Cell Motility and Spreading Are Suppressed by HOXA4 in Ovarian Cancer Cells: Possible Involvement of β1 Integrin

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Abstract
HOX genes are transcription factors that control morphogenesis, organogenesis and differentiation. Increasing evidence suggests that HOX genes play a role in ovarian cancer progression; however few studies have defined functional roles and mechanisms of action. We showed previously that HOXA4 expression is increased in invasive, compared to noninvasive, epithelial ovarian tumors. However, HOXA4 suppressed cell migration suggesting that elevated HOXA4 expression in invasive tumors constitutes a homeostatic response. In the present study, we used siRNA and forced-expression in multiple cell lines to define the role of HOXA4 in the regulation of transwell migration/invasion and cellular/colony morphology. Knockdown of endogenous HOXA4 increased migration, but not Matrigel invasion, of OVCAR-8 and OVCAR-3 cells. HOXA4 knockdown also increased cell spreading on plastic or fibronectin, reduced cell-cell adhesion, and increased filopodia in two- and three-dimensional cultures. These changes were not associated with significant changes in αv or β3 integrin and E- or N-cadherin. However, down-regulation of HOXA4 significantly reduced β1 integrin protein levels within cell colonies and cell aggregates, but not of single, nonadherent cells. It had no effect on β1 integrin, α5 integrin, or fibronectin mRNA levels. Conversely, overexpression of HOXA4 in CaOV-3 cells suppressed transwell migration and increased β1 integrin protein levels. Our results confirm that HOXA4 inhibits cell motility, show that it suppresses cell spreading and filopodia formation while enhancing cell-cell adhesion, and suggest a role for β1 integrin in mediating these changes. These observations support the hypothesis that overexpression of HOXA4 in invasive ovarian tumors is a homeostatic, invasion-suppressive response. (Mol Cancer Res 2009;7(9):1425–37)

Introduction
Ovarian carcinomas are the fifth most common cause of cancer death in women and the leading cause of death among gynecological malignancies. Five-year survival rates can be as high as 90% for patients presenting with localized disease, however the majority of patients (70%) present with disseminated disease for which the average five-year survival rate is only 30% (1). This is due in part to the mode of dissemination of epithelial ovarian cancers which involves seeding of the abdominal cavity with peritoneal implants and subsequent migration/invasion of the tumor cells into the peritoneum and underlying organs. The major subtypes of epithelial ovarian carcinoma are distinguished by their patterns of histological differentiation, in particular by the acquisition of complex Müllerian duct-derived epithelial characteristics. The molecular mechanisms underlying this paradoxical differentiation of epithelial ovarian cancers are poorly understood but may involve HOX genes (2-6).

The mammalian HOX gene family consists of 39 genes organized in four clusters (A-D) on four chromosomes in 13 paralogous groups. HOX genes encode morphoregulatory transcription factors that specify positional identity during development and regulate differentiation, motility, adhesion and proliferation in adult tissues. Accumulating evidence suggests that aberrantly expressed HOX genes may play important roles in the biology of a variety of solid tumors and hematological malignancies (7-9). However, although numerous studies have documented differences in HOX gene expression between normal and neoplastic tissues, relatively few studies have defined specific functional roles and mechanisms of action for HOX genes in cancer.

The expression of several HOX genes is altered in ovarian carcinomas compared to normal ovarian surface epithelium (2, 10) or normal ovaries (11). Overexpression of HOXB7 in immortalized ovarian surface epithelial cells enhances cellular proliferation (10), whereas overexpression of antisense to HOXB7 and HOXB13 suppresses transwell invasion of SKOV-3 ovarian cancer cells (11). Human HOXB13 has also been shown to enhance the growth of SKOV-3 and OVCAR-5 cells in vitro, and to promote the growth of a genetically defined mouse ovarian cancer cell line in vitro and in vivo (12). Ectopic expression of HOXA7 enhances the epithelial phenotype of immortalized ovarian surface epithelial cells (2), and promotes the abilities of Hoxa9, Hoxa10 and Hoxa11 to induce Müllerian-like differentiation in an experimental ovarian cancer mouse model (3). In a previous study, we used cDNA microarray analysis to demonstrate increased expression of HOXA4 and HOXA7 in ovarian carcinomas compared to benign or low malignant potential serous ovarian tumors (5). More recently,
we examined the expression of HOXA4, HOXA7 and HOXA9 in ovarian surface epithelial cells and ovarian cancer cell lines, and investigated the influence of culture conditions and growth patterns on their expression. Knockdown of HOXA4 was not associated with changes in proliferation, rather, scratch assays demonstrated that HOXA4 suppresses cellular motility in ovarian surface epithelial cells and ovarian cancer cell lines by antagonizing the pro-migratory effects of epidermal growth factor (EGF) receptor ligands (6). In the present study, we used small interfering RNA (siRNA) and forced-expression to investigate the role of HOXA4 in the regulation of transwell chemotactic migration/invasion and cellular/colony morphology in multiple ovarian cancer cell lines. Our results reveal an important role for HOXA4 in suppressing ovarian cancer cell motility and spreading, and suggest that this may be achieved by modulating cell-cell adhesion and β1 integrin protein levels. The paradoxical relationship between increased HOXA4 expression with progression from noninvasive to invasive ovarian tumors, and its inhibitory effect on cell migration and dispersion, suggest that the increased expression of HOXA4 in ovarian carcinomas may constitute a homeostatic response to aberrant cell behavior.

Results

Endogenous HOXA4 Suppresses Transwell Migration, but not Matrigel Invasion, of Ovarian Cancer Cells

In previous studies, we used scratch assays to demonstrate that HOXA4 suppresses cellular motility in ovarian cancer cells, in part, by antagonizing the pro-migratory effects of EGF receptor ligands (6). Based on these results we hypothesized that the effects of HOXA4 may extend to the regulation of invasiveness. Chemoinvasion assays using transwell cell culture inserts coated with reconstituted basement membrane...
Western blots showed that endogenous HOXA4 protein levels varied greatly among ovarian cancer cell lines, with OVCAR-8 and OVCAR-3 cells having the highest levels of expression (Fig. 1A, upper panel). OVCAR-8 and OVCAR-3 cells were transfected with siRNA for 48 hr and seeded in transwell inserts coated with (invasion) or without (migration) Matrigel. HOXA4 siRNA downregulated HOXA4 expression in OVCAR-8 and OVCAR-3 cells (Fig. 1A, lower panel), and significantly increased transwell migration, but not invasion, of both cell lines (Fig. 1B and C). In a previous study, we used scratch assays to show that HOXA4 siRNA has no effect on the motility of HOXA4-negative SKOV-3 cells (6). These results suggest that endogenous HOXA4 suppresses chemotactic migration, but not invasion, of ovarian cancer cells.

Next, studies were carried out with the EGF receptor inhibitor AG1478 to further characterize the effects of HOXA4 on transwell migration. To avoid possible confounding chemotactic effects, AG1478 was applied to the upper and lower chambers of the transwell system. Treatment with 1 M AG1478 significantly reduced basal migration of OVCAR-8 cells and partially inhibited the stimulatory effects of HOXA4 siRNA (Fig. 2). The possibility that the decreased migration induced by AG1478 could be due to a reduction in cell proliferation can be eliminated because short-term treatment with AG1478 for 48 hr does not affect cell proliferation (MTT assay, data not shown). It has been reported that 1 M AG1478 can inhibit ErbB4 (14), however our previous results have shown that HOXA4 siRNA enhances EGF receptor phosphorylation and scratch assay migration following treatment with exogenous EGF (6). Since EGF is not a ligand for ErbB4 we feel that the ability of AG1478 to inhibit HOXA4 siRNA-induced migration is most likely related to its ability to inhibit EGF receptor, although we cannot exclude a role for ErbB4. In addition to corroborating our previous findings, these results suggest that this could be part of a general mechanism of action for HOXA4 that applies to diverse migratory situations.

Endogenous HOXA4 Suppresses Cell Spreading and Enhances Cell-Cell Adhesion

In light of the effects of HOXA4 down-regulation on cell motility, we investigated the effects HOXA4 siRNA on cell/ colony morphology and actin cytoskeleton organization (Fig. 3). OVCAR-8 and OVCAR-3 cells were transfected with siRNA for 72 hr and morphology was assessed by phase contrast microscopy. As shown in Fig. 3A, cell spreading was enhanced by treatment with HOXA4 siRNA in both OVCAR-8 and OVCAR-3 cell lines. Control cells grew in tightly packed epithelial clusters whereas cells treated with HOXA4 siRNA grew in larger, flatter colonies. To confirm this observation we used image analysis to quantify the mean surface area per cell for colonies of varying size. Knockdown of endogenous HOXA4 increased the surface area per cell, and this increase was independent of colony size (Fig. 3B). Next, we examined the effects of HOXA4 siRNA on the actin cytoskeleton by using rhodamine conjugated phalloidin to detect F-actin. In control cells, F-actin was localized at the intercellular junctions (Fig. 3C). Consistent with the spreading phenotype, OVCAR-8 cells transfected with HOXA4 siRNA displayed an increase in filopodia and F-actin was more dispersed within the cytoplasm.

To determine whether changes in cell-cell adhesion were involved in the cell spreading caused by HOXA4 siRNA, OVCAR-8 cells were transfected with siRNA for 72 hr and cell suspensions were maintained in slow gyroratory shaker culture (favouring cell aggregation) for a further 48 hr. The formation of loose cell aggregates (2 cells or >2 cells) or compact spheroids (equal to or greater than 26 μm in diameter) was quantified microscopically. As shown in Fig. 4, treatment with HOXA4 siRNA significantly reduced the formation of multicellular structures (aggregates + spheroids) compared to cells transfected with control siRNA. Taken together, these results suggest that endogenous HOXA4 suppresses cell spreading, in part by enhancing cell-cell adhesion, and thus maintains a compact epithelial growth morphology.

Effects of HOXA4 Knockdown on Morphology in Three-dimensional Culture

Interactions between epithelial cells and the microenvironment, in particular the extracellular matrix, are critical for tissue morphogenesis and differentiation (15). In this context, three-dimensional cell cultures allow for a physiologically relevant modeling of cell behaviour in vitro. We therefore tested the effects of HOXA4 siRNA on the morphology of OVCAR-8 cells in three-dimensional Matrigel cultures. Cells were transfected for 48 hr, seeded in three-dimensional cultures and morphology was examined after 4 days by phase contrast microscopy. OVCAR-8 cells grew as compact cell masses (Fig. 5). Consistent with the results obtained in monolayer cultures, cells transfected with HOXA4 siRNA displayed an

**FIGURE 2.** HOXA4 siRNA-stimulated migration of OVCAR-8 cells is partially inhibited by the EGF receptor inhibitor AG1478. OVCAR-8 cells were transfected for 48 h with Lipofectamine alone (Lipo), control siRNA (siCon), or HOXA4 siRNA (siA4). Trypsinized cells were seeded in transwell inserts (as described in the Materials and Methods section) and cultured for a further 48 h in the absence (DMSO) or presence of the EGF receptor inhibitor AG1478 (1 μM). Medium with 10% FBS was used as a chemoattractant agent and migrating cell nuclei were stained with Hoechst and counted using fluorescence microscopy. Pooled results are presented as the mean ± SEM of three independent experiments and significant differences are indicated by different letters (P < 0.05).
increase in filopodia that in some cases linked separate colonies.

**Down-Regulation of HOXA4 Does Not Affect E- or N-cadherin**

Down-regulation of E-cadherin, and on occasion N-cadherin, plays a critical role in the loss of cell-cell adhesion and the acquisition of migratory and/or invasive properties by epithelial cells (16). In light of the effects of HOXA4 down-regulation on cell motility, spreading and cell-cell adhesion, we hypothesized that HOXA4 may regulate the expression of E- or N-cadherin. As shown in Fig. 6A, total cellular levels of E- and N-cadherin were not altered in OVCAR-8 cells transfected with HOXA4 siRNA (pool or two individual duplexes). Moreover, immunofluorescent staining of E-cadherin confirmed these results and suggested no change in the localization of E-cadherin to sites of cell-cell contact (Fig. 6B).

**Knockdown of Endogenous HOXA4 Reduces Protein, but not mRNA, Levels of β1 Integrin**

The integrin family of glycoproteins form heterodimeric receptors for extracellular matrix molecules and are crucial regulators of cell adhesion and migration (17, 18). In ovarian cancer, β1 integrins participate in the initial adhesion of ovarian
Knockdown of endogenous HOXA4 reduces cell-cell adhesion. OVCAR-8 cells were transfected for 72 h with control siRNA (siCon) or HOXA4 siRNA (siA4). Cell suspensions were maintained in slow gyratory shaker culture (as described in the Materials and Methods section) for a further 48 h and the formation of loose cell aggregates or compact spheroids was quantified microscopically. Pooled results are presented as the mean ± SEM of three independent experiments and significant differences are indicated by different letters (P < 0.05).

FIGURE 4.

Effects of HOXA4 siRNA on the morphology of OVCAR-8 cells in three-dimensional Matrigel cultures. Cells were transfected for 48 h with control siRNA (siCon) or HOXA4 siRNA (siA4). Trypsinized cells were seeded in three-dimensional cultures (as described in the Materials and Methods section) and morphology was examined after 4 d by phase contrast microscopy. Scale bar: 100 μm.

To further examine the relationship between HOXA4, β1 integrin and cell spreading, we next investigated the effects of changes in cell-substratum and/or cell-cell adhesion on HOXA4 siRNA-mediated changes in β1 integrin protein levels and cell spreading (Fig. 8). OVCAR-8 cells were transfected with siRNA for 72 h and trypsinized cells were re-seeded on plastic, fibronectin or maintained in fast gyratory shaker culture (minimizes cell aggregation and abolishes cell-substratum adhesion). Alternatively, transfected cells were re-seeded on agarose to maximize cell aggregation in the absence of cell-substratum adhesion. Cells were re-seeded in medium depleted of extracellular matrix molecules to minimize the confounding effects of serum-derived fibronectin and vitronectin. Down-regulation of HOXA4 reduced β1 integrin protein levels in cells re-seeded on plastic, fibronectin or agarose, but not in cells maintained in fast gyratory shaker culture (Fig. 8A). Cells treated with control siRNA displayed increased spreading when re-seeded on fibronectin (Fig. 8B), however this was
not associated with reduced \( \beta_1 \) integrin protein levels (Fig. 8A). Interestingly, treatment with HOXA4 siRNA increases the spreading of cells re-seeded on either plastic or fibronectin (Fig. 8B). Moreover, the effects of HOXA4 siRNA on cell spreading seem to be additive to those of fibronectin. Taken together, these results suggest that the ability of endogenous HOXA4 to maintain elevated \( \beta_1 \) integrin protein levels may be related to its ability to enhance cell-cell adhesion, as it appears to be relatively insensitive to manipulations of cell-substratum adhesion.
Overexpression of HOXA4 Suppresses Transwell Migration and Increases β1 Integrin, but not in HOXA4-Negative Cell Lines

Thus far, we have used a loss-of-function approach to demonstrate that endogenous HOXA4 suppresses chemotactic transwell migration and maintains elevated β1 integrin protein levels (Figs. 1C, 6C, D and 8A). These studies were complemented by gain-of-function experiments using full-length HOXA4 cDNA. HOXA4 was ectopically expressed in cell lines where it was undetectable by Western blot (SKOV-3, OVCAR-5 and A2780; see Fig. 1A). Alternatively, HOXA4 was overexpressed in CaOV-3 cells, which express low levels of HOXA4. We initially investigated the effects of transient transfection of HOXA4 on β1 integrin protein levels and transwell migration (Fig. 9A and B). Strong ectopic expression of full-length HOXA4 in SKOV-3 and OVCAR-5 cells had no effect on β1 integrin protein levels (Fig. 9A) or transwell migration (Fig. 9B, SKOV-3 data not shown). In contrast, overexpression of HOXA4 in CaOV-3 cells increased β1 integrin protein levels, and significantly decreased transwell migration (Fig. 9A and B). To verify the ineffectiveness of ectopic expression, we stably expressed HOXA4 in SKOV-3 and A2780 cells (Fig. 9C and D). Stable expression of HOXA4 was confirmed by RT-PCR using transient transfection and/or endogenous expression in OVCAR-8 cells as positive controls (Fig. 9C). Similar to the results from transient transfections, stable ectopic expression of HOXA4 in SKOV-3 and A2780 cells does not affect transwell migration (Fig. 9D). Results from the overexpression of HOXA4 in CaOV-3 cells are consistent with the results obtained using siRNA, and suggest that endogenous expression of HOXA4 suppresses chemotactic transwell migration and increases β1 integrin protein levels. However, our data also indicate that the function of HOXA4 is dependent on cell context, as ectopic expression in HOXA4-negative cell lines was not effective.

Discussion

Accumulating evidence suggests that HOX genes play a role in ovarian cancer development and/or progression (2, 3, 5, 10-12). However, functional roles and putative mechanisms of action for individual HOX genes remain poorly understood. In a previous study, we used scratch assays to demonstrate that HOXA4 suppresses cellular motility in ovarian cancer cells (6). In the present study, we used siRNA and forced-expression to define the role of HOXA4 in the regulation of chemotactic migration/invasion and cellular/colony morphology. We also demonstrate that modulation of HOXA4 regulates β1 integrin protein levels, but has no effect on αv or β3 integrin and E- or N-cadherin. Furthermore, our studies suggest a tumor-suppressive, homeostatic role for HOXA4 in ovarian neoplastic progression that may be linked to morphologic epithelial differentiation.

The ability of HOX genes to regulate migration and/or invasion has been demonstrated in a variety of cancers, including lung cancer (23), melanoma (24), breast cancer (25-27), endometrial cancer (28), and cervical cancer (29). In ovarian cancer, pro-invasive roles for HOXB7 and HOXB13 have been demonstrated in SKOV-3 cells (11). In contrast, this study shows that knockdown of HOXA4 in OVCAR-8 and OVCAR-3 cells increases transwell chemotactic migration, whereas overexpression in CaOV-3 cells suppresses migration. The ability of

![FIGURE 7](#) Knockdown of endogenous HOXA4 does not affect β1 integrin, α5 integrin or fibronectin mRNA levels. OVCAR-8 cells were transfected for 24, 48, 72 or 96 h with Lipofectamine alone (Lipo), control siRNA (siCon), or HOXA4 siRNA (siA4). HOXA4, β1 integrin (ITGβ1), α5 integrin (ITGα5) and fibronectin 1 (FN1) mRNA levels were analyzed by semi-quantitative RT-PCR (normalized to GAPDH). Combined results (mean ± SEM) from two independent experiments are given as a percentage change with respect to a time-matched Lipofectamine control.
HOXA4 siRNA to increase transwell migration but not chemoinvasion through Matrigel of two relatively non-invasive cell lines (OVCAR-8 and OVCAR-3) suggests that the effects of HOXA4 may be restricted to the regulation of cell motility, and are not likely to involve the modulation of protease secretion and/or activity. On the other hand, in more invasive cell lines (i.e. with greater protease secretion and/or activity) it is likely that HOXA4-induced changes in migration would lead to changes in invasion, as motility is required for invasion. The suppressive effects of endogenous HOXA4 on cell spreading are also consistent with a less motile phenotype. The process of cell spreading is at least partially analogous to cell migration, and our results suggest that endogenous HOXA4 acts to restrict cell spreading and maintain a compact epithelial growth morphology, at least in part by enhancing and/or maintaining cell-cell adhesion. Results from two- and three-dimensional cultures of siRNA-treated cells suggest that cell dispersion may also be limited by inhibiting the formation of filopodia. Filopodia are important mediators of cell adhesion and migration because they contain adhesion molecules, such as integrins and cadherins, and receptors for signaling molecules (30). Together these data provide convincing evidence that endogenous HOXA4 contributes to a non-motile phenotype and thus may play an inhibitory role in ovarian cancer invasiveness.

The acquisition of migratory and/or invasive properties by epithelial cells is often associated with reductions in cell-cell adhesion, particularly that mediated by E-cadherin (16). The regulation of E- or N-cadherin expression by HOX proteins has been previously demonstrated for HOXD3 (23), HOXA7 (2), and HOXA10 (28). On the other hand, Hoxa4 null or over-expressing mouse embryos had no change in N-cadherin (31). Our slow gyration shaker culture results suggest that endogenous HOXA4 enhances and/or maintains cell-cell adhesion. This is supported by our siRNA data showing that cell spreading is enhanced throughout the colony, rather than just on the periphery, implying a colony-wide increase in cell-substratum contact that would necessitate a reduction in cell-cell contact. Similarly, enforced expression of HOXA10 in endometrial cancer cells promotes cell-cell adhesion and suppresses invasiveness by inducing the expression of E-cadherin (28). In contrast, although we observed significant changes in cell-cell adhesion, cell spreading and migration in HOXA4 siRNA-treated cells, these do not appear to be associated with changes in the expression or localization of E- or N-cadherin. Future studies will be required to elucidate the mechanism of HOXA4 effects on cell-cell adhesion, however β1 integrin is known to participate in a variety of intercellular adhesive structures (18, 32). Nevertheless, cell migration can occur in the presence of cadherin-mediated cell-cell junctions through collective cell migration, a process ubiquitous in development, tissue repair and tumor invasion (32).

Integrin family proteins are important mediators of cell migration and invasion, not only because they regulate cell-substratum adhesion, but also because they send and receive signals that regulate these processes (17). Our data suggest that endogenous HOXA4 suppresses cell motility and spreading while maintaining elevated β1 integrin protein levels. Although this may appear contradictory, the relationship between migration and cell-substratum adhesiveness is biphasic and depends not only on integrin expression level, but also on extracellular matrix ligand level and integrin binding affinity (17, 33). Thus, elevated integrin expression can inhibit motility in situations of high integrin binding affinity or ligand concentration. Moreover, elevated integrin expression may suppress cell motility and spreading by enhancing cell-cell adhesion. In this context, it is tempting to speculate that the effects of endogenous HOXA4 on cell motility, spreading and cell-cell adhesion may lead to the suppression of intraperitoneal dissemination and invasion. It should be noted that our study has yet to fully...
resolve which α integrin(s) or extracellular matrix ligand(s) are
mediating the effects of HOXA4. Moreover, it is still uncertain
whether the changes in β1 integrin are responsible for, or the
product of, the phenotypic changes observed. Interestingly, en-
forced expression of HOXB4 (a paralog of HOXA4) in human
neonatal keratinocytes decreased adhesion to plastic and was
associated with reductions in the surface expression of CD44
and α2 integrin (34). CD44 also participates, in concert with
β1 integrins, in the adhesion of ovarian cancer cells to perito-
neal mesothelial cells (19, 20). A detailed analysis of the effects
of HOXA4 on integrin and extracellular matrix adhesion pro-
files in ovarian cancer cells awaits future studies. Nonetheless,
our data do support the hypothesis that HOXA4 regulates the
adhesive properties of ovarian cancer cells.

Although the regulation of integrins by HOX genes has been
described in other cancer cells (23, 26, 35), the present study is
the first to show this in ovarian cancer cells. Moreover, our data
are unique in that they suggest an indirect form of regulation
that is likely occurring at a post-transcriptional level. Though
indirect, the effects of HOXA4 on β1 integrin levels do appear
to be specific since HOXA4 siRNA reduced β1 integrin levels
in OVCAR-8 and OVCAR-3 cells whereas overexpression of
HOXA4 increased β1 integrin levels in CaOV-3 cells. In addi-
tion, not only is the relationship between HOXA4 siRNA-
mediated cell spreading and reductions in β1 integrin protein
consistent, but it suggests a mechanism that may be distinct
from spreading induced by enhanced cell-substratum adhesion.
In this study, fibronectin enhanced the spreading/flattening of
control cells compared to growth on plastic, but this was not
associated with reductions in β1 integrin protein. Thus, cell
spreading is not necessarily associated with decreased levels
of β1 integrin, at least in the case of fibronectin-coating which
most likely increases spreading by enhancing cell-substratum
adhesion. In contrast, HOXA4 siRNA-mediated cell spreading
is consistently associated with decreased β1 integrin protein
levels and appears to be relatively insensitive to manipulation
of cell-substratum adhesion (i.e. the effects are observed on plas-
tic, in the presence of high or low levels of serum-derived vi-
tronectin, and on fibronectin). However, the effects of HOXA4
siRNA on β1 integrin are abolished when both cell-cell and
cell-substratum adhesion are removed by fast gyratory shaker
culture. Since manipulation of cell-substratum adhesion did
not affect the ability of HOXA4 siRNA to induce spreading
or decrease β1 integrin, then perhaps it is the loss of cell-cell
adhesion that abolishes the effect of HOXA4 siRNA on β1 in-
tegrin protein levels. Indeed, maximizing cell-cell adhesion in

FIGURE 9. Overexpression of HOXA4 suppresses transwell migration and increases β1 integrin protein levels, but not in HOXA4-negative cell lines. A and B. Effects of transient transfection of HOXA4 in cells with no detectable HOXA4 (SKOV-3 and OVCAR-5) or low endogenous HOXA4 (CaOV-3). A. Cells were transfected for 48 h with control vector (LacZ) or vector containing full-length HOXA4 cDNA, and total cellular levels of β1 integrin (ITGβ1) were analyzed by Western blot. B. Cells were transfected for 48 h, trypsinized, seeded in transwell inserts (as described in the Materials and Methods section) and cultured for a further 24 h. Medium with 10% FBS was used as a chemotactic agent and migrating cell nuclei were stained with Hoechst and counted using fluorescence microscopy. Pooled results are presented as the mean ± SEM of three independent experiments and significant differences are indicated by different letters (P < 0.05). C and D. Stable expression of HOXA4 in cells with no detectable HOXA4 does not affect transwell migration. C. RT-PCR analysis of HOXA4 expression in SKOV-3 (upper) and A2780 (lower) cell lines stably transfected with control vector (LacZ) or HOXA4 vector. Transient transfection and endogenous expression in OVCAR-8 cells were used as positive controls. D. Transwell migration (24 h) of stably transfected cells. Pooled results are presented as the mean ± SEM of five (SKOV-3) and two (A2780) independent experiments.
the absence of cell-substratum adhesion was able to restore the effects of HOXA4 siRNA on β1 integrin. Together with our slow gyratory shaker culture results suggesting that endogenous HOXA4 enhances and/or maintains cell-cell adhesion, these data support the hypothesis that the effects of HOXA4 on cell spreading, motility and β1 integrin may be related to its effects on cell-cell adhesion. If endogenous HOXA4 exerts its effects through cell-cell adhesion, that might explain why the effects of HOXA4 siRNA on cell spreading seem to be additive to those of farnesin. At this point we can only speculate about possible mechanisms, however there is now abundant evidence to suggest that membrane trafficking pathways that recycle adhesion receptors via endosomal compartments contribute to migration, and that certain pathways are attuned to transporting particular integrins (36-38). Indeed, endocytosis of β1 integrin is important for L1 cell adhesion molecule-induced migration of HEK293 cells (39). Perhaps HOXA4-induced changes in β1 integrin levels reflect a shift in the surface integrin profile via the selective regulation of specific αβ integrin heterodimers.

Interestingly, our study also suggests that HOXA4 functions in a cell context-dependent fashion. Knockdown or overexpression of HOXA4 was associated with changes in cell motility, spreading and β1 integrin levels (OVCAR-8, OVCAR-3 and CaOV-3), whereas ectopic expression in HOXA4-negative cell lines was not (SKOV-3, OVCAR-5 and A2780). The importance of contextual information in contributing to the functional specificity of HOX proteins is well recognized, however the mechanisms underlying the integration of contextual information remain elusive (40, 41). Our study suggests that HOXA4-negative cell lines may be lacking a component(s) which is important for the function of HOXA4. We have previously reported that HOXA4 expression is proportional to morphologic epithelial differentiation in ovarian cancer cells (6). This is supported herein by the finding that endogenous HOXA4 maintains a compact epithelial growth morphology by suppressing cell spreading and enhancing cell-cell adhesion. These data raise the possibility that the expression and function of HOXA4 may be linked to epithelial differentiation. Our results suggest that the increase in expression of HOXA4, which functions as an inhibitor of cell dispersion and motility, in serous ovarian carcinomas represents a homeostatic response to aberrant cell behaviour (i.e. increased cell dissociation and motility). Future studies will be required to fully characterize the pathological significance of HOXA4 expression in ovarian tumors of differing grade and histological subtype. These functions suggest that HOXA4 acts as an ovarian epithelial tumor suppressor whose mechanism of action and clinical significance warrant further investigation.

Materials and Methods

Cell Culture

Normal human ovarian surface epithelial cells were obtained during laparoscopic procedures for nonmalignant gynecological conditions and informed consent was obtained in accordance with institutional regulations. Ovarian surface epithelial cell cultures were established as previously described (42), and were maintained in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma-Aldrich, Oakville, ON) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Logan, UT). OVCAR-8, OVCAR-5 and A2780 ovarian cancer cell lines were kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). OVCAR-3, CaOV-3 and SKOV-3 ovarian cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Unless otherwise specified, ovarian cancer cell lines were maintained in M199/MCDB105 supplemented with 5% FBS. All cells were grown at 37°C in a humidified 5% CO2/air atmosphere.

Knockdown of HOXA4

All siRNAs were purchased from Thermo Fisher Scientific (Lafayette, CO). The sequences of siGENOME SMARTpool siRNA duplexes targeting HOXA4 were as follows (sense, 5'-3'): GCA AGG AGC CCG UGG UGU AUU, AAG AUG CGA UCC UCC AAU UUU, CUA CAU CGA GCC CAA GUU CUU and GCC CAC ACG CUC UGU UUG UUU. Initial studies utilized siCONTROL non-targeting siRNA #2 which was replaced with ON-TARGETplus siCONTROL non-targeting pool. Unless otherwise specified, OVCAR-8, OVCAR-3 and SKOV-3 cells were plated at densities of 9,500, 14,000 and 4,200 cells/cm2, respectively. Cells were transfected one day after seeding using Lipofectamine RNAiMAX and Opti-MEM I according to the manufacturer's instructions (Invitrogen, Burlington, ON). Cells were transfected with 70 nM control or HOXA4 siRNA pools or with 17.5 nM individual HOXA4 siRNA duplexes. As an additional control, cells were mock transfected with Lipofectamine alone. We have previously demonstrated transfection efficiencies ≥ 80% for OVCAR-8, OVCAR-3 and SKOV-3 cells using siGLO Green fluorescent oligonucleotides (6).

Antibodies and Western Blots

The specificity of the rabbit polyclonal HOXA4 antibody (Abcam, Cambridge, MA) has been previously demonstrated (6), and was further validated in the present study by the detection of exogenously expressed HOXA4. The antibody was diluted 1:40,000 and the size of HOXA4 is ~39 kDa. Mouse monoclonal antibodies for E-cadherin, N-cadherin, αV integrin and β1 integrin (all from BD Biosciences, Mississauga, ON) were diluted 1:40,000, 1:20,000, 1:500 and 1:40,000, respectively. Rabbit monoclonal β3 integrin antibody (Epitomics, Burlingame, CA) was diluted 1:1,000. Goat polyclonal actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:8,000. All antibodies were diluted in TBS with 5% (w/v) skim milk and 0.1% (v/v) Tween-20. Cells were washed with ice-cold PBS and lysed with cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4 and 1 μg/mL leupeptin; Cell Signaling Technology, Danvers, MA) containing freshly added protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were sonicated, centrifuged for 30 min at 20,000 g and supernatants were collected and stored at -70°C until used. Protein concentrations were quantified using the DC assay (Bio-Rad Laboratories, Mississauga, ON) with bovine serum albumin as a standard. Equal amounts of protein (20 μg) were subjected to reducing SDS polyacrylamide gel electrophoresis prior to being...
Transwell Migration/Invasion Assays

Cell culture inserts (24-well, pore size 8 μm; BD Biosciences) were seeded with 75,000 cells in 500 μL of medium with 0.1% FBS. Un-coated inserts were used for migration assays whereas inserts pre-coated with growth factor reduced Matrigel (40 μL, 1 mg/mL; BD Biosciences) were used for invasion assays. Medium with 10% FBS (750 μL) was added to the lower chamber and served as a chemotactic agent. Non-migrating/intruding cells were wiped from the upper side of the membrane and cells on the lower side were fixed in cold methanol (-20°C) and air dried. Cell nuclei were stained with the membrane and cells on the lower side were fixed in cold methanol and post-fixed for 5 min in 20% HCl to block endogenous peroxidase activity. The membranes were then washed thoroughly with PBS and permeabilized for 5 min in PBS containing 0.1% (v/v) Triton X-100. All coverslips were washed three times with PBS prior to blocking for 1 hr at room temperature with Protein Block (1% non-fat dry milk, 2% bovine serum albumin in PBS). For E-cadherin immunostaining, coverslips were fixed for 20 min in cold methanol (-20°C) and post-fixed for 5 min in cold methanol/acetone (1:1, v/v). For β1 integrin immunostaining, coverslips were fixed for 10 min at room temperature in PBS with 4% (v/v) paraformaldehyde, washed three times and permeabilized for 5 min in PBS with 0.05% (v/v) Triton X-100. All coverslips were washed three times with PBS prior to blocking for 1 hr at room temperature with Protein Block (serum-free; DAKO, Mississauga, ON). E-cadherin antibody was diluted 1:500 in Protein Block and incubated for 1 hr at room temperature. β1 integrin antibody was diluted 1:100 in cell aggregation and formation of compact spheroids (10× objective). At least 150 single cells, loose aggregates or spheroids were counted for each siRNA treatment in each of three separate experiments.

Transwell migration assays were performed using 24-well Transwell inserts (BD Biosciences) on an inverted phase contrast microscope (10× objective) three days after transfection. At least 150 single cells, loose aggregates or spheroids were counted for each siRNA treatment in each of three separate experiments.

Analysis of Cell Spreading

OVCAR-8 and OVCAR-3 cells were plated at densities of 6,300 and 9,500 cells/cm², respectively. Morphology was assessed by phase contrast microscopy (10× objective) three days after transfection using a Nikon Eclipse TE300 microscope equipped with a digital camera (QImaging, Surrey, BC). Image analysis software (Northern Eclipse 6.0; Empix Imaging, Mississauga, ON) was used to quantify the mean surface area per cell (μm²/cell) for colonies of varying size (between 5 and 40 cells per colony). Within this range of colony sizes, colony surface area increases linearly as the number of cells increases (regression analysis, OVCAR-8 r² = 0.58, OVCAR-3 r² = 0.73). In addition, surface area per cell does not change considerably as the number of cells per colony increases (OVCAR-8 r² = 0.054, OVCAR-3 r² = 0.014). Results are presented as mean ± SEM (≥9 colonies per colony size, ≥45 colonies and ≥905 cells in total for each siRNA treatment) and are representative of at least three independent experiments.

Suspension Cultures

For the analysis of cell-cell adhesion, OVCAR-8 cells were transfected for 72 hr, trypsinized and maintained in slow gyulatory shaker culture which favours cell aggregation (>70% of the cells in multicellular structures). Cells (1 × 10⁶) were suspended in 6 mL of medium with 10% FBS, placed in 50 mL Teflon Erlenmeyer flasks and shaken at 37°C for 24 hr at 100 rpm to allow for recovery from trypsinization, followed by another 24 hr at 60 rpm. Aliquots of the cell suspensions were removed after 24 and 48 hr and analyzed microscopically for cell aggregation and formation of compact spheroids (10× objective). At least 150 single cells, loose aggregates or spheroids were counted for each siRNA treatment in each of three separate experiments.

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Three-dimensional Cultures

Three-dimensional cultures were prepared using a modified protocol described by Carrió et al. (26). Briefly, transfected OVCAR-8 cells were trypsinized and seeded (30,000 cells per well in 24-well plates) on top of 200 μL of polymerized growth factor reduced Matrigel (10.6 mg/mL) in 250 μL of medium without serum. After 30 min of incubation at 37°C, 250 μL of medium with 10% FBS and 1 mg/mL Matrigel was added on top of the cells. The morphology of the three-dimensional cultures was examined after 4 days by phase contrast microscopy (10× objective). Results are representative of two separate experiments with duplicate wells.

Immunofluorescence and Filamentous Actin Staining

OVCAR-8 cells were seeded on glass coverslips at a density of 6,300 cells/cm², transfected with siRNA for 72 hr and washed once with medium without serum prior to fixation. For E-cadherin immunostaining, coverslips were fixed for 20 min in cold methanol (-20°C) and post-fixed for 5 min in cold methanol/acetone (1:1, v/v). For β1 integrin immunostaining, coverslips were fixed for 10 min at room temperature in PBS with 4% (v/v) paraformaldehyde, washed three times and permeabilized for 5 min in PBS with 0.05% (v/v) Triton X-100. All coverslips were washed three times with PBS prior to blocking for 1 hr at room temperature with Protein Block (serum-free; DAKO, Mississauga, ON). E-cadherin antibody was diluted 1:500 in Protein Block and incubated for 1 hr at room temperature. β1 integrin antibody was diluted 1:100 in cell aggregation and formation of compact spheroids (10× objective). At least 150 single cells, loose aggregates or spheroids were counted for each siRNA treatment in each of three separate experiments.

Fast gyrotary shaker culture, which minimizes cell aggregation (60% single cells, very few loose aggregates of >4 cells and no compact spheroids), was used in studies examining the effects of changes in cell-substratum and/or cell-cell adhesion. To minimize the confounding effects of serum-derived fibronectin and vitronectin, medium with 10% FBS was depleted of extracellular matrix molecules by incubating thin (2.5 mm) layers of medium in tissue culture dishes at 37°C for 2 hr as previously described (22). Transfected OVCAR-8 cells (2.4 × 10⁶) were suspended in 15 mL of depleted medium, placed in 50 mL Teflon Erlenmeyer flasks and shaken (200 rpm) for 5 hr at 37°C to allow for recovery from trypsinization. After recovery, two-thirds of the cells were re-seeded on plastic or fibronectin-coated dishes (human plasma fibronectin, 5 μg/cm²; Sigma-Aldrich), and the remaining third were maintained in fast gyrotary shaker culture (200 rpm). After 20 hr of culture, morphology was assessed by phase contrast microscopy and the cells were lysed for Western blot analysis as described above.

To maximize cell aggregation in the absence of cell-substratum adhesion, OVCAR-8 cells were transfected for 72 hr, suspended in depleted medium and re-seeded (6 × 10⁵ cells per 35 mm dish) on agarose-coated dishes (1% w/v, 2.3 mL per dish). Cells were lysed for Western blot analysis after 20 hr of culture. Cell viability in suspension cultures was assessed prior to lysis by Trypan blue exclusion and non-viable cells were ≤7% in all experiments.

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Protein Block and incubated overnight at 4°C. After three washes with PBS, coverslips were incubated for 1 hr at room temperature with Alexa Fluor 594 goat anti-mouse (Invitrogen) diluted 1:800 in Protein Block. Coverslips were washed three more times with PBS and cell nuclei were stained with Hoechst 33258. For filamentous actin (F-actin) staining, coverslips were fixed, permeabilized and blocked as described above for β1 integrin immunostaining. F-actin was stained with rhodamine-conjugated phalloidin (Invitrogen) diluted in Protein Block (1 U/mL) for 20 min at room temperature. All coverslips were washed a final three times with PBS, mounted with Gelvatol and examined using a Zeiss Axiopt photomicroscope (40× or 40× oil immersion objectives).

RT-PCR
Cells were washed once with ice-cold PBS and total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Mississauga, ON). cDNA was reverse transcribed from 2 μg of total RNA using the First-Strand cDNA Synthesis Kit and NotI-d(dT)18 primers (GE Healthcare Bio-Sciences, Baie d’Urfé, QC), and 133 ng was used as template for PCR. All primers spanned at least one intron so as to detect specific mRNA sequences. Cycle tests and cDNA titrations were performed to ensure a linear range of amplification. Primer sequences (5′-3′), annealing temperatures, cycle numbers and product sizes were as follows: HOX4 (58°C, 28 cycles, 226 bp) CCC TGG ATG AAG AAG ATC CA and TCC ACT TCA TTC TCC GGT TC; β1 integrin (60°C, 22 cycles, 271 bp) TTG CAG TTG GTG CAT CAC TGA TT and AGC TCC TTG TAA ACA GCA TGA A; α5 integrin (61°C, 26 cycles, 279 bp) GCA GCT CCT ATG GAC CAG CAG AGT TA and TCC CGC TGC AAG AAA GTC TT; fibronectin 1 (63°C, 28 cycles, 553 bp) CAC TTC TAG AAA TAG ATG CAA CGA TCA and CTG AAC CAA AAC AGT GTG GTC GTG TT; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 60°C, 18 cycles, 373 bp) TTG CAG TTG GTG GTG AAC CA and TGG CAG GTT TTT CTA GAG AAC GGT GC. Following an initial 5 min at 94°C, each cycle consisted of denaturation at 94°C for 45 seconds, annealing at the specified temperature for 30 seconds and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 10 min, and all PCR reactions were carried out in a Mastercycler Personal thermal cycler (Eppendorf, Mississauga, ON). PCR products were analyzed by gel electrophoresis, quantified by densitometry (Biometra – BioDocAnalyze 1.0; Montreal Biotech, Kirkland, QC) and normalized to GAPDH.

Cloning and Expression of HOX4
Full-length HOX4 (980 bp) was amplified by RT-PCR using the FasIlSafe PCR Selection Kit (Epigence Biotechnologies, Madison, WI) and the following primers (5′-3′): TTG CAC TTC ACA AAT TAA TGA CCA TGA GC and TTA TAT GGA GGA GGA AAC GAG TGT GGA. Gel purified PCR product was cloned into the pcDNA3.1 vector using the TOPO TA Expression Kit (Invitrogen) and confirmed by DNA sequencing (Child & Family Research Institute DNA Sequencing Core Facility, Vancouver, BC). pcDNA3.1..LacZ was used as a control vector. Cells at 80% confluency were transfected with FuGENE HD (Roche, Laval, QC) and Opti-MEM I according to the manufacturer’s instructions. Stably transfected cells were generated by selection for 2-3 weeks with 600 μg/mL Geneticin (G-418, Invitrogen).

Statistical Analysis
Results from a minimum of three independent experiments performed on separate cell passages were pooled and analyzed by one-way ANOVA. If the ANOVA analysis showed that a significant difference existed among the groups, the Student–Newman-Keuls (SNK) test for multiple comparisons of means was performed to identify the treatment groups that were different from one another. Means were considered statistically different if P < 0.05 and are indicated by different letters.

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

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References


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Cell Motility and Spreading Are Suppressed by HOXA4 in Ovarian Cancer Cells: Possible Involvement of β1 Integlin

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