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Lineage Infidelity of MDA-MB-435 Cells: Expression of Melanocyte Proteins in a Breast Cancer Cell Line

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ABSTRACT

The origin of cell lines is critical in defining cell type-specific biological functions. Several reports (D. T. Ross *et al.*, Nat Genet 2000;24:227–35; G. Ellison *et al.*, J Clin Pathol Mol Pathol 2002;55:294–9) suggested that the MDA-MB-435 cell line, a cell line extensively used for studying breast cancer biology, has a gene expression pattern most compatible with melanocyte origin. However, we demonstrate that MDA-MB-435 cells express breast-specific or epithelial-specific markers. Also, MDA-MB-435 cells were induced to express breast differentiation-specific proteins and secrete milk lipids as observed in other well-established breast cancer cell lines. Notably, MDA-MB-435 cells also expressed melanocyte-specific proteins as did another highly aggressive breast cancer cell line. MDA-MB-435 xenograft tissue sections stained entirely positive for epithelium-specific markers but only partially positive for melanocyte-specific markers. Thus, MDA-MB-435 is most likely a breast epithelial cell line that has undergone lineage infidelity.

INTRODUCTION

Cells that coexpress protein markers of different cell lineages are considered to have undergone lineage infidelity (1). Acute leukemias that do not adhere to myeloid or lymphoid lineages but instead display elements of both phenotypes are the most frequently encountered examples of such mixed-lineage tumors (2).

Lineage infidelity has also been seen in breast carcinomas that express melanoma-associated genes (3, 4). In one study, the melanoma and ductal carcinoma components of a breast carcinoma have been shown to share the same genetic alterations; therefore, it was suggested that the breast carcinoma and malignant melanoma components may have originated from a single cell clone with the breast carcinoma diverging to melanoma through genetic instability (4). The finding of a single tumor of breast origin showing evidence of both mammary epithelial and melanocytic differentiation was described as a loss of genomic repression resulting in cell development achieved by two different differentiation pathways (3).

In one previous report, the breast cancer cell line MDA-MB-435 was clustered with melanoma cell lines based on the expression of certain melanocyte genes, and it was thus suggested that the MDA-MB-435 cell line may have originated from melanoma (5). Recently, another report categorized two MDA-MB-435 sublines (MDA-MB-435S and MDA-MB-435-HGF) as of melanocytic origin because these sublines did not express breast cancer genes (*pS2*, mammaglobin, and prolactin inducible proteins) but expressed genes commonly expressed by melanocytes (6). On the other hand, there have been other reports that MDA-MB-435 cells produce milk lipid droplets on induction of differentiation (7). These seemingly conflicting findings led us to hypothesize that MDA-MB-435 cells are of breast epithelium origin but have undergone dedifferentiation to a melanocyte phenotype as a result of genetic instability. To test this hypothesis and to elucidate the highly malignant behavior of advanced breast cancers, we analyzed MDA-MB-435 cells for breast epithelial and melanocyte-related differentiation properties. Our data confirmed that MDA-MB-435 cells indeed originated from breast epithelium, although they expressed melanocyte-specific genes, indicating that the MDA-MB-435 breast cancer cells underwent lineage infidelity during tumor progression.

MATERIALS AND METHODS

Materials. Antibodies for β-casein (Ab-1, Clone F20.14), epithelial membrane antigen (EMA; Clone GP1.4), GP100 (Clone HMB45), cytokeratin (pan-keratin Ab-1, Clone AE1/AE3), keratin 8 (Ab-2, Clone M20), keratin 19 (Clone A53-B/A2.2.6), α-lactalbumin (Ab-1, Clone F20.16), melan-A (Clone M2–9E3), microphthalmia transcription factor (MITF; Ab-1, Clone C5), and tyrosinase (Clone T311) were obtained from NeoMarkers, Inc. (Fremont, CA). Antihuman β-actin was obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase-labeled antimouse secondary antibody was obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture and Treatments. The MDA-MB-435 and Hs578T human breast cancer cell lines were gifts from Dr. Janet Price, University of Texas M. D. Anderson Cancer Center, Houston, TX, human melanoma cell lines WM266–4 and MEWO were gifts from Dr. Menashe Bar-Eli, University of Texas M. D. Anderson Cancer Center, C8161 (8) was a gift from Dr. Jim Klostergaard, University of Texas M. D. Anderson Cancer Center, and WM1205-Lu was a gift from Dr. Meenhard Herlyn, The Wistar Institute, Philadelphia, PA (9). MCF-7, BT-474, SKBr-3, MDA-MB-231, and BT-549 cells were obtained from the American Type Culture Collection (Manassas, VA). All of the cells, except for the SKBr3 and Hs578T, were cultured in DMEM-F12 supplemented with 10% serum and penicillin/streptomycin. SKBr3 cells were grown in McCoy's media supplemented with 10% serum and penicillin/streptomycin. Hs578T cells were grown in HAM's F-12K media supplemented with 10% serum, penicillin/streptomycin, and 5% nonessential amino acids. In some experiments, cells were untreated or treated with vitamin E succinate (5 μg/ml; Sigma) or heregulin-β1 (HRGβ1; 10 ng/ml; NeoMarkers) to induce differentiation.

Oil Red O Staining. Cells were cultured at a density of 1.5×10^3 on a tissue culture glass slide in 10% fetal bovine serum/DMEM F-12 media. Cells were untreated or treated with 5 μg/ml vitamin E succinate or 10 ng/ml HRGβ1 for 72 h. Cells were washed with PBS, fixed with 3.7% formaldehyde at room temperature, and washed again with PBS before being stained with 0.5% Oil Red O (Sigma) in isopropanol. Cells were counterstained with hematoxylin and rinsed with water. Slides were preserved with Crystal/Mount mounting media (Biomeda Corp., Foster City, CA) for observation.

Preparation of Cell Lysates. Cells were starved overnight in serum-free media and then were incubated with 10 ng/ml HRGβ1 or 5 μg/ml vitamin E, or were left untreated for 72 h. After treatment, the cells were washed with PBS and lysates were collected with lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40]. The protein levels were quantified using the BCA Protein Assay kit (Pierce, Rockford, IL). Protein lysates were boiled for 5 min in reducing sample buffer before separation by SDS-PAGE.

Western Blot Analysis. Western blots were performed as previously reported (10, 11). Signals were detected using the enhanced chemoillumines-

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Note: S. Sellappan and R. Grijalva contributed equally to the study.

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cence detection system (Amersham Corp., Arlington Heights, IL). All Western blot membranes except for β-casein and α-lactalbumin were blocked in a 5% dry milk/PBS-1 × PBS + 0.05% Tween-20 (PBST) solution. The β-casein and α-lactalbumin membranes were blocked in a 5% BSA/PBST solution, as described previously (12).

Immunofluorescence. To detect Melan-A and Tyrosinase proteins by immunofluorescence staining, 2×10^4 cells were plated in 300 μl of complete media on each chamber of a four-chamber culture slide (Falcon 354114). Cells were grown overnight, washed two times with PBS, and fixed with 4% paraformaldehyde/PBS for 1 h. Cells were washed three times for 5 min in PBS, then permeabilized for 10 min with 0.3% Triton X-100/PBS. Cells were washed three times for 5 min in PBS and then blocked with protein blocking reagent (DAKO X0909) for 20 min at room temperature with moderate shaking. The cells were incubated overnight at 4°C with primary antibody diluted 1:100 (tyrosinase) and 1:50 (melan-A) in 3% BSA/0.1% NP40/PBS. Cells were washed three times for 5 min in PBS and then were incubated for 2 h at room temperature with goat antimouse Texas Red-conjugated secondary antibody (Molecular Probes A-11032) diluted 1:200 in 3% BSA/0.1% NP40/1× PBS. Cells were washed three times for 5 min in PBS and were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min. The cells were washed two times for 5 min with PBS. To maintain signal intensity, the slides were treated with an antifade kit (Molecular Probes S-746). The chambers were then removed, and a coverslip was overlayed and sealed onto the culture slide. Slides were photographed with a Nikon Eclipse E400 microscope with 100X oil immersion objective linked to a Sensys digital camera (Photometrics Ltd.). Exposure times for the 4′,6-diamidino-2-phenylindole stain was 50 ms and for the Texas Red-conjugated antibodies was 150 ms. Images were optimized followed by color alignment with MetaMorph software (Universal Imaging).

Immunohistochemistry of Tumor Xenografts. To generate the MDA-MB-435 xenografts, we injected MDA-MB-435 cells into the mammary fat pad of severe combined immunodeficient mice (Taconic Labs, Germantown, NY) as described previously (13). To generate the WM266−4 melanoma xenografts, BALB/c nude mice were given s.c. injections of WM266−4 cells (Animal Productions Area of the National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). Breast carcinoma samples were derived from estrogen receptor-negative breast-invasive ductal carcinoma of $T_2N_{1b}M_0$ stage. Tumors were excised when they reached 10 mm in diameter and then were fixed in formalin and embedded in paraffin. A representative tissue block for serial sections was chosen from tumor xenografts for immunohistochemical staining. After deparaffinization and rehydration, 5-μm sections were subjected to heat-induced epitope retrieval in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. Nonspecific binding was blocked by treatment with normal horse serum at room temperature. The slides were then incubated with primary monoclonal antibodies against cytokeratin (1:100 dilution), HMB45 (1:40 dilution), EMA (1:500 dilution), or tyrosinase (1:80 dilution) at room temperature. Immunodetection was performed with the labeled streptavidin-biotin (LSAB)2 system (DAKO, Carpinteria, CA), as described previously (14). 3,3′-diaminobenzidine (DAB) was used for color development, and hematoxylin was used for counterstaining. Negative control slides were processed without primary antibody and were included for each staining.

Double-labeling immunohistochemistry was performed with the labeled-streptavidin-biotin-2 system and two different horseradish peroxidase substrates (3,3′-diaminobenzidine and VectorVIP). Anti-EMA monoclonal antibody (1:500 dilution) was used as the first primary antibody. After 3,3′-diaminobenzidine color development (*brown*), the slides were washed in PBS followed by incubation with anti-HMB45 monoclonal antibody (1:40 dilution) as the second primary antibody at room temperature. VectorVIP substrate (SK-4600; Vector Laboratories, Burlingame, CA) was used for second color development (*purple*). The slides were then dehydrated and mounted for observation.

RESULTS

Expression of Breast Epithelial-Specific and Epithelial-Specific Protein Markers by MDA-MB-435 Cells. During the induction of functional differentiation, breast epithelial cells undergo morphological changes, produce lipid droplets, and express breast epithelium-specific proteins, including β-casein and α-lactalbumin (15–17). Epithelial cells, which include breast tissue, specifically express EMA and cytokeratins (18, 19). To determine whether the MDA-MB-435 cell line is able to produce breast epithelial-specific proteins and express epithelial-specific markers, we performed Western blot analyses of breast epithelial and epithelial markers on cell lysates from MDA-MB-435 cells (Fig. 1, *arrowhead*) along with MCF-7, BT-474, SKBr-3, MDA-MB-231, BT-549, and Hs578T breast cancer cell lines and WM1205-Lu, C8161, WM266−4, and MEWO melanoma cell lines. The MDA-MB-435 cell line expressed the breast epithelial cell-specific protein β-casein and α-lactalbumin, as did the other breast cancer cell lines (Fig. 1). In addition, MDA-MB-435 cells also expressed the epithelial-specific markers EMA, keratin 19, and pan-keratin (Fig. 1). MDA-MB-435 cells had no detectable keratin 8 signal, similar to the highly aggressive breast cancer cell lines MDA-MB-231, BT-549, and Hs578T (Fig. 1).

On the other hand, none of the melanoma cell lines expressed any of the breast epithelial-specific proteins (Fig. 1). However, the melanoma cell lines weakly expressed the general epithelial marker keratin 19 and pan-keratin (Fig. 1). Our results indicated that the MDA-MB-435 cells expressed all of the breast epithelial and some of the epithelial-specific proteins examined, although some of these were expressed at lower levels than those in other well-differentiated breast cancer cell lines.

Previous studies have shown that HRGβ1 or vitamin E can induce some breast tumor cell lines to differentiate and express the proteins found during normal breast epithelial cell differentiation (7, 20). To determine whether differentiating agents further induce the breast epithelial cell-specific marker β-casein expression in MDA-MB-435 cells, the MDA-MB-435, the MCF-7, and the melanoma cell lines were treated with HRGβ1 (10 ng/ml). Compared with untreated cells, HRGβ1 treatment produced a slight increase in the expression of the breast epithelial-specific protein β-casein in the MDA-MB-435 cell line and in the MCF-7 breast cancer cell line. The melanoma cell lines again did not express β-casein with or without HRGβ1 (Fig. 2).

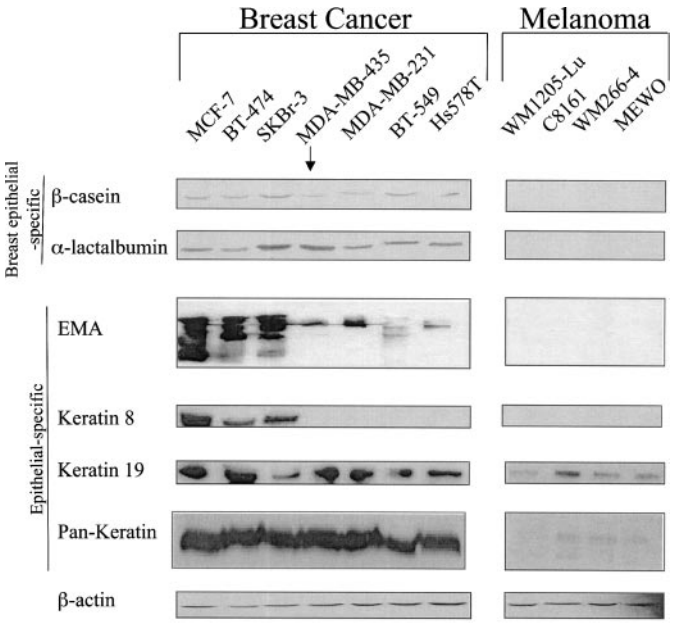

Fig. 1. MDA-MB-435 cells express breast epithelium-specific and epithelial-specific markers. β-casein, α-lactalbumin, epithelial membrane antigen (*EMA*), keratin 8, keratin 19, and pan-keratin expression levels in the indicated breast cancer and melanoma cell lines were analyzed by immunoblotting with specific antibodies recognizing each of these proteins, respectively. β-actin was used as a loading control. Data are representative of three independent experiments.

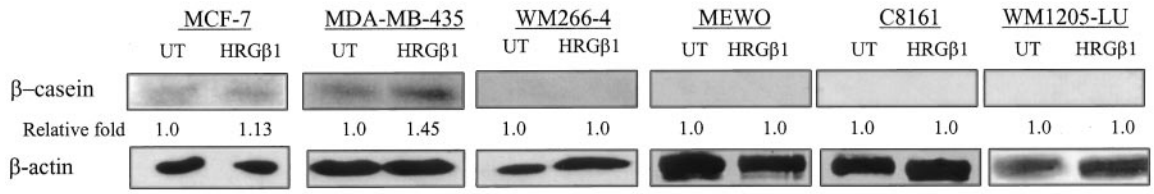

Fig. 2. Induction of breast epithelial-specific milk protein (*β-casein*) by heregulin-β1 (*HRGβ1*). Cells of indicated cell lines were untreated (*UT*) or treated with HRGβ1 (10 ng/ml) for 72 h. Immunoblotting was performed on cell extracts with anti-β-casein antibody. β-actin was used as a loading control. Data are representative of three independent experiments. *Relative fold*, the relative fold of the intensity of the induced signal *versus* the uninduced signal was measured by setting the intensity of the uninduced signal as l.0.

Production of Milk Lipids and Morphological Changes by MDA-MB-435 Cells. The production of lipid droplets is another indicator of differentiated breast epithelial cells (21), and the induction of lipid droplet formation in cultured breast cell lines by differentiating agents has also been reported (22). To determine whether MDA-MB-435 cells are capable of lipid droplet formation, MDA-MB-435 cells and the MCF-7 breast cancer cell line, as well as the melanoma cell lines, were treated with HRGβ1 (10 ng/ml) or vitamin E (5 μg/ml). Lipid droplets, which appeared on Oil Red O staining, were observed in both untreated and treated MDA-MB-435 cells and MCF-7 breast cancer cells (Fig. 3). Treatment of MDA-MB-435 cells with HRGβ1 and vitamin E produced slight increases in lipid droplet formation. This is consistent with previous reports that MCF-7 breast cancer cells treated with HRGβ1 and vitamin E also produced increased lipid droplet formation (7, 15). However, no lipid droplets were detected in three of the four melanoma cell lines with or without HRGβ1 or vitamin E treatment (Fig. 3). The highly aggressive and dedifferentiated WM1205-Lu cells showed a very low amount of lipid staining, which may indicate a mesenchymal to epithelial transition (MET). Taken together, these results indicated that the MDA-MB-435 cancer cell line synthesizes components of breast milk, similar to that seen in MCF-7 breast cancer cells, which is one of the characteristics of breast epithelial cells.

Differentiated breast epithelial cells also frequently demonstrate altered cell morphology. In our study, HRGβ1- and vitamin E-treated MDA-MB-435 and MCF-7 cells exhibited an enlarged cytoplasm, cell flattening, and increased cell volume (Fig. 3). These morphological changes have been reported in both breast and melanoma cell lines by treatment with other differentiation-inducing agents (23, 24).

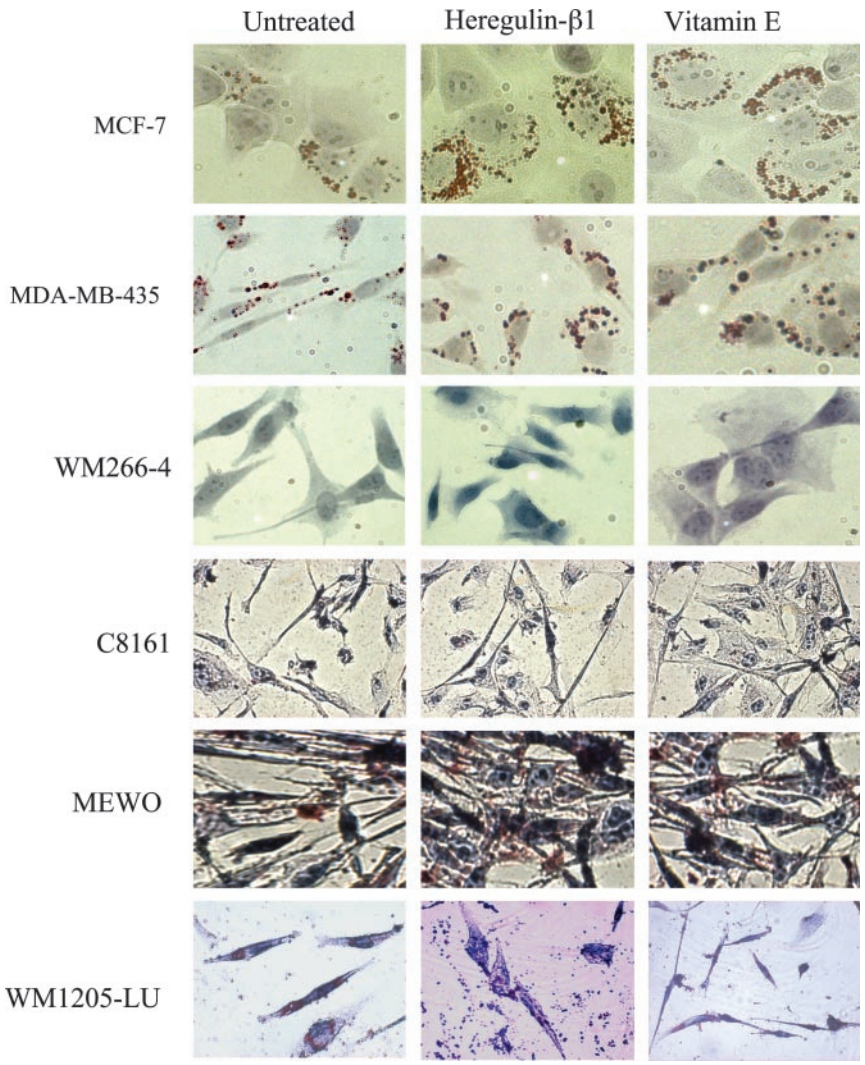

Fig. 3. MDA-MB-435 and MCF-7 breast cancer cells, but not the melanoma cell lines, produce neutral lipids in the absence (*Untreated*) or presence of differentiation reagents [10 ng/ml heregulin-β1 or 5 μg/ml vitamin E succinate (*Vitamin E*)]. After cells were incubated with or without differentiation reagents for 72 h, neutral lipids were visualized by Oil Red O staining. *Red*, neutral lipids. Slides were observed and photographed at $\times$400.

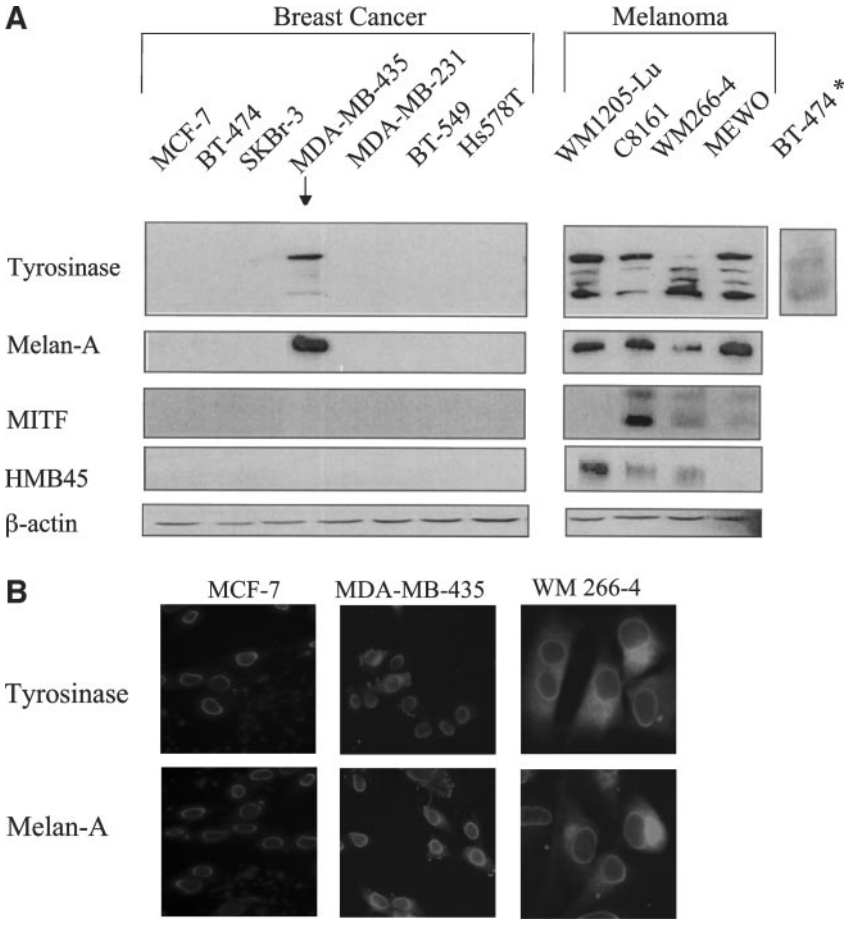

Fig. 4. MDA-MB-435 cells express some of the melanocyte-specific proteins. *A*, tyrosinase, melan-A, microphthalmia transcription factor (*MITF*), and HMB45 antigen protein levels were analyzed in cell extracts (30 μg) from the indicated breast cancer and melanoma cell lines by immunoblotting with their respective antibodies. *BT-474∗*, longer exposure of tyrosinase expression in BT-474 cells. β-actin was used as the loading control. Data are representative of three independent experiments. *B*, immunofluorescent images of partial expression of tyrosinase and melan-A by the MDA-MB-435 cell line, the MCF-7 breast cancer cell line, and the melanoma cell line WM266–4.

Expression of Melanocyte-Specific Protein Markers by MDA-MB-435 Cells. Although our data support the breast epithelial origin of the MDA-MB-435 cancer cells, a cDNA microarray-based expression profile study showed that the MDA-MB-435 cells express certain melanocyte genes (5), and another recent study showed that it expresses melanoma-specific genes (6). We, therefore, examined whether MDA-MB-435 cells, which express breast epithelium-specific markers, also coexpress functional melanocyte-specific proteins (Fig. 4).

These melanocyte-specific proteins included tyrosinase, the key enzyme required for melanin biosynthesis and used as a marker for melanocytes (25); melan-A, a melanocyte differentiation antigen recognized by autologous cytotoxic T cells and expressed in 50–60% of melanoma cell lines (26); MITF, a basic helix-loop-helix-leucin zipper tissue factor implicated in pigmentation (27); and HMB45, a highly specific antibody that recognizes an oncofetal glycoconjugate associated with immature melanosomes (28). The detection of all four of the melanoma-specific proteins have been used in the clinical diagnosis of melanoma. In addition, tyrosinase and HMB45 antigen have been used as biomarkers to distinguish malignant melanoma from other malignancies (29). All four of the melanoma cell lines expressed the melanocyte-specific proteins tyrosinase and melan-A, whereas most of the breast cancer cell lines did not (Fig. 4*A*). None of the breast cancer cell lines, including the MDA-MB-435 cells, expressed the melanoma markers MITF or HMB45, whereas three of the four melanoma cell lines expressed MITF and HMB45 (Fig. 4*A*). The incomplete expression of these melanocyte-specific proteins in melanoma cell lines can be attributed to the suggestion that the loss of expression of these antigens occurs as a defense mechanism that the melanoma cell uses to escape immunosurveillance (30).

Interestingly, although MDA-MB-435 cells did not express HMB45 antigen nor MITF, they expressed tyrosinase and melan-A at levels lower than or comparable with those in the melanoma cell lines. To further evaluate the presence of both tyrosinase and Melan-A in the MDA-MB-435 cell lines, we performed immunofluorescence staining on the MDA-MB-435 cells, along with the MCF-7 breast cancer cell line and the melanoma cell line WM266–4, with antibodies against tyrosinase and Melan-A. We found that a subpopulation of the MDA-MB-435 cell line expressed these proteins, whereas the melanoma cell lines had a homogenous expression of tyrosinase and melan-A (Fig. 4*B* and data not shown). Thus, the MDA-MB-435 cell line does express some melanocyte-specific proteins (Fig. 4*A*). Notably, another breast cancer cell line, BT-474, also expressed the melanocyte-specific proteins tyrosinase, albeit at lower levels (Fig. 4*A*, *BT-474∗, longer exposure*).

Coexpression of Breast Epithelium and Melanocyte-Specific Markers by MDA-MB-435 Xenografts. MDA-MB-435 cancer cell lines demonstrate high degrees of genetic instability in cell culture (10). To determine whether the coexpression of epithelial-specific and melanocyte-specific proteins by MDA-MB-435 cells can be detected *in vivo* and not only in cell cultures, we examined tissue sections from an MDA-MB-435 tumor xenograft grown in the mammary fat pad of severe combined immunodeficient mice, a WM266–4 melanoma xenograft grown over the right scapular region of BALB/c nude mice, and a human breast carcinoma from a patient who underwent a mastectomy. We examined the expression of the epithelial-specific proteins cytokeratin and EMA and the melanoma-specific proteins HMB45 antigen and tyrosinase in each tissue section by immunohistochemical analyses (Fig. 5*A*). As expected, the human breast carcinoma sample stained positive for cytokeratin and EMA but negative

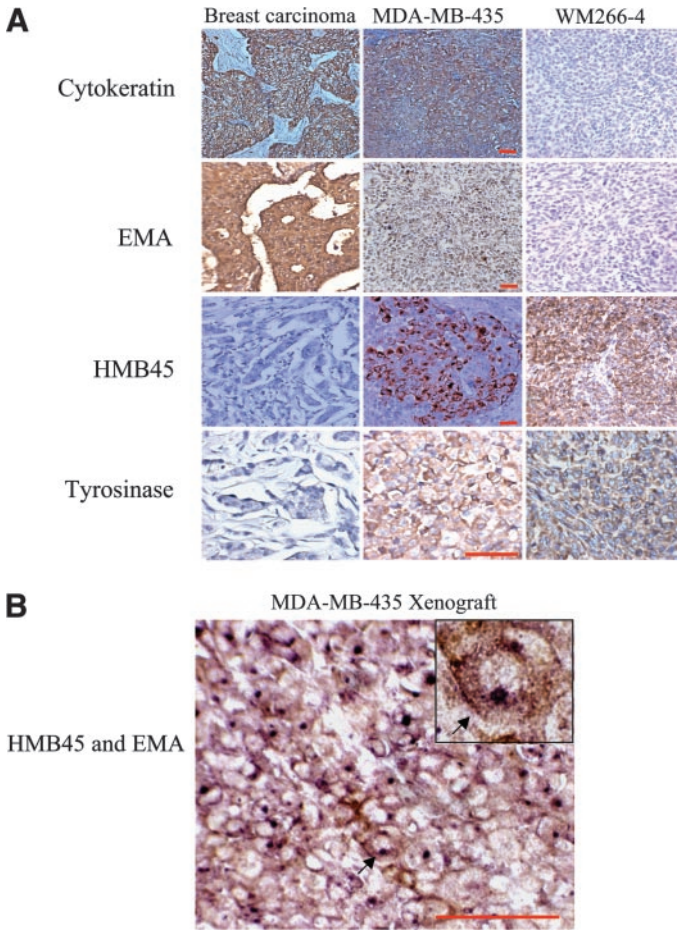

Fig. 5. Expression of epithelial- and melanocyte-specific markers by MDA-MB-435 xenografts *in vivo*. *A*, tissue sections from human breast carcinoma, MDA-MB-435 cell xenograft, and WM266−4 melanoma cell xenograft were deparaffinized and immunostained with cytokeratin, epithelial membrane antigen (*EMA*), HMB45, or tyrosinase antibodies (×400 for tyrosinase; all others original magnification, ×100). *B*, a subpopulation of cells in the MDA-MB-435 xenograft express EMA and melanocyte-specific HMB45 antigen in the cytoplasm. A tissue section from MDA-MB-435 xenograft was stained with EMA as the first primary antibody and with 3,3′-diaminobenzidine (DAB) color development, followed by staining with HMB45 as the second primary antibody and with Vector VIP (no. SK-4600) for color development. *Arrow*, the representative cell that clearly shows colocalized HMB45 (*purple*, paranuclear dot-like and diffuse cytoplasmic expression) and EMA (*brown*, diffuse cytoplasmic expression) protein staining. *Insert*, a double-staining cell. ×400 or ×1000 (*insert*). *Scale bar*, relative magnification compared with *A*.

for HMB45 antigen and tyrosinase. On the other hand, the melanoma xenograft tissue stained positive for the HMB45 antigen and tyrosinase but negative for cytokeratin and EMA. Notably, the vast majority of the cell population from the tissue of the MDA-MB-435 xenograft stained positive for cytokeratin and EMA. Interestingly, a small region of the tissue section stained positive for the HMB45 antigen, and most cells expressed tyrosinase at a lower level than did the melanoma xenograft, indicating that a subpopulation of the cells expressed melanocytic proteins *in vivo*. To further determine whether the MDA-MB-435 cells expressing melanocytic proteins are the same cells that express epithelial markers, we examined whether EMA expression (*brown staining*) can be detected in the same cells that expressed HMB45 antigen (*purple staining*) by double-labeling immunochemistry. Indeed, the EMA-expressing MDA-MB-435 cells also expressed HMB45 antigen, and both HMB45 and EMA were expressed in the cytoplasm (Fig. 5, *B* and *insert*). Taken together, these data indicate that MDA-MB-435 is a breast epithelial cell line that has not strictly adhered to a breast cell lineage but has gained melanocyte lineage characteristics.

DISCUSSION

Breast-Specific Features of MDA-MB-435 Cells. In this study, we demonstrated that the MDA-MB-435 cell line exhibits the hallmark features of breast epithelial cells. It expressed breast-specific milk components, including milk proteins and milk lipids. Although the expression levels of β-casein and EMA were lower in MDA-MB-435 cells than in some of the other breast cancer cell lines examined, these breast epithelial- or epithelial-specific proteins were consistently expressed by MDA-MB-435 cells. In addition, we can readily detect expression of α-lactalbumin, keratin 19, and pan-keratin in MDA-MB-435 cells as in other breast cancer cells, confirming that it has the features of breast epithelial tissue. The production of milk lipid droplets by MDA-MB-435 cells also showed that MDA-MB-435 is a breast epithelial cell line. Together, these findings indicate that MDA-MB-435 cells originate from breast cancer cells and retain the key features of breast cancer cells.

Reduced levels of some breast epithelial proteins are not uncommon in highly advanced breast cancer, from which MDA-MB-435 cells were established. As we and others have shown (31–33), the MDA-MB-435 cell line, established from a highly advanced breast cancer, exhibits the characteristics of a highly malignant tumor cell line: it is capable of forming tumors in the mammary fat pad and metastasizes in nude mice. MDA-MB-435 cells do not have a detectable level of the normal epithelial marker E-cadherin (34), an adhesion molecule that plays a significant role in normal epithelial differentiation. However, this is expected, because E-cadherin is frequently lost during the progression of human epithelial cancers, including breast cancers (35). Likewise, MDA-MB-435 does not express the estrogen receptor and is hormone unresponsive, which are indicators of poorly differentiated and highly malignant breast tumor cells (36, 37). Therefore, it is likely that the loss (*e.g.*, of keratin-8) or reduction (*e.g.*, β-casein and EMA) of epithelial-specific proteins and breast epithelial-specific proteins is a result of dedifferentiation that involves the loss of some breast epithelial-specific or epithelial-specific differentiation markers, as observed in some advanced malignant neoplasms (38). For example, EMA is an indicator of a more mature epithelial phenotype and is found more consistently in well- and moderately differentiated neoplasms, whereas low EMA expression correlates with higher tumor grade and worse disease-free prognosis (39). However, despite the advanced malignant characteristics of MDA-MB-435 cells, the cell line still retains the fundamental and functional biomarkers of mammary epithelial cells, as our data showed.

It is well known that as cells of epithelial origin progress through the epithelial to mesenchymal transition, they lose their keratin expression (40). On the other hand, it is also known that as melanoma cells progress through the MET, they begin to express keratin (41). Consistent with this, our Western blot analysis demonstrates that all four of the melanoma cell lines express low or intermediate levels of keratin 19 (Fig. 1). Interestingly, we also found that the WM1205-Lu melanoma cell, which is the most aggressive and highly dedifferentiated melanoma line of the four melanoma lines, produces low amount of lipids, another indication of MET.

Melanocytic Proteins Expressed by MDA-MB-435 Cells. In our study, the MDA-MB-435 cell line expressed the specific melanocyte proteins tyrosinase and melan-A; this is consistent with previous reports that found similar gene expression patterns in melanoma cell lines and the MDA-MB-435 cell line, but not in other breast cancer cell lines (5, 6). This resulted in the categorization of the MDA-MB-435 cell line in the same gene cluster with melanoma cell lines and further categorized it as being of melanocytic origin. However, specific breast epithelial proteins, such as β-casein and α-lactalbumin,

were not examined, and our data clearly indicates that MDA-MB-435 cells are breast epithelial cells because they retain the fundamental and functional markers of breast epithelial cells, including β-casein and α-lactalbumin. The discrepancy between the classification of MDA-MB-435 cells as of melanocytic *versus* breast epithelial origin is further complicated by the fact that multiple MDA-MB-435 sublines exist and the MDA-MB-435 cell lines used in the previous studies may be different sublines from that of our MDA-MB-435 cell line.

Notably, MDA-MB-435 cells do not express the melanocyte-specific markers MITF and HMB45 antigen in cell culture, unlike most of the melanoma cell lines. Interestingly, the highly aggressive WM1205-Lu cells are known to have undergone MET (42). Although WM1205-Lu has also lost MITF expression, they do not express any of the breast-specific markers or most of the epithelial-specific markers we examined. This clearly distinguishes the aggressive melanoma cell line WM1205-Lu that underwent MET from the advanced breast cancer cell line MDA-MB-435 that underwent epithelial to mesenchymal transition.

Our *in vivo* data added key information in support of the hypothesis that MDA-MB-435 cells originate from breast epithelial tissue and gain aberrant melanocyte markers as opposed to originating from melanocyte tissue and gaining breast epithelial markers. In particular, the vast majority of cells in the MDA-MB-435 xenograft stained positive for the epithelial markers cytokeratin and EMA, but only a subpopulation of cells stained positive with the HMB45 antibody that recognizes melanocyte-specific proteins. We did not, however, detect melanocytic proteins with the HMB45 antibody in cell lysates of MDA-MB-435. It may be that melanocyte protein expression levels in the cells are below the detection limits of Western blot analysis or, alternatively, that *in vivo* growth alters expression of specific proteins. Together, our data suggest that a subpopulation of MDA-MB-435 cells that have undergone lineage infidelity exist within the entire population of breast epithelial cells, and this may account for the expression of melanocyte markers observed by us and others. If MDA-MB-435 had originated from melanocyte tissue, then the reverse findings would have been seen: a minor portion of cells would have expressed cytokeratin and EMA, whereas a majority of cells would have expressed tyrosinase and the HMB45 antigen. Notably, the original MDA-MB-435 cell stock obtained by Brinkley *et al.* (43) was derived from a pleural effusion in a patient who had extensive breast adenocarcinoma with deposits in the breast and breast lymphatics. There was no report of melanoma before or after breast carcinoma diagnosis, and the patient died one year after diagnosis. The clinical data of this patient is consistent with our notion that MDA-MB-435 cells originated from an advanced breast cancer.

The expression of melanocytic proteins by the breast cancer cell line MDA-MB-435 establishes its lack of strict adherence to a breast cell lineage. Dedifferentiation, indicated by the loss of differentiation markers, characterizes advanced malignant neoplasms. Here we show that the gain of aberrant differentiation markers is also characteristic of a dedifferentiated cell line that arose from an advanced malignant neoplasm. Interestingly, the heterogeneous expression of the two melanoma markers, tyrosinase and melan-A, indicates that not all of the MDA-MB-435 cells in a single sample have undergone the same degree of epithelial to mesenchymal transition.

These findings are in agreement with previous reports that describe the expression of atypical cell-specific markers in various advanced malignancies such as melanoma and chronic myeloid leukemia (44, 45). Moreover, MDA-MB-231 human breast cancer cells have also been shown to express markers of the endothelial lineage (46). Together, our data indicate that the MDA-MB-435 breast cancer cell line may serve as an excellent cell model for future studies of the lineage infidelity phenotype as well as other biological phenotypes of highly malignant and dedifferentiated breast cancers. We conclude that the MDA-MB-435 cell line is of breast cancer origin that may be used as an excellent model for studies of highly malignant and dedifferentiated breast cancers.

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