CLINICAL & EXPERIMENTAL METASTASIS

HOXD3-Overexpression Increases Integrin αvβ3 Expression and Deprives E-cadherin while It Enhances Cell Motility in A549 Cells

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Running Title: HOXD3 induces integrin αvβ3-dependent motility

Abstract

We have previously shown that transduction of *HOXD3*, one of homeobox genes, into human lung cancer A549 cells enhances cell motility, invasion and metastasis. In the present study, we examined the roles of integrin β3 which was up-regulated by *HOXD3*-overexpression in the *HOXD3*-induced motility of A549 cells. We first established integrin β3-transfectants and compared their motile activity to those of the *HOXD3*-transfected, control-transfected and parental cells by three different assays. The integrin β3-transfectants as well as the *HOXD3*-transfectants formed heterodimer with integrin αv subunit, and showed highly motile activities assessed by haptotaxis or phagokinetic track assay compared to the control transfectants or parental cells. *In vitr*o wound-healing assay revealed that migratory activities were graded as the *HOXD3*-transfectants > the integrin β3-transfectants > the control transfectants or parental cells. E-cadherin was expressed in the integrin β3-transfectants but not expressed in the *HOXD3*-transfectants. An addition of function-blocking antibody to E-cadherin into the wound-healing assay promoted the migratory activity of the integrin β3-transfectants, suggesting that E-cadherin prevented the cells from dissociating from the wound edges. These results indicate that increased expression of integrin αv β3 and loss of E-cadherin by *HOXD3*-overexpression are responsible for the enhanced motility and dissociation.

Key words: chemotaxis, E-cadherin, HOXD3, integrin αvβ3, migration, phagokinesis, wound healing assay.

Introduction

The metastatic process is a series of sequential steps in which tumor cells are released from the primary tumor and disseminated to distant sites, where they proliferate to form new tumor foci [1, 2]. Dynamic cell movement observed in tumor metastasis is similar to that observed during embryonic morphogenesis, although there is a difference that tumor cells move, destroying normal tissues, whereas normal cells move, maintaining homeostasis in morphogenesis. We hypothesized therefore that metastatic tumor cells might undergo at least a part of the genetic program of embryonic morphogenesis.

Homeobox-containing genes are well known as master control genes in development [3]. One family of human homeobox-containing genes is *HOX* whose prototype is *HOM* in *Drosophila* [4]. Thirty-nine *HOX* genes are clustered in a similar arrangement of 13 paralog groups on four different chromosomal/genomic regions, *HOXA*, *B*, *C*, and *D* and encode transcription factors [4-7]. They are expressed in a spatiotemporal manner during embryonic morphogenesis, each regulating a group of genes involved in modeling a specific segmental architecture. Their expression has also been detected in normal adult tissues with characteristic patterns, suggesting their possible role in the maintenance of normal tissue homeostasis [8, 9].

HOX gene products control the expression of a set of morphogenesis-associated genes, one of which is cell adhesion molecules. Accumulated evidence indicates that several adhesion molecules are target genes for *HOX* gene products. For example, experiments by co-transfection into NIH3T3 cells indicate that Xenopus *HoxC6*, *HoxB9* and *HoxB8* modulate promoter activity of mouse neural cell adhesion

molecule (N-CAM) [10, 11]. Human melanoma cells whose *HOX C* locus genes are silent show high levels of integrin α2β1, α5β1, α6β1 and intercellular cell adhesion molecule (ICAM) whereas those whose *HOX C* locus genes are actively expressed show low levels of ICAM-1 and lack α2β1, α5β1, α6β1 [12]. Ectopic expression of *HOXA7* induces E-cadherin expression in immortalized ovarian surface epithelial cells [13]. We have provided evidence that transduction of *HOXD3* gene into human erythroleukemia HEL cells leads expression of integrin β3 which forms heterodimer with αIIb [14]. Boudreau et al. also have observed that integrin β3 is one of the target genes for *HOXD3* in their experiment using human endothelial cells[15]. Further, our previous study has shown that human lung cancer A549 cells overexpressing *HOXD3* gene enhance the expressions of N-cadherin and integrins such as α3, α4 and β3 subunits, and eliminate E-cadherin expression [16]. It is reasonable that some HOX genes regulate directly or indirectly the expressions of cell adhesion molecules because the molecules play an important role in cell-cell and cell-matrix recognition which leads cell proliferation, migration, differentiation and death in morphogenesis. Thus, the aberrant expression of *HOX* genes in tumor cells is thought to induce the invasive and metastatic phenotype through the altered expression of cell adhesion molecules.

In the present study, we report that integrin β3, which is up-regulated by *HOXD3*-overexpression, functioned as a major motility-promoting molecule and that loss of E-cadherin expression by *HOXD3*-overexpression may facilitate cell scattering.

Materials and methods

Antibodies and reagents A mouse monoclonal antibody to integrin β3 (RUU-PL7F12) was purchased from Becton Dickinson (San Jose, CA). Mouse monoclonal antibodies to integrin β1 (P5D2), integrin α4 (P4C2), integrin αvβ3 (LM609), integrin αvβ5 (P1F6), integrin αvβ6 (E7P6) and normal mouse IgG were from Chemicon International (Temecula, CA). LM609 recognizes αvβ3 heterodimer and inhibits adhesion of cells to vitronectin through αvβ3 [17]. A mouse monoclonal antibody to E-cadherin (clone 36) for immunoblotting was from Transduction Laboratories (Lexington, KY). A mouse monoclonal antibody to E-cadherin (HECD-1) for blocking E-cadherin dependent cell-cell contact was from Takara Shuzo (Ohtsu, Japan). A fluorescein-5-isothiocyanate-conjugated goat polyclonal antibody to mouse IgG was from Cappel (Aurora, OH). A peroxidase-conjugated sheep polyclonal antibody to mouse Ig was from Amersham (Little Chalfont, England). Bovine plasma vitronectin was from Yagai (Yamagata, Japan). Bovine plasma fibronectin, mouse laminin-1, Lipofectamine PLUS and TRIzol were from Gibco BRL (Grand island, NY). Human fibrinogen was from Sigma (St. Louis, MO). Bovine serum albumin was from Boehringer Mannheim (Mannheim, Germany). Hygromycin B was from Wako (Tokyo, Japan). G418 sulfate was from Cellgro (Herndon, VA). The Transwell chamber and culture dishes were from Costar (Cambridge, MA). Tissue culture plates and dishes were from Becton Dickinson (Lincoln Park, NJ).

Cells and cell culture Human lung cancer A549 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). The cells were grown on tissue culture dishes in a 1: 1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12) supplemented with 5% of heat-inactivated fetal bovine serum (FBS). HOX+1 and HOX+2 were *HOXD*3-overexpressing clones isolated from the parental cells which had been

transfecetd with the *HOXD3* expression vector pMAMneo-*HOX4A*. Neo1 and Neo2 were control clones isolated from the parental cells which had been transfected with only pMAMneo vector. The HOX+1, HOX+2, Neo1 and Neo2 cells were grown in DME/F12 containing 5% FBS and 400 μg/ml of G418 sulfate. All cell lines were cultured in a CO_2 incubator (5% CO_2 and 95% air).

Construction of plasmids Full length cDNA encoding the human integrin β3 subunit was kindly provided by Dr. David A. Cheresh (The Scripps Research Institute, La Jolla, CA) [18]. From this cDNA, the region of integrin β3 containing translation-starting codon to stop codon was amplified by PCR with the use of the forward primer 5'-CGGGAGGCGGACGAGATGCGAGC-3' and the reverse primer 5'-GCCCCGAAATCCCTCCCCACAAATACTG-3'. The PCR product was subcloned into the multi-cloning site of the pTARGET (pTARGET-Int β3) (Promega. Madison, WI). The pTARGET-Int β3 was digested with *Xho*I and *Sal*I and subcloned into the multi-cloning site of pcDNA3.1Hyg(+) (Invitrogen,) which had been digested with *Xho*I (pcDNAHyg(+)-Intβ3).

Transfection and cell cloning. The transfection of pcDNA3.1Hyg(+)-Int β3 or pcDNA3.1Hyg(+) into Neo1 cells was performed with Lipofectamine PLUS according to the manufacturer's instruction. The cells stably transfected with the pcDNA3.1Hyg(+)-Int β3 or pcDNA3.1Hyg(+) were selected by their resistance to both 400 μg/ml of G418 sulfate and 100 U/ml of hygromycin B. G418 sulfate- and hygromycin B-resistant cells which had been transfected with the pcDNA3.1Hyg(+)-Int β3 were expanded, and the cells expressing integrin β3 on their cell surface were enriched by magnetic cell sorting using MACS goat anti-mouse IgG microbeads system (Miltenyi Biotec, Bergisch Gladbach, Germany) and the integrin β3-specific antibody RUU-PL7F12. After the magnetic cell sorting, the cells were cloned by the

limiting dilution method. The integrin β3 expression of the cloned cells was analyzed by flow cytometry using RUU-PL7F12 monoclonal antibody. We isolated three clones which expressed integrin β3 at the similar level to those in HOX+1 and HOX+2 cells and designated them Neo1 β3-1, Neo1 β3-2, Neo1 β3-3. Neo1Hyg1 and Neo1Hyg2 were cloned from G418 sulfate- and hygromycin B-resistant cells which had been transfected with pcDNA3.1Hyg(+). The Neo1 β3-1, Neo1 β3-2, Neo1 β3-3, Neo1Hyg1 and Neo1Hyg2 cells were grown in DME/F12 containing 5% FBS and 400 μg/ml of G418 sulfate and 100 U/ml hygromycin B.

Semiquantitative duplex RT-PCR analysis. For RT-PCR analysis, total RNA was extracted from monolayer cultures of each cell line with Trizol¸ according to the manufacturer's instruction. Three μg of total RNA sample was subjected to cDNA synthesis for 2 h at 37°C in 50 microL of reaction mixture containing 4 U/μL of Molony murine leukemia virus reverse transcriptase, 10 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.5 μM dNTP and 2 μM random primer. PCR amplification of cDNA was performed in 50 μL of reaction mixture containing 1 μL of cDNA sample, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.125 U/μL of *Taq* polymerase and different primer sets (10 nM each). Co-amplification of the specific gene and human β-actin gene, as an internal control, was achieved by using two primer sets in a single reaction mixture. Each primer was designed to encompass an exon junction for prevention of templating possibly contaminated genomic DNA. The sense/antisense primers for PCR were designed as follows: (a) HOXD3 (434 bp), 5'-AGGATCCTGGTCTGAAACTCAGAGC-3'/5'-ACTCGAGTTCATCCGCCGGTTCTGGAACCA-3'; (b) integrin β3 (526 bp), 5'-CTCCATCCAAGTGCGGCAGGTGGAG-3'/5'-CATGACACTGCCCGTCATTAGGCTGGAC-3'; β-actin (236 bp/530 bp), 5'-

ACCTCATGAAGATCCTCACCGAGCG-3' or 5'-TCTACAATGAGCTGCGTGTGGCTCC-3'/5'-AGGAAGGAAGGCTGGAAGAGTGCCTC-3'. PCR products were electrophoresed in a 2.5% agarose gel and intensity of the bands was observed under a UV illuminator.

Flow cytometry. The cultured cells were harvested by trypsinization and washed twice with PBS containing 0.065% sodium azide. The cells were incubated with primary antibodies for 1 h at 4°C, and then with secondary antibodies conjugated with FITC. After incubation, the cells were washed three times with PBS containing 0.065% sodium azide. The stained cells were resuspended in 1 ml of PBS containing 0.065% sodium azide and analyzed with the use of FACSCalibur (Becton Dickinson, San Jose, CA).

Haptotaxis assay. Haptotaxis assay was performed by using Transwell chambers by the same method described in our previous report [16]. The lower surface of the membranes with 8-μm pores of Transwell chambers was coated with 10 μg each of vitronectin, fibronectin, fibrinogen or laminin-1. Six hundreds μL of DME/F12 containing 0.1% BSA was placed into the lower compartment of the Transwell chambers, and then 100 μl of the cell suspension ($2x10^5$ cells/ml in DME/F12 containing 0.1% BSA) was placed into the upper compartment. After 6 h-incubation, each membrane was fixed with 10% neutral-buffered formalin and stained in Giemsa solution. After the cells attached to the upper side of the membrane were removed by wiping with a cotton swab, those attached to the lower side of the membrane were counted under a microscope. Haptotactic activity was evaluated by the number of cells per field at x200 magnification (mean ± SD, n = 20).

Phagokinetic track assay. Phagokinetic track assay was performed to assess the random cell motility by the same method described in our previous report [16].

Briefly, the gold particle-coated coverglasses were placed in 35-mm tissue culture dishes, and two thousand tumor cells suspended in DME/F12 supplemented with 1% fetal bovine serum (FBS) were added into each dish. After 24 h-incubation, the coverglasses were fixed for 30 min in 10% neutral-buffered formalin and mounted on slide glasses by using Gelmount (Biomeda, Foster City, CA). The phagokinetic tracks were observed with Nikon microscope. The area cleared of gold particles by randomly selected 20 cells was measured with an apparatus for image analysis (Nikon Cosmozone R500) connected to the microscope at a magnification of x200.

***In vitro* wound-healing assay**. *In vitro* wound-healing assay for measuring cell migrating activity was carried out by the method reported by Boyer et al. [19]. Briefly, confluent cell monolayers were obtained after 48 h of culture in DME/F12 supplemented with 5% fetal bovine serum (FBS). They were gently scratched with a Gilson pipette yellow tip, and extensively rinsed with DME/F12 medium to remove all cellular debris. This procedure left a cell-free area of substratum ("wound"). Then, the cultures were allowed to grow in DME/F12 supplemented with 1% FBS. After making "wound", the cultures were observed with a phase contrast inverted microscope and photographed.

For determining the roles of E-cadherin in the *in vitro* wound healing, the cells were seeded on Chamber Slide with 8 chambers (Nalge Nunc, Naperville, IL). At confluent point of the cell monolayers, DME/F12 containing 5% FBS was replaced with PBS containing 2 mM $MgCl_2$ and 2 μM $CaCl_2$. After 2-h incubation, the PBS was replaced with DME/F12 supplemented with 1% FBS, and simultaneously 100 microg/ml of anti-E-cadherin antibody or normal mouse IgG was added. Immediately after the addition of antibodies, the monolayers were scratched for the assay.

Immunoblot analysis. Tumor cells (2 x 10^6/dish) were seeded on 100-mm tissue culture dish in DME/F12 supplemented with 5% FBS. After 24 h incubation, the cells were washed twice with 10 ml of cold PBS, and then harvested with a cell scraper in lysis buffer (20 mM Hepes, pH 7.6, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM Na_3VO_4, 5 μg/ml Pepstatin A, 5 μg/ml Leupeptin). The cell lysates were centrifuged at 20,000 x*g* for 10 min at 4°C. The supernatants were collected and protein concentration was determined by modified Bradford assay (Bio-Rad protein assay, Bio-Rad, Hercules, CA). Equal amounts of protein were separated by SDS-PAGE in 7.5% polyacrylamide gel and electrotransferred to Immobilon membranes (Millipore, Bedford, MA). The membranes were blocked in TBS-T (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20) with 5% skim milk overnight at 4°C, and then incubated with primary antibodies for 1 h at room temperature. The membranes were incubated next with horseradish peroxidase-conjugated antibodies for 1 h at room temperature, and developed by the Enhanced Chemiluminescent Detection System (Amersham Pharmacia, Little Chalfont, UK).

Results

Expressions of *HOXD3* and integrins in integrin β 3-overexpressing A549 cells. First we examined expression of *HOXD3* gene and integrin β3 gene in the cell lines used in this study by RT-PCR analysis. It was confirmed that the cloned transfectants with *HOXD3* gene (HOX+1 and HOX+2) expressed both HOXD3 and integrin β3; those with integrin β3 gene (Neo1 β3-1, Neo1 β3-2 and Neo1 β3-3) did only integrin β3; parental and the control transfectants (Neo1 and Neo1Hyg1 and Neo1Hyg2) did neither of them (Figure 1A & B). Cell surface expressions of integrins were analyzed

by flow cytometry using antibodies to integrins β3, αvβ3, αvβ5, αvβ6, α4 and β1. The integrin β3-overexpressing cells as well as the *HOXD*3-overexpressing cells showed the formation of αvβ3 heterodimer (Figure 2). The expression of integrin αvβ5 tended to decrease in the *HOXD3*- and the integrin β3-overexpressing cells compared to the control cells (Figure 2). No cell lines showed the expression of integrin αvβ6 (data not shown). The control cells showed no expression of integrin α4 subunit whereas the *HOXD3*-overexpressing and the integrin β3-overexpressing cells newly showed the expression of integrin α4 (although the expression levels of integrin α4 in the integrin β3-overexpressing clones were fairly low) (Figure 2). The integrin β1 expression was not affected by the integrin β3 transduction (Figure 2).

Haptotactic activity of the integrin β3-overexpressing A549 cells. As the *HOXD3*-overexpressing A549 cells increased their haptotactic activities to vitronectin, fibrinogen and fibronectin [16], we examined the haptotactic activities of the integrin β3-overexpressing cells. Haptotactic activities of the integrin β3-overexpressing cells as well as the *HOXD3*-overexpressing cells to vitronectin and fibrinogen, which are of ligands highly specific to αvβ3, were significantly higher than those of the parental and the control transfectant cells (Figure 3A, B). The haptotactic activity of the integrin beta 3-overexpressing cells to fibronectin, which is a ligand for αvβ3, was significantly lower than that of the *HOXD3*-overexpressing cells although significantly higher than that of the parental and the control transfectant cells (Figure 3C). However, no significant difference was observed in haptotactic activity to laminin which is not a ligand for αvβ3 among all cell lines tested (Figure 3D).

Phagokinetic activity of the integrin β3-overexpressing A549 cells. Unlike haptotaxis assay, phagokinetic track assay was performed in the absence of specific attractants for cell migration such as extracellular matrix components. And thus cell

motility assessed by phagokinetic track assay shows non-directed motility, so called random motility. As shown in Figure 4A and B, the integrin β3-overexpressing cells, as well as the *HOXD3*-overexpressing cells, showed high motile activities compared to the parental and the control transfectants. There was no difference in cell motility between the integrin β3-overexpressing cells and the *HOXD3*-overexpressing cells. An addition of the blocking antibody to integrin αvβ3 (LM609) into the assay reduced the phagokinetic activities of both HOXD3- or integrin β3-overexpressing cells to the level of the control transfectants (Figure 4C).

In vitro wound-healing activity of the integrin β3-overexpressing A549 cells. Both haptotaxis and phagokinetic track assays started in the condition without cell-cell adhesion whereas *in vitro* wound-healing assay proceeded in the condition with cell-cell adhesion. A confluent monolayer was scratched with a micropipette tip, which left a cell-free area of substratum ("wound"). Cells on the wound edges migrated toward the wound. Cell migration distances were chronologically measured with a micrometer. Both the integrin β3-overexpressing cells and the *HOXD3*-overexpressing cells migrated faster than the parental and the control transfectants (Figure 5A). In the earlier stage after starting the assay, the migratory distance of the integrin β3-overexpressing cells was significantly shorter than that of the *HOXD3*-overexpressing cells (Figure 5A, B).

Roles of E-cadherin in in vitro wound-healing activity. In the wound-healing assay, we observed that migratory activity of the integrin β3-overexpressing cells was low in the early stage of the assay compared to that of the *HOXD3*-overexpressing cells. We earlier reported that the *HOXD3*-overexpression led the loss of E-cadherin expression in A549 cells [16]. *In vitro* experiments on cultured cells indicate E-cadherin has invasion-suppressing properties [20-22]. Therefore, we next examined

the expression and function of E-cadherin in the integrin β3-overexpressing cells. Immunoblot analysis revealed that the integrin β3-overexpressing cells retained the expression of E-cadherin although the *HOXD3*-overexpressing cells lost it (Figure 6A). To investigate whether the E-cadherin suppresses the cell migration from the wound edges, we added an antibody to human E-cadherin (HECD) which blocks the cell-cell contact through E-cadherins to the wound healing assay. As shown in Figure 6B, the migratory activity of the integrin β3-overexpressing cells treated with HECD-1 antibody significantly increased compared to the non-treated cells, but the increase was not as high as that of the *HOXD3*-overexpressing cells.

Discussion

We have previously shown that *HOXD3*-overexpression enhances cell motility and increases expression of adhesion molecules such as integrin β3, α3 and α4 in human lung cancer A549 cells [16]. Following that, we focused on the roles of integrin β3 in the enhanced cell motility of the *HOXD3*-overexpressing cells in the present study. We first established cell clones transfected with integrin β3, and then compared their cell motile behavior to those of the HOXD3-overexpressing clones and the control clones (neomycin- or neomycin/hygromycin-resistant clones).

Our present study demonstrated that integrin β3 transduced into A549 Neo1 cells formed heterodimer with integrin αv subunit. The transduction of *HOXD3* or integrin β3 decreased the expression of integrin αvβ5. The parent and transfectants analyzed here showed no expression of integrin αvβ6 on their cell surface (unpublished data). Further our previous report documented that *HOXD3*-overexpression did not have any effect on the expression of endogenous αv subunit [16]. Taken together, we speculate

that the αv subunit dimerizing with β3 subunit is, at least partially, derived from alphav subunit of endogenous integrin αvβ5 although we further need to examine the expression of integrin αvβ1 and αvβ8.

We think that mainly the integrin αvβ3 was responsible for the enhanced motility by *HOXD3*- or β3-overexpression although we have to consider that the decreased expression of integrin avb5 may have caused the enhanced motility. As expected, integrin β3-transfectants as well as *HOXD3*-transfectants showed highly haptotactic activities to vitronectin and fibrinogen which were ligands specific for integrin αvβ3, compared to the control transfectants. Haptotaxis of integrin β3-transfectants to fibronectin was higher than that of the control transfectants and lower than the *HOXD3*-transfectants. Fibronectin is a ligand for a variety of integrins such as α3β1, α4β1, α5β1 and αvβ3 [23]. The *HOXD3*-transfectants expressed α3β1, α4β1 and αvβ3 at higher levels than the control transfectants. The integrin β3-transfectants expressed α3β1 and αvβ3 at the same levels as the *HOXD*3-transfectants; however, the expression of α4β1 slightly increased by the integrin β3-transduction. The difference in the haptotactic activity to fibronectin between the *HOXD3*- and integrin beta 3-transfectants may result from the different levels of alpha4beta1 expression.

It was unexpected that the αvβ3 expression gave A549 cells higher motility when assessed by phagokinetic track assay. This assay is often used to measure random motility, namely, motility without direction [24, 25]. In this study, the cells were seeded on coverglasses coated with BSA and gold particles in DME/F12 containing 1% FBS. It should be noted that αvβ3 binds to a ligand(s) to transduce outside-in signal for motility. The ligands for αvβ3 in FBS are bovine fibronectin and vitronectin [23, 26]. We added fibronectin or vitronectin at almost comparable concentrations to those in 1% FBS into the FBS-free assay medium; however, neither

of them could stimulate phagokinetic motility of the integrin β3- or HOXD3-transfectants (unpublished data). It is well known that FBS contains several potent motility-stimulating factors, some of which are lysophosphatidic acid (LPA), insulin, epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), platelet derived growth factor (PDGF), and hepatocyte growth factor (HGF) [27]. LPA, EGF and HGF are unlikely to be a motility-stimulating factor for A549 cells because we have evidence that these molecules do not stimulate the phagokinetic motility of any of the transfectants used here (unpublished data). Of the above-mentioned motility-stimulating factors, receptors of insulin and PDGF associate with integrin αvβ3 and potentiate the biological activity of their ligands [28-30]. Therefore, it is possible that the receptors activated by insulin or PDGF which are present in the FBS form a complex with αvβ3, which acts on phagokinetic motility. We need further study to clarify the association of αvβ3 with these growth factor receptors in the cell motility enhanced by integrin β3- or *HOXD*3-transduction.

In vitro wound healing assay revealed that the integrin β3-transfectants migrated faster than the control transfectants and slower than the *HOXD3*-transfectants. This wound healing assay was started in the condition maintaining cell-cell contact unlike the haptotactic or phagokinetic track assay in which measures cell motility was measured in the condition where the cells were apart from each other. We showed here that *HOXD3*-transduction led to the loss of E-cadherin expression whereas integrin β3-transduction did not affect the E-cadherin expression. Dysfunction of the E-cadherin-mediated cell adhesion system plays an important role in acquisition of more malignant phenotype by tumor cells [31]. Experimental biological approaches also show that E-cadherin functions as an invasion-suppressor [20-22]. In the present study, the addition of function-blocking antibody to E-cadherin into the assay

increased migratory activity of the integrin β3-transfectants, suggesting that E-cadherin plays a role in preventing the tumor cells from migrating from wound edge. However, the treatment with anti-E-cadherin antibody could not recover the migratory activity of the integrin β3-transfectants to the level of that of the *HOXD3*-transfectants. The *HOXD3*-transfectants are thought to operate additional system(s) to accelerate or brake the migration, although we need further investigation to demonstrate it. The anti-E-cadherin antibody treatment did not accelerate the migration of the control transfectants. We assume that disruption of cell-cell contact through the E-cadherins no longer affected the migration of the control transfectants since they were intrinsically less motile. Taken together, it is most likely that the increased expression of integrin αvβ3 and loss of expression of E-cadherin strongly contributed to the enhancement of various types of cell motility and dissociation in the *HOXD3*-overexpressing A549 cells.

As previously shown, *HOXD3*-overexpressing A549 cells convert latent TGF-beta into active one, which activates the TGF-β-signaling in an autocrine manner [32]. The stimulation with TGF-β enhances haptotaxis of A549 cells to type I collagen but not to vitronectin, fibrinogen or fibronectin [32]. Thus we suppose that *HOXD3* enhances cell motility through the TGF-β-dependent and –independent pathways in A549 cells. Further, TGF-β did not affect the expression of integrin β3 in either A549 parental cells or the control transfectants, and TGF-β-stimulated haptotaxis to type I collagen was not prevented by anti-αvβ3 antibody (unpublished data). Therefore, in the present study, we found that the TGF-β-independent pathway which enhanced cell motility was the integrin αvβ3-dependent one. It is noteworthy that one molecule (HOXD3) controls the two distinct pathways to stimulate the different types of cell motility.

Taken together with our previous study indicating that *HOXD3* directly or indirectly regulates transcriptions of metastasis-related genes such as matrix metalloproteinase-2, urokinase-type plasminogen activator, E-cadherin, desmoglein and others [16, 32], *HOXD3* can be a master gene of metastasis in A549 cells. Namely, *HOXD3* commands a number of soldiers which are engaged in metastasis. *HOXD3* is possibly a good target in prevention and treatment of invasion and metastasis.

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FIGURE LEGENDS

Figure 1 Expressions of *HOXD3* and integrin β3 in parental A549 cells and the cells transfected with either *HOXD3* or integrin β3 gene. Total RNA was extracted from the cultured cells; after reverse transcription, a duplex polymerase chain reaction (PCR) was performed with the primers for *HOXD3* or integrin β3 and for β-actin . After the PCR amplification, the samples were electrophoresed and stained with ethidium bromide. *Hae*III-digested phiX174 DNA was used as size markers.

Figure 2 Expressions of integrins on the surface of parental A549 cells and the cells transfected with either *HOXD3* or integrin β3 gene. The cells were incubated with monoclonal antibodies to each integrin and then reacted with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (thick lines). Fluorescence intensity was analyzed by flow cytometry. The abscissa and the ordinate indicate fluorescence intensity and cell number, respectively. Thin lines indicate fluorescence intensity of the cells treated with FITC-conjugated anti-mouse IgG alone.

Figure 3 Haptotaxis of parental A549 cells and the cells transfected with either *HOXD3* or integrin β3 gene to extracellular matrix components. Haptotaxis assay was performed by using Transwell chambers. The lower surface of the membranes of Transwell chambers was coated with 10 microg of vitronectin (A), fibrinogen (B), fibronectin (C) or laminin-1 (D). The cell suspension was placed in the upper compartment of the chamber. After 6 h-incubation, migrated cells were counted by using a microscope. The columns represent mean +/- standard deviation in randomly selected 20 fields per well at x200 magnification. $*p < 0.01$ compared to the parent

and the cells transfected with neomycin-resistant gene or neomycin- and hygromycin-resistant genes; #$p < 0.01$ compared to the cells transfected with integrin β3 gene (by one-way ANOVA followed by Scheffe's F analysis as a post hoc test).

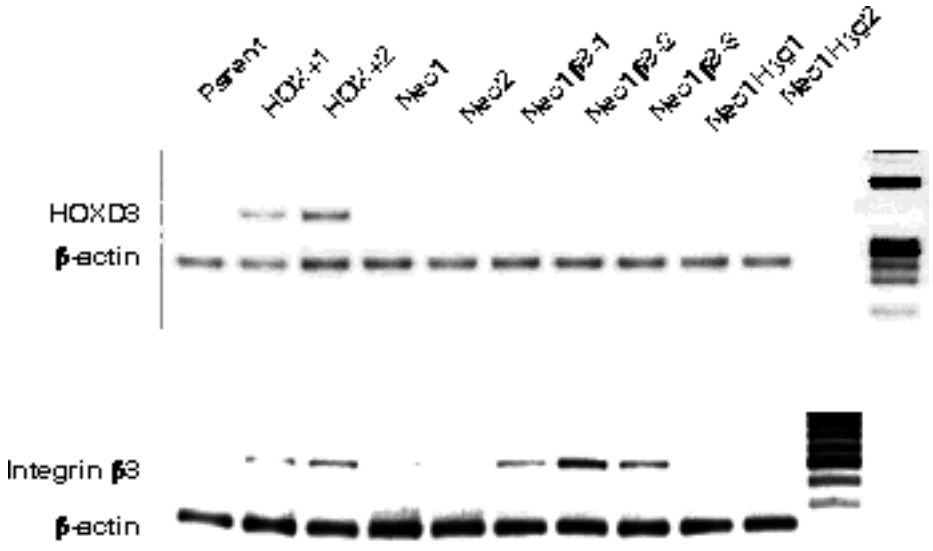
Figure 4 Phagokinetic activity of parental A549 cells and the cells transfected with either *HOXD3* or integrin β3 gene. The phagokinetic tracks were observed under a microscope after 24 h-incubation. The area cleared of gold particles by 20 cells was measured with the use of an image analyzer connected to the microscope. A: Representative photomicrographs of each cell line taken under dark-field illumination (x100). B: The columns represent mean values of track areas +/- standard deviation of randomly selected 20 cells. C: The assay was performed in a gold-particle-coated chambered-coverglass (8-well) with either anti-αvβ3 antibody (closed), anti-β1 antibody (hatched) or normal mouse IgG (shaded). Non-treated control (open). *$p < 0.01$ compared to the parent and the cells transfected with neomycin-resistant gene or neomycin- and hygromycin-resistant genes; #$p < 0.01$ compared to non-treated, normal IgG-treated or anti-integrin β1 antibody-treated cells (by one-way ANOVA followed by Scheffe's F analysis as a post hoc test).

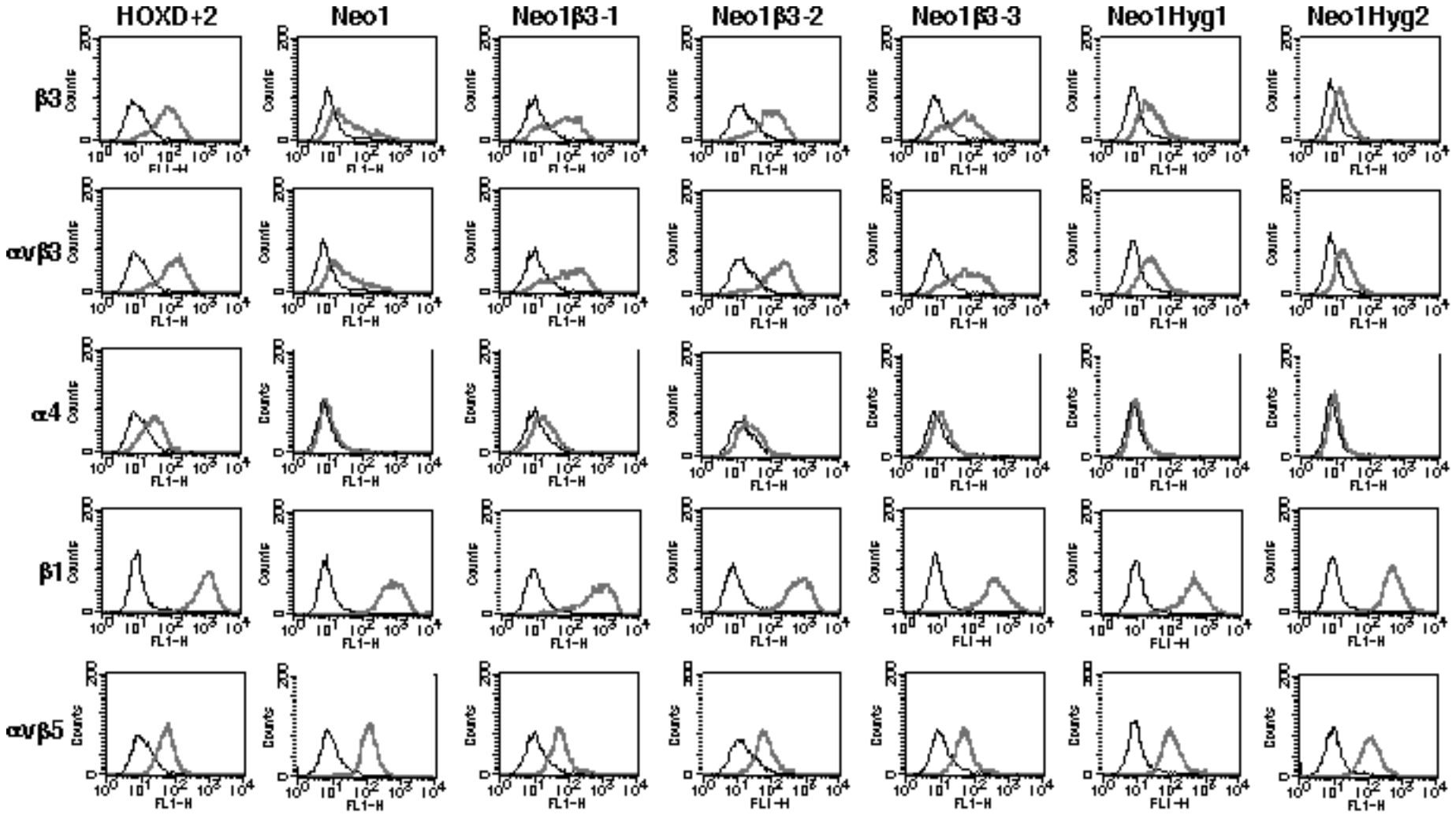
Figure 5 Migratory activities of parental A549 cells and the cells transfected with either *HOXD3* or integrin β3 gene in an *in vitro* wound healing assay. A: Confluent monolayers of the cells were scraped with a plastic pipet tip to create a wound at 0 h. After 12 h, numerous individual *HOXD3*- or integrin β3-overexpressing cells migrated into the wound in contrast to the control cells (Parent, Neo1, Neo2, Neo1Hyg1 and Neo1Hyg2). Representative photomicrographs of each cell line taken under a phase contrast inverted microscope (x100). B: After making a wound, the

distance between the two wound edges was chronologically measured at 10 to 18 sites with a micrometer under the microscope (x100). The migratory activity was evaluated as migration distance which was calculated by [distance between the two wound edges at 0 h - distance between the two wound edges at n h]/2. Data are shown as mean +/- standard deviation. *p* value were less than 0.01 in the comparison between any of HOXD3-overexpressing cell lines (HOX+1 or HOX+2) and any of integrin β3-overexpressing cell lines (Neo1β3-1, Neo1 β3-2 or Neo1 β3-3) or between the former and any of control cell lines (parent, Neo1, Neo2, , Neo1Hyg1 or Neo1Hyg2) at 1 to 5 h, between any of the HOXD3-overexpressing cell lines and any of the control cell line at 1 to 24 h, and between any of the integrin β3-overexpressing cell lines and any of the control cell line at 10 to 24 h (by one-way ANOVA followed by Scheffe's F analysis as a post hoc test).

Figure 6 A: Expressions of E-cadherin in parental A549 cells and the cells transfected with either *HOXD3* or integrin β3 gene. E-cadherin expression was detected by immunoblot analysis. The membrane was blotted with an antibody to E-cadherin and a horseradish peroxidase-conjugated sheep anti-mouse Ig antibody, and then developed with the use of reagents obtained from the Enhanced Chemiluminescent Detection System. B: Recovery of migratory activities of the cells transfected with integrin β3 gene by the treatment with anti-E-cadherin antibody. *In vitro* wound healing assay was performed by the same method described in Figure 5. Data represent cell migration distances 6 h after starting the assay. *In vitro* wound healing assay was performed in the presence of either anti-E-cadherin antibody (closed), normal mouse IgG (shaded) or no antibody (open) by the method described in MATERIALS AND METHODS. $*p < 0.01$ compared to the non-treated cells or

the cells treated with normal mouse IgG; #$p < 0.01$ compared to HOX+1 cells treated with or without antibodies (by one-way ANOVA followed by Scheffe's F analysis as a post hoc test).


Parent
HOX-1
HOX-2
Neo1
Neo2
Neo1β3-1
Neo1β3-3
Neo1β3-5
Neo1His1
Neo1His2
HOXD8
β-actin
Integrin β3
β-actin


HOXD+2
Neo1
Neo1β3-1
Neo1β3-2
Neo1β3-3
Neo1Hyg1
Neo1Hyg2
β3
αvβ3
α4
β1
αvβ5
Counts
FL1-H

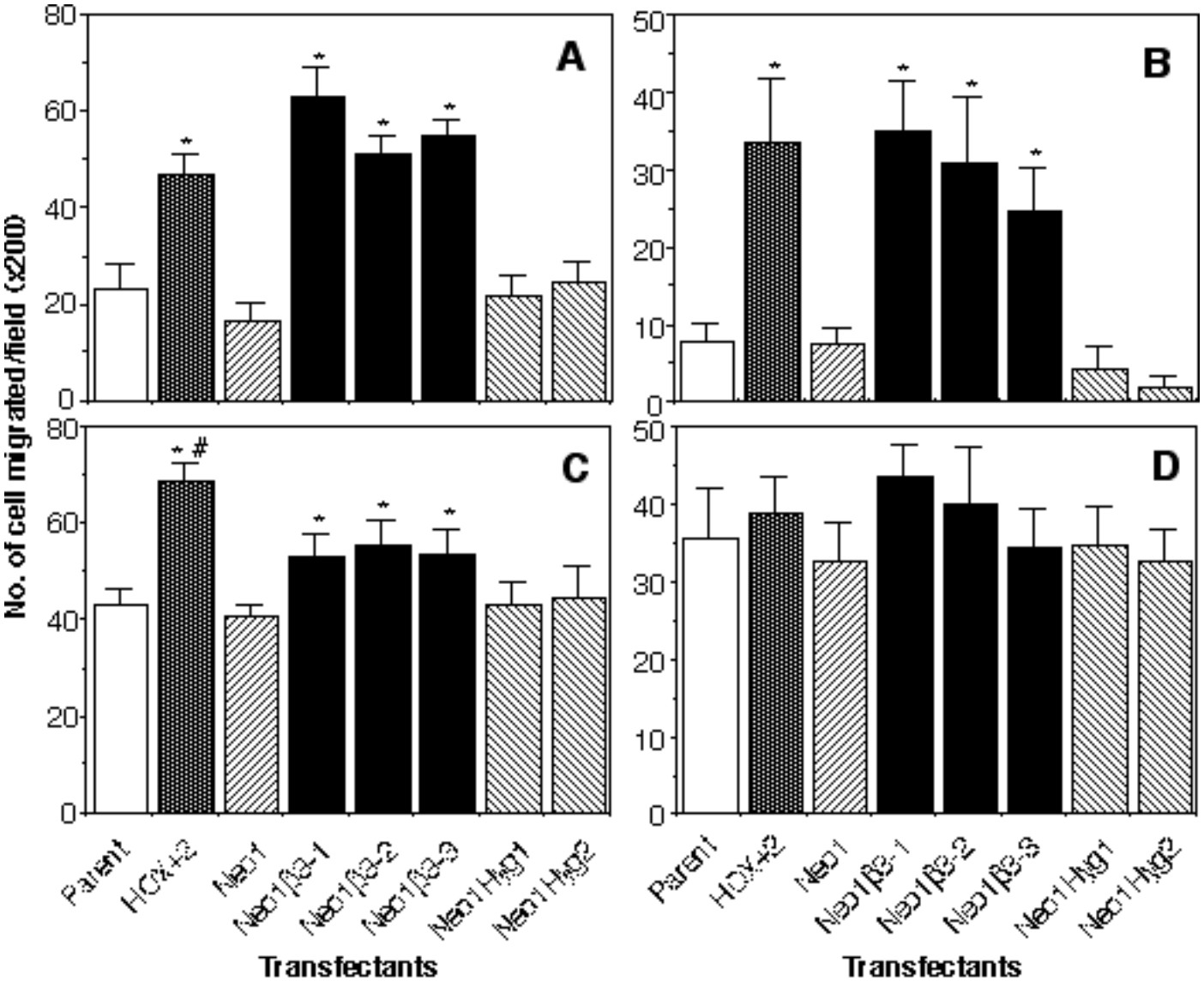
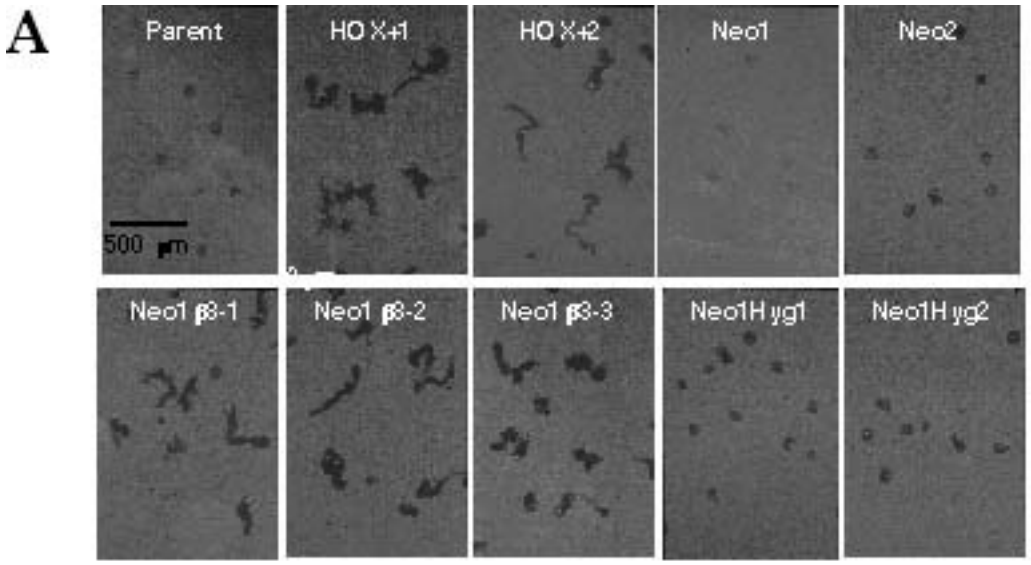
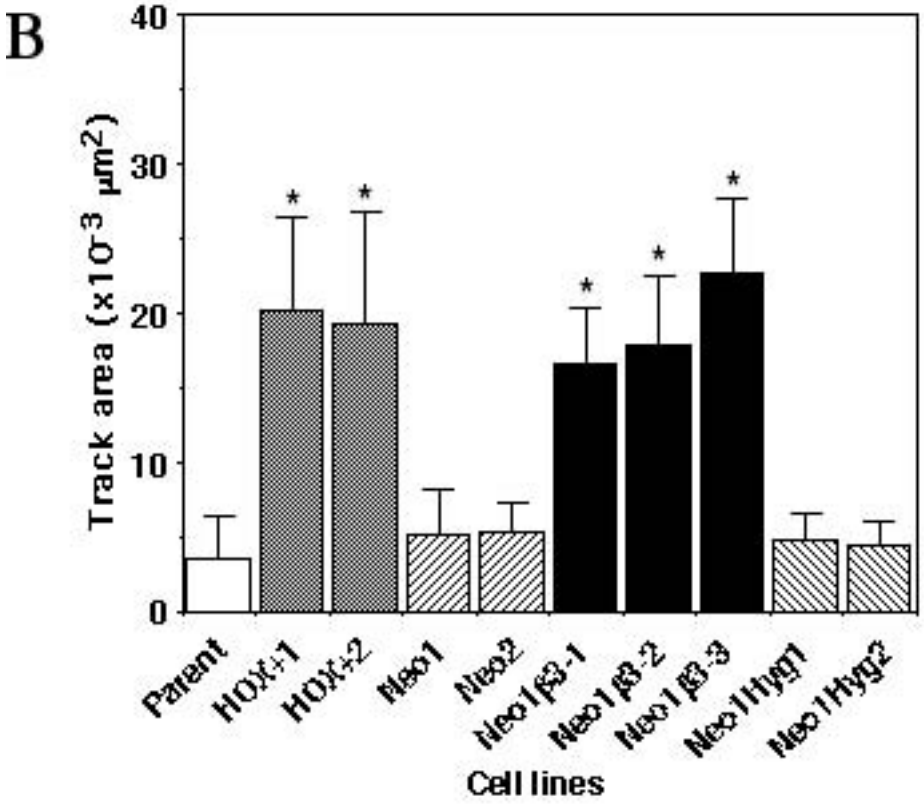
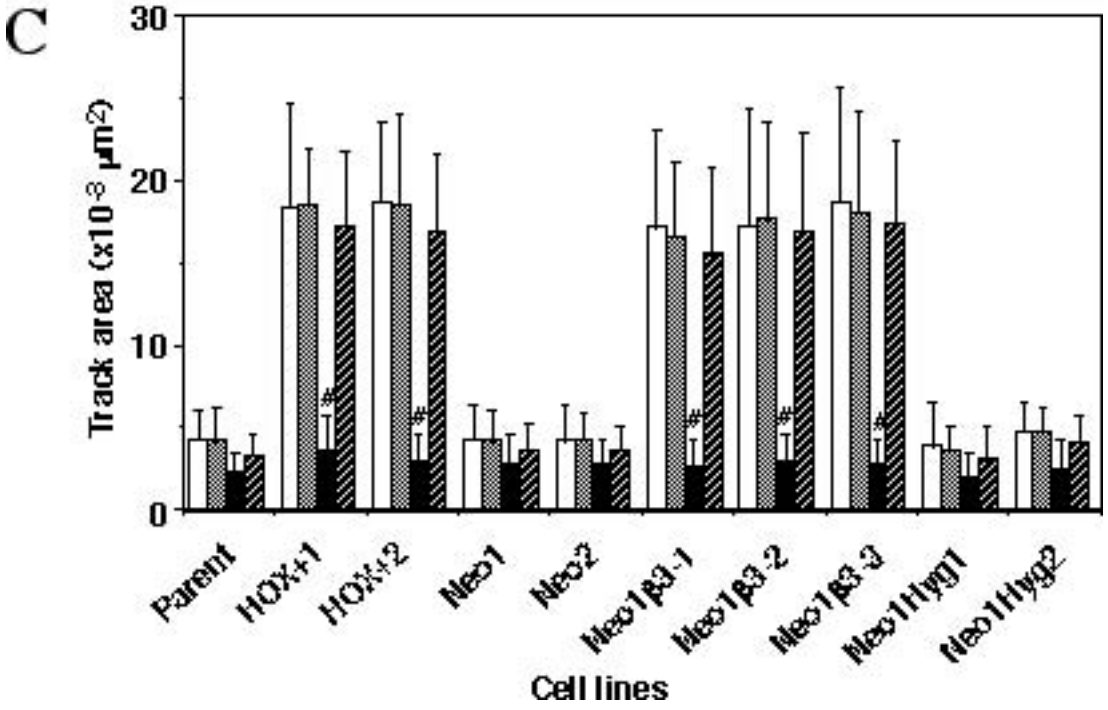
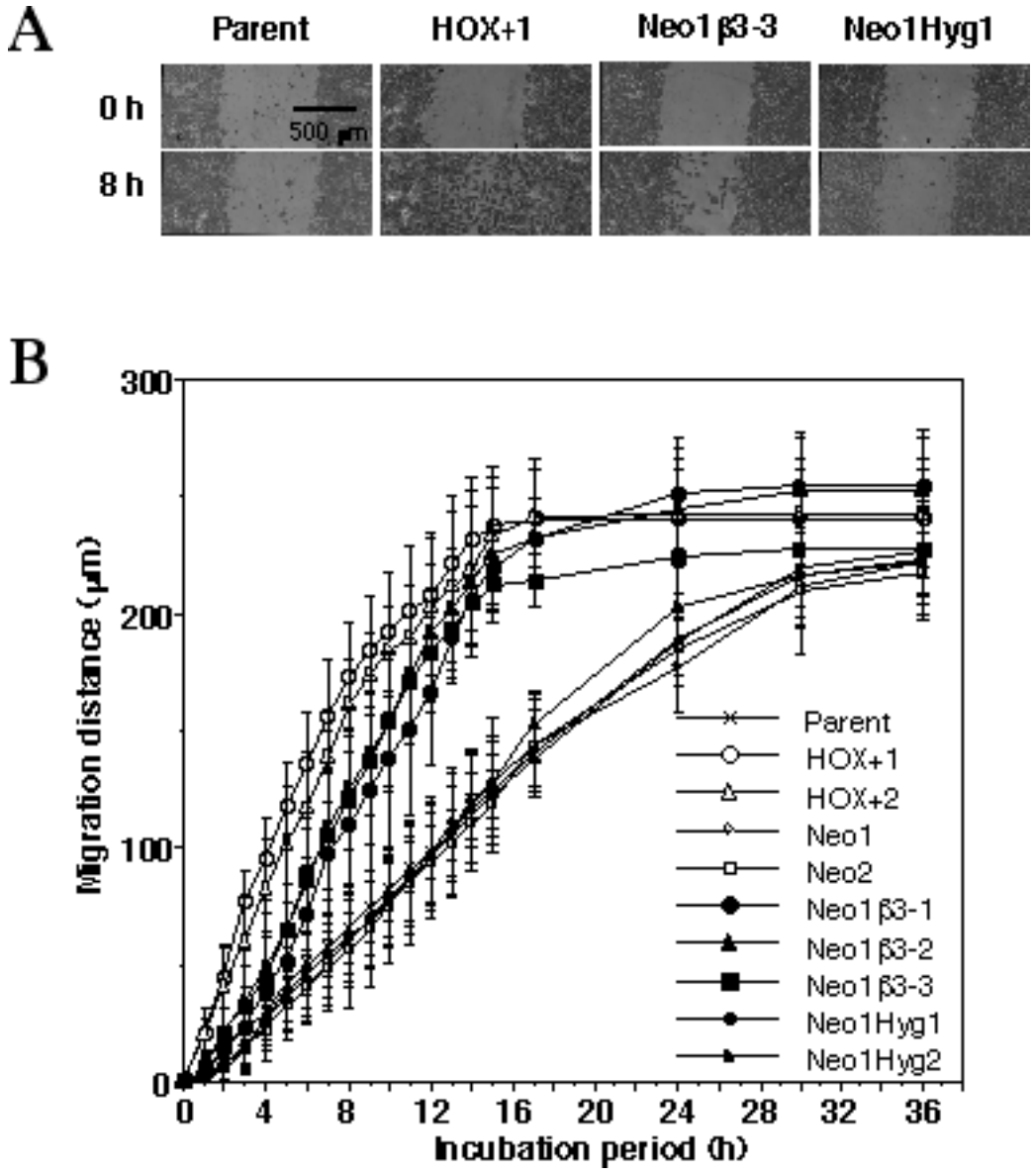


Figure 3 Ohta et al.


A
Parent
HOX+1
HOX+2
Neo1
Neo2
500 μm
Neo1 β3-1
Neo1 β3-2
Neo1 β3-3
Neo1Hyg1
Neo1Hyg2


B
Track area (x10-3 μm2)
40
30
20
10
0
*
Parent
HOX+1
HOX+2
Neo1
Neo2
Neo1β3-1
Neo1β3-2
Neo1β3-3
Neo1Hyg1
Neo1Hyg2
Cell lines


C
Track area (x10-3 μm2)
30
20
10
0
#
Parent
HOX+1
HOX+2
Neo1
Neo2
Neo1β3-1
Neo1β3-2
Neo1β3-3
Neo1Hyg1
Neo1Hyg2
Cell lines


A
Parent
HOX+1
Neo1β3-3
Neo1Hyg1
0 h
8 h
500 μm
B
Migration distance (μm)
Incubation period (h)
0
100
200
300
0
4
8
12
16
20
24
28
32
36
Parent
HOX+1
HOX+2
Neo1
Neo2
Neo1β3-1
Neo1β3-2
Neo1β3-3
Neo1Hyg1
Neo1Hyg2

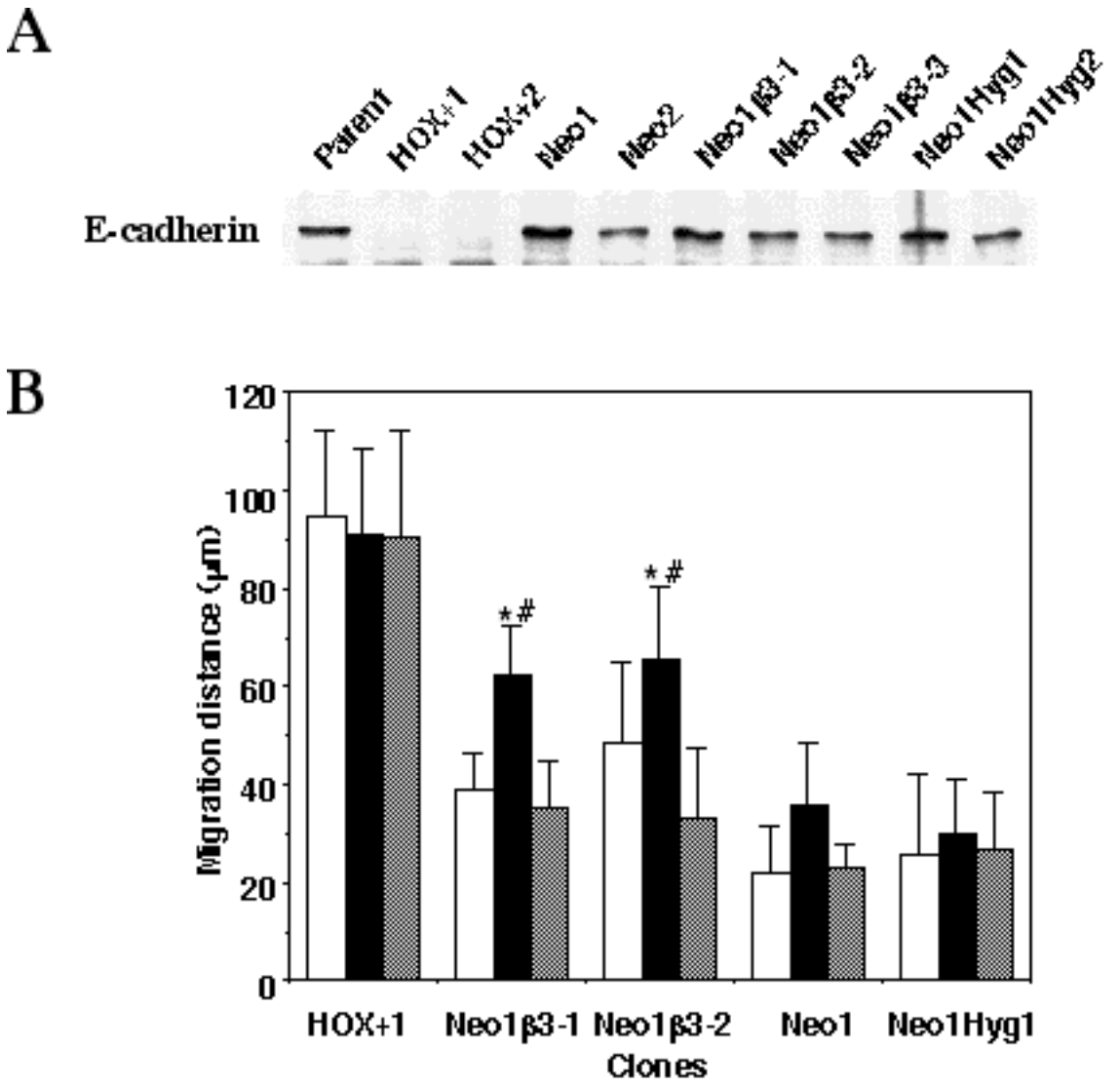


Figure 6 Ohta et al.