression upon a reduction in HP1 levels (56, 65). Therefore, it would be beneficial to identify genes in which expression levels depend on HP1\(^{\text{Hsc}}\) dimerization and to evaluate the role they play in invasion and metastasis. These genes and the pathways in which they are involved, would offer new insight into the process of metastasis and possibly provide new markers for diagnosis and prognosis.

Acknowledgments—We thank Greta L. Schriff and Karrie A. Hines for structural modeling. Greg Hannon for RNAi reagents, and Frank Rauscher III for HP1\(^{\text{Hsc}}\) antibodies.

REFERENCES

1. Price, J. E. (1990) \textit{Cancer} \textbf{66}, Suppl. 6, 1313–1320
2. Guo, W., and Giancotti, F. G. (2004) \textit{Nat. Rev. Mol. Cell. Biol.} \textbf{5}, 816–826
3. Debies, M. T., and Welch, D. R. (2001) \textit{J. Mammary Gland Biol. Neoplasia} \textbf{6}, 441–451
4. Hayes, D. F., Isacs, C., and Stearns, V. (2001) \textit{J. Mammary Gland Biol. Neoplasia} \textbf{6}, 441–451

4 Rebecca T. Marquez, Laura E. Norwood, Timothy J. Moss, and Lori L. Wallrath, unpublished data.
5 Karrie A. Hines and Lori L. Wallrath, unpublished data.
