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Role of Id-2 in the Maintenance of a Differentiated and Noninvasive Phenotype in Breast Cancer Cells[1]

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ABSTRACT

Id proteins are inhibitors of basic helix-loop-helix transcription factors and generally stimulate cell proliferation and inhibit differentiation. We have shown that ectopic expression of Id-1 in murine mammary epithelial cells resulted in loss of differentiation and gain of invasive and proliferative abilities. Moreover, Id-1 was highly expressed in aggressive breast cancer cells in culture and in biopsies from infiltrating carcinomas. In contrast to Id-1, we found that, *in vitro* and *in vivo*, Id-2 mRNA and protein were up-regulated as mammary epithelial cells lost proliferative capacity and initiated differentiation. We further determined that this up-regulation of Id-2 was a necessary step toward a fully differentiated phenotype in breast cells. Here we show that one of the components of the extracellular matrix network, laminin, is responsible for the increase in Id-2 expression during differentiation. We also show that Id-2 expression is inversely correlated with the rate of proliferation in murine mammary epithelial cells and that Id-2 is expressed at a higher level in differentiated human breast cancer cells in comparison with very aggressive and metastatic cells. When reintroduced in aggressive breast cancer cells, Id-2 is able to reduce their proliferative and invasive phenotypes and decrease their level of matrix metalloproteinase 9 secretion as well as increase syndecan-1 expression. Moreover, little Id-2 protein expression is detectable in human biopsies from aggressive and invasive carcinomas in comparison with *in situ* carcinomas. In conclusion, Id-2 expression not only follows a pattern opposite to that of Id-1 during mammary gland development and breast cancer progression but also appears to act as an important protein for the maintenance of a differentiated and noninvasive phenotype in normal and transformed breast cells.

INTRODUCTION

In vivo, growth and differentiation of the mammary gland progress through several stages during puberty, and ultimately, full function is achieved during lactation, *i.e.*, expression and secretion of milk proteins. At birth, female mice possess mammary glands composed of a simple ductal system. At 3 weeks of age, which is the onset of puberty, the immature glands begin to grow rapidly. There is intense mitotic activity within the stem cells of the end buds. In 4–8-week-old mice, extensive branching morphogenesis occurs, originating at the tips of the end buds. DNA synthesis still occurs during the first half of pregnancy (1). During the second half of pregnancy, the levels of casein mRNA increase as mammary epithelial cells cease proliferation. In the lactating gland (2, 3), the secretory lobules developed from the branching ducts become filled with milk. The entire gland then remodels during involution.

It is well documented that the decrease in DNA synthesis observed during late pregnancy and lactation is coupled to the functional differentiation of the mammary gland. However, little is known about the molecular mechanisms that coordinate growth and differentiation, as well as the tightly regulated and transient invasive behavior of the normal epithelial cells (4).

bHLH[3] transcription factors are key regulators of lineage- and tissue-specific gene expression in a number of mammalian and nonmammalian organisms. bHLH proteins act as obligate dimers, dimerizing through HLH domains and binding DNA through the composite basic domains to regulate the transcription of target genes containing E-boxes (CANNTG) in their promoters. Id (inhibitor of DNA binding) proteins dimerize with bHLH proteins, but because Id proteins lack basic domains, Id-bHLH heterodimers fail to bind DNA. Thus, Id proteins are dominant negative regulators of bHLH function (5). Constitutive expression of Id proteins has been shown to inhibit the differentiation of various cell types (6, 7). Four members of the Id gene family have been described thus far: (*a*) Id-1; (*b*) Id-2; (*c*) Id-3; and (*d*) Id-4. The different family members localize to different chromosomes and show marked differences in their pattern of expression and function (8, 9).

A role for HLH Id proteins in the differentiation of mammary epithelial SCp2 cells was first suggested by our finding that Id-1 expression declined to undetectable levels when the cells were induced to differentiate in culture (10). A similar decline was observed in lactating mammary epithelial cells *in vivo* (11). Further support for the importance of Id-1 was obtained when SCp2 cells were transfected with a constitutively expressed Id-1 cDNA. The transduced cells lost the ability to differentiate, proliferated, and became invasive (12, 13). Moreover, high Id-1 levels correlated with invasiveness in breast cancer cells in culture and in human breast cancer biopsies, and when a noninvasive breast cancer cell line was transfected with Id-1, it became invasive (14, 15).

Unlike Id-1, which is expressed during proliferation and is able to suppress differentiation in all cell types examined, the data on Id-2 are much less consistent. The Id-2 protein contains a HLH motif similar to that of Id-1 [90% identity (16, 17)], but the two proteins differ markedly in the rest of their sequence. Id-1 and Id-2 are encoded by unlinked genes. A 1.3-kb mRNA encodes the Id-2 protein of 134 amino acids. Id-2, as well as Id-1, was first identified as an inhibitor of differentiation because it was down-regulated during the differentiation of a variety of cell types (7, 18). In addition, overexpression of Id-2 inhibited the differentiation of myoblasts (19) and led to a stage-specific developmental block early in thymopoiesis (20). However, inconsistent with the role for Id-2 as an inhibitor of differentiation, Id-2 mRNA levels increased markedly during the differentiation of myeloid precursors (such as HL-60) to either granulocytes or macrophages (21). Id-2 gene expression was also maintained during embryonic stem cell-derived hematopoietic differentiation (22). It has been reported recently that mice deficient in Id-2 were devoid of lymph nodes and Peyer's patches and displayed disturbed differentiation of natural killer cells (23). Surprisingly, these Id-2-deficient

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[3] The abbreviations used are: bHLH, basic helix-loop-helix; HLH, helix-loop-helix, ECM, extracellular matrix; MMP, matrix metalloproteinase; FBS, fetal bovine serum; DCIS, ductal carcinoma(s) *in situ*; PRL, prolactin.

mice also exhibited, in addition to the above-mentioned phenotypes, a lactation defect (24).

These results suggest that in some tissues, Id-2, unlike Id-1, may promote differentiation. Consistent with this, we have recently reported studies of murine mammary epithelial cells in culture and *in vivo* (11) suggesting that Id-2 may promote cell differentiation of mammary gland, possibly by inhibiting the activity of bHLH involved in stimulation of cell proliferation. Based on the pattern of Id-2 expression during mammary epithelial cell differentiation *in vitro* and mammary gland development *in vivo*, we proposed that Id-2 was acting as an activator, rather than an inhibitor, of mammary differentiation (11). To complete differentiation, according to this hypothesis, mammary epithelial cells would have to switch off Id-1 and turn on Id-2 expression, and these two events appear to be tightly coordinated.

In the mammary gland, expression of milk protein genes is controlled by cross-talk between signals derived from the basement membrane protein, laminin, and the lactogenic hormone, PRL (10). Although the mechanisms of hormone regulation in this system are being investigated, the mechanism of ECM signaling is still poorly defined. Here we show that the ECM protein laminin, and not the lactogenic hormones, is responsible for the increase in Id-2 expression during differentiation. We also report that Id-2 expression is inversely correlated with the rate of proliferation in murine mammary epithelial cells. Furthermore, we show that Id-2 is expressed at a high level in the more differentiated human breast cancer cells. On the other hand, its level of expression is very low in the more aggressive and metastatic breast cancer cells. However, when Id-2 is constitutively expressed in these aggressive breast cancer cells, their proliferative and invasive abilities are significantly reduced. Likewise, Id-2 protein expression is low or undetectable in human biopsies from grade III invasive carcinomas. However, Id-2 is detectable in most of the *in situ* carcinomas as well as in the least aggressive invasive carcinomas investigated.

The results suggest that Id-2 expression follows a pattern opposite to that of Id-1 during mammary gland differentiation as well as breast cancer cell progression and that it appears to act as a potent differentiating protein. We therefore propose that Id-2 expression, in contrast to Id-1 expression, serves as a good prognostic indicator for breast cancer.

MATERIALS AND METHODS

Retroviral Vectors and Virus Production. The full-length mouse Id-2 cDNA, as described previously (11), was cloned into a pLXSN retroviral vector in a sense and antisense orientation. The full-length human Id-2 cDNA, a kind gift from Dr. Eiji Hara (Manchester, United Kingdom), was also cloned into a pLXSN vector in a sense orientation. These viral vectors were then packaged in TSA-54 cells (Cell Genesis, Foster City, CA) using calcium phosphate. Twenty-four h after transfection, culture medium (containing infectious virus) was harvested twice at 4-h intervals and frozen at −80°C. Viral titers were determined by reverse transcriptase activity.

Cell Culture and Retroviral Infection. Human breast cancer cell lines MCF-7, T47D, MDA-MB231, and MDA-MB436 were purchased from the American Type Culture Collection. All breast cancer cell lines were grown in RPMI 1640 containing 10% FBS and insulin (5 μg/ml; Sigma). For experiments using serum-free medium, FBS was omitted. For the infection experiments, approximately 8 reverse transcriptase units of either pLXSN or pLXSN-Id-2 retrovirus were mixed with 5 ml of medium containing 4 μg/ml Polybrene and added to MDA-MB231 cells in 100-mm dishes. Cells expressing the retroviral genes were selected in neomycin and pooled.

RNA Isolation and Northern Analysis. Total RNA was isolated and purified as described by Chomczynski and Sacchi (25). RNA was size fractionated by electrophoresis through denaturing formaldehyde-agarose gels and transferred to nylon membrane (Hybond-N; Amersham Corp.). The blots were hybridized with ^{32}P-labeled probes prepared by random oligonucleotide priming, washed, and exposed to Kodak XAR-5 film for autoradiography. β-Casein and Id-1 probes were described previously (12). The mouse Id-2 probe was as described previously (11), and the human Id-2 probe was a kind gift from Dr. Eiji Hara.

Western Analyses. Cells were rinsed twice with PBS, scraped, and lysed at 37°C overnight. Lysates were heated at 95°C for 10 min to inactivate proteases. Lysates were clarified by centrifugation, supernatants were collected, and protein concentration was measured. Proteins were separated by SDS-PAGE using 12% acrylamide gels and blotted onto nitrocellulose membranes. Blots were probed with anti-β-casein (12), anti-Id-2 (Santa Cruz Biotechnology), anti-syndecan-1 (H-174 and C-20; Santa Cruz Biotechnology), or anti-actin (Chemicon) antibodies. Membranes were incubated with horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology), and binding was detected using enhanced chemiluminescence.

Boyden Chamber Invasion Assays. Assays were performed in modified Boyden chambers with 8-μm-pore filter inserts for 24-well plates (Collaborative Research). Filters were coated with 10–12 μl of ice-cold Matrigel (11 mg/ml protein; Collaborative Research). MDA-MB231 cells (50,000 cells/well) were added to the upper chamber in 200 μl of serum-free medium. Cells were assayed in triplicate or quadruplicate, and the results were averaged. The lower chamber was filled with 300 μl of conditioned medium from fibroblasts (26). After a 20-h incubation, cells were fixed with 2.5% glutaraldehyde in PBS and stained with 0.5% toluidine blue in 2% Na_2CO_3. Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy.

Anchorage-Dependent Growth Assays. Liquefied 2% agarose was mixed with an equal volume of 2× DMEM:Ham's F-12 growth medium lacking serum and supplemented with insulin (10 μg/ml) and gentamicin [100 μg/ml (2× medium)]. One ml of the mixture was layered onto 35-mm dishes to create a 1% agarose base. Liquefied 0.6% agarose was mixed with an equal volume of 2× medium, and 10 ml of this solution were mixed with 1 ml of growth medium containing 1×10^5 MDA-MB231 cells to yield 1×10^4 cells/ml in 0.27% agarose; 1 ml of this cell suspension was layered on top of the 1% agarose base, and 1 ml of DMEM:Ham's F-12 containing 10% FBS was added. The cells were incubated for 2–3 weeks. Counts were performed according to the size of the colonies.

Zymography. Proliferating MDA-MB231 cells (1×10^6 cells in 100-mm dishes) were shifted to serum-free medium for 2–3 days, at which time they were given 10 ml of fresh serum-free medium. Forty-eight h later, the conditioned medium was collected and concentrated 10–15-fold using 10 kDa cutoff filters (Millipore, Bedford, MA). The concentrated medium was analyzed on gelatin substrate gels, as described by Fisher and Werb (27). Briefly, gels consisted of 8–10% polyacrylamide and 3 mg/ml gelatin (Sigma). Concentrated conditioned medium was mixed with nonreducing Laemmli sample buffer and incubated at 37°C for 15 min. After electrophoresis, the gels were incubated for 1 h in 2.5% Triton X-100 at room temperature, followed by 24–48 h in substrate buffer [100 mM Tris-HCl (pH 7.4) and 15 mM $CaCl_2$]. The gels were stained with Coomassie Blue for 30 min and destained with 30% methanol/10% acetic acid.

CellStat System to Monitor Cell Proliferation. To monitor the cellular proliferation of mouse mammary epithelial SCp2 cells, we used the apparatus kindly provided by CellStat Technologies Inc. (Belmont, CA; Ref. 28). This noninvasive, nondestructive system allows a continuous cell monitoring. The CellStat system monitors the electrical signal (voltage) produced through the culture medium by the metabolic activity occurring in the cells, and the intensity of the signal is proportional to the cell count. The system was previously validated by counting cells in replicate wells using a hemocytometer. In our experiments, 15,000 cells were plated per well in 24-well plates. Each cell population (control, Id-2 sense and Id-2 antisense mouse mammary SCp2 cells) was plated in 6 wells. Cell growth was measured every 60 min, and the readings from the 6 wells/cell population were averaged.

[^{3}H]Thymidine Labeling. MDA-MB231 cells cultured in 0.5% or 2% serum were given [^{3}H]thymidine (10 μCi/ml; 60–80 Ci/mmol; Amersham) for the last 7 or 16 h of the experiments, whereupon they were fixed with methanol/acetone (1:1) and stained with 4′,6-diamidino-2-phenylindole. [^{3}H]Thymidine labeling was developed as described previously (12). The percentage of labeled nuclei was calculated by comparing the number of [^{3}H]thymidine-labeled nuclei with the number of 4′,6-diamidino-2-phenylin-

dole-stained nuclei in a given field, using phase-contrast and fluorescence microscopy.

Immunohistochemistry. Formalin-fixed paraffin-embedded mammary gland tissue sections (11) as well as 48 paraffin-embedded human tumor biopsies (obtained from California Pacific Medical Center) were used to determine Id-2 protein expression. Slides were dewaxed, rehydrated, placed in a container containing 1 liter of 0.01 M citrate buffer (pH 6.0), microwaved at 700 W for 20 min, allowed to remain in the hot citrate buffer for 15 min, and cooled down in running cold water. The slides were washed in deionized water and incubated in 10% nonfat dry milk for 30 min at room temperature, washed in TBS, and incubated with 1 μg/ml anti Id-2 antibody (Santa Cruz Biotechnology) overnight at 4°C. The slides were washed in TBS, incubated with biotinylated swine antirabbit $F(ab')_2$ (1:400) for 30 min, and then incubated with 1:500 streptavidin-horseradish peroxidase for 30 min. After washing in TBS, peroxidase was visualized by incubation in 0.5 mg/ml diaminobenzidine-4HCl and 0.03% hydrogen peroxide in TBS. Finally, the slides were counterstained with hematoxylin.

RESULTS

Id-2 Expression in Murine Mammary Epithelial Cells and Its Regulation by Laminin. We previously examined Id-2 expression during normal mouse mammary gland development *in vivo* using Western analysis (11). Id-2 protein expression was correlated with β-casein and inversely correlated with Id-1 protein expression, *i.e.*, Id-2 was expressed when cells differentiated during the second part of pregnancy and during lactation. Because proteins were isolated from a whole tissue, it was important to confirm that Id-2 was expressed by the epithelial component of the mammary gland. We therefore performed immunohistochemistry using an Id-2-specific antibody. A strong signal corresponding to Id-2 protein was detected at day 12 of lactation in the nuclei of differentiated epithelial cells (Fig. 1*A, c*). A signal was already detectable at day 18 of pregnancy (Fig. 1*A, b*), and no signal was detectable from proliferating and invasive epithelial cells from 7-week-old virgin mice (Fig. 1*A, a*).

We previously used Western analysis to determine Id-2 expression during mammary gland development (11). We found that Id-2 protein expression increased after day 12 of pregnancy. We now show in Fig. 1*A, e* the expression of Id-2 mRNA at various stages of development. Id-2 mRNA expression also increased at day 12 of pregnancy, with a peak of expression detectable at day 18 of pregnancy. The increase in both Id-2 mRNA and protein expression during mid-pregnancy therefore corresponded to a time when differentiation occurs and milk proteins such as β-casein begin to be expressed.

During mammary gland differentiation, the epithelial cells receive some extracellular signals that arrest cell growth and initiate their differentiation process (milk production). Among these different signals are the lactogenic hormones as well as the proteins from the ECM (29). We reported previously (11) that murine mammary epithelial SCp2 cells stop proliferation and differentiate when treated with hormones and the ECM component laminin and that Id-2 is up-regulated during this process.

We now show that the crucial component responsible for the increase of Id-2 expression is laminin, which represents about 90% of the ECM, and not any of the lactogenic hormones (Fig. 1*B, a*). SCp2 cells were cultured in serum-free medium for 2 days before treatment for 2 additional days with or without laminin and with or without PRL or hydrocortisone. RNA was isolated and analyzed for Id-2 and β-casein expression. Upon addition of laminin, Id-2 mRNA was clearly up-regulated independently of the absence or presence of lactogenic hormones (Fig. 1*B, a, Lanes 2, 4,* and *6*).

However, we detected some basal levels of Id-2 mRNA in cells cultured in plain medium without any lactogenic hormone, with hydrocortisone only, or with both hydrocortisone and PRL (Fig. 1*B, a, Lanes 1, 3,* and *5*, respectively). We previously determined (11) that, after serum starvation, Id-1 expression was down-regulated, and Id-2 mRNA levels increased, independently of the presence of extracellular signals. As a potential explanation, we proposed that some bHLH proteins, previously sequestered by Id-1, became available to up-regulate Id-2 expression. To confirm this hypothesis, we compared Id-2 expression (Fig. 1*B, b*) in mammary epithelial SCp2 cells that constitutively express Id-1 (SCp2-Id-1 cells) and were subsequently infected with either control pLXSN (Fig. 1*B, b, Lane 1*) or pLXSN-ITF2 (Fig. 1*B, b, Lane 2*) vector. ITF2 is an Id-1-interacting bHLH transcription factor, and we previously determined (11) that its overexpression could reverse the phenotypes induced by constitutive Id-1 expression, *i.e.*, reduction of proliferation as well as reestablishment of milk secretion in SCp2-Id-1-LXSN-ITF2 cells. Here we show that the effects of ITF2 overexpression on the reestablishment of a more normal phenotype are correlated with the up-regulation of Id-2 expression in SCp2-Id-1-LXSN-ITF2 cells (Fig. 1*B, b, Lane 2*).

Using Id-2 antisense constructs, we have previously shown (11) that Id-2 was necessary for mammary epithelial cells to differentiate in culture. Murine mammary epithelial SCp2 cells were infected with either pLXSN (control), pLXSN-Id2-sense, or pLXSN-Id2 antisense constructs, and cells expressing reduced levels of Id-2 protein were unable to express the milk protein β-casein. Using the same three populations of infected cells, we now show Id-2 is not only necessary for differentiation but also acts as a strong regulator of the cell cycle. Cell proliferation was measured using the CellStat system. Cells infected with Id-2 sense constructs grew more slowly than control cells (Fig. 1*C*). Conversely, cells infected with Id-2 antisense constructs grew more quickly than control cells. Id-2 was therefore able to reduce the rate of cell proliferation when expressed in murine mammary epithelial cells. Because Id-2, when overexpressed in mammary epithelial cells, triggers a high level of differentiation and milk secretion (11), it is unlikely that this reduction of proliferation could be explained by an induction of apoptosis. Moreover, Id-2-overexpressing cells maintained a high level of viability using the technique of trypan blue exclusion (data not shown).

Id-2 Expression in Human Breast Cancer Cell Lines. Because the loss of proper regulation of proliferation and differentiation is an important aspect of cancer progression, we next investigated the role of Id-2 in human breast cancer cells. We first determined Id-2 expression in human breast cancer cell lines in culture. We used the T47D and MCF-7 cell lines that display nonaggressive and differentiated characteristics in culture (in the absence of estrogen), and the two aggressive and metastatic MDA-MB231 and MDA-MB436 cell lines. Id-2 mRNA was detectable in T47D cells cultured in serum-free conditions. However, Id-2 expression was undetectable in the two aggressive cell lines MDA-MB231 and MDA-MB436, which express high levels of Id-1 (Fig. 2*A*). These results therefore confirmed the inverse correlation between the expression of Id-1 and Id-2 in human breast cancer cells that we previously detected in mouse mammary epithelial cells. This inverse relationship is particularly clear in Fig. 2*B*, where the levels of Id-2 mRNA increased in MCF-7 cells upon serum withdrawal, whereas the levels of Id-1 mRNA decreased. Therefore, we hypothesized that Id-2 can only be expressed when Id-1 is down-regulated. As a potential explanation, we propose that some bHLH proteins such as ITF2, previously sequestered by Id-1, up-regulate Id-2 expression at the promoter level through their binding to E-boxes.

Constitutive Expression of Id-2 Reduces the Aggressive Phenotype of Human Metastatic Breast Cancer Cells. We hypothesize that aggressive breast cancer cells have acquired the ability to constitutively express Id-1 gene (14) and therefore have lost the ability to

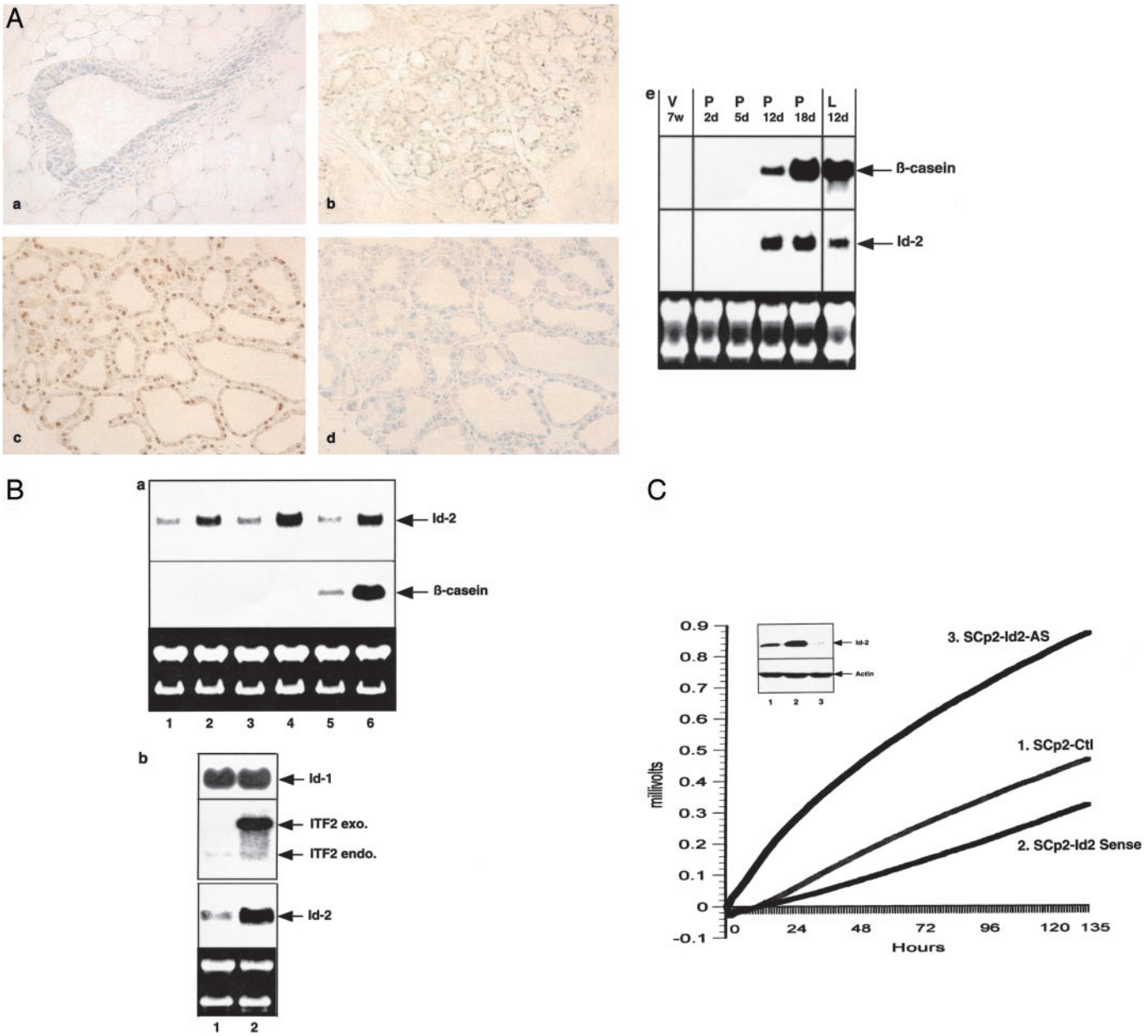

Fig. 1. *A*, Id-2 expression in mouse mammary glands. In *a–d*, Id-2 protein expression was detected by immunohistochemistry. *a*, virgin, 7 weeks of age; *b*, 18 days after onset of pregnancy; *c* and *d*, 12 days after onset of lactation (the control using Id-2 blocking peptide is shown in *d*). In *e*, total RNA was extracted from glands at different stages of development (*V*, virgin; *P*, pregnant; *L*, lactation), and Northern analyses using cDNA probes for mouse β-casein and Id-2 were performed. *B*, Id-2 expression in mouse mammary epithelial SCp2 cells in culture. In *a*, SCp2 cells were cultured in serum-free medium or treated with laminin and/or lactogenic hormones for 2 days. Total RNA was extracted and analyzed by Northern blot for Id-2 and β-casein. *Lane 1*, no lactogenic hormone and no laminin; *Lane 2*, laminin only; *Lane 3*, hydrocortisone only; *Lane 4*, hydrocortisone and laminin; *Lane 5*, hydrocortisone and PRL; *Lane 6*, hydrocortisone, PRL, and laminin. In *b*, SCp2 cells transfected with Id-1 were subsequently infected with an LXSN-control vector (SCp2-Id-1-LXSN-ctl in *Lane 1*) or an LXSN-ITF2 vector (SCp2-Id-1-LXSN-ITF2 in *Lane 2*). Northern analyses using cDNA probes for Id-1, ITF2, and Id-2 were performed. *C*, reduction of cell proliferation of SCp2 cells infected with a pLXSN-Id-2-sense-expressing vector (*SCp2-Id2 Sense*) and increased cell growth of SCp2 cells infected with a pLXSN-Id-2-antisense-expressing vector (*SCp2-Id2-AS*). *SCp2-Ctl* represents the cells infected with an empty pLXSN construct. Each cell population was plated at 15,000 cells/well in 6 wells. Cell growth was measured every 60 min, and the readings from the 6 wells/cell population were averaged. *Inset*, a Western blot comparing the levels of Id-2 protein in the three populations.

express Id-2, which may explain why these cells are aggressive. The phenotype of MDA-MB231 is more invasive and aggressive than the phenotype of MDA-MB436 cells; we therefore chose to focus on the MDA-MB231 cells to determine the effects of Id-2 overexpression on the reduction of aggressiveness. We compared the level of aggressiveness of MDA-MB231 cells that constitutively express high levels of Id-2 protein with the level of aggressiveness of control MDA-MB231 cells, which lack Id-2. The MDA-MB231 cell line was infected with either pLXSN-control or pLXSN-Id2-sense constructs. Cells infected with pLXSN-Id2-sense expressed the Id-2 protein at a much higher level than control cells (Fig. 3*A*). We then quantified the levels of invasion and migration of MDA-MB231-control cells compared with MDA-MB231-Id2 sense cells (Fig. 3*B*). Cells expressing high levels of Id-2 were 4-fold less invasive than the control cells.

We used zymography to further characterize the mechanisms by which Id-2 overexpression reduced the invasive ability of metastatic breast cancer cells. We show that the levels of expression of MMP-9 (92-kDa gelatinase) were reduced in cells expressing Id-2 protein (Fig. 3*C*), representing a potential explanation for the reduction in the invasive phenotype of MDA-MB231 cells. We also compared the levels of syndecan-1 protein expression between MDA-MB231-control and MDA-MB231-Id2 sense cells. As described further in the

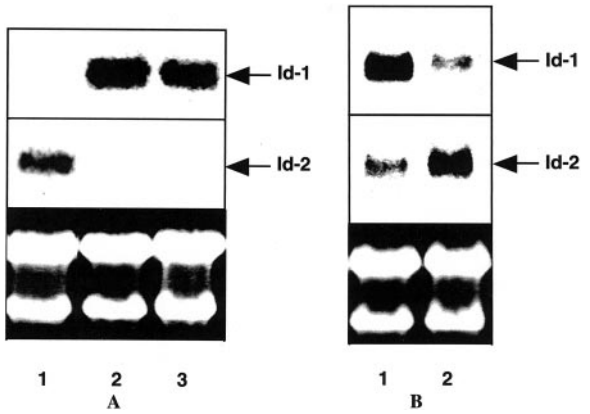

Fig. 2. *A*, Id-1 and Id-2 mRNA expression in human breast cancer cell lines. Cells were cultured in serum-free medium for 48 h before RNA was extracted and subjected to Northern blotting. *Lane 1*, nonaggressive T47D cancer cells; *Lane 2*, highly aggressive and metastatic MDA-MB231 cells; *Lane 3*, aggressive and metastatic MDA-MB436 cells. *B*, Id-1 and Id-2 mRNA expression in MCF-7 cells growing in 10% FBS (*Lane 1*) and in MCF-7 cells cultured in serum-free medium for 24 h (*Lane 2*).

"Discussion," syndecan-1 is a cell surface proteoglycan that has been implicated in a number of cellular functions, and its suppression has been shown to be associated with increased invasive potential and dysregulated growth of breast epithelial cells. We determined that syndecan-1, which was expressed at a low level in MDA-MB231-control cells, was significantly up-regulated in MDA-MB231-Id2 sense cells (Fig. 3*D*).

Next, we determined the effects of Id-2 on the regulation of cell proliferation. MDA-MB231-control cells and MDA-MB231-Id2-sense cells were plated on coverslips, cultured in either 0.5 or 2% serum, and labeled with methyl-[^{3}H]thymidine for 7 or 16 h, and the labeling index of the two cell populations was determined. MDA-MB-231-Id2-sense cells consistently showed a significant reduction of their capacity for proliferation compared with control cells (Fig. 3*E*). Finally, we also determined that the ability of the MDA-MB231-Id2 sense cells to grow in an anchorage-independent growth assay was strongly reduced (Fig. 3*F*). The majority of the MDA-MB231-control cells formed large colonies (>25 μm in diameter), whereas the majority of the MDA-MB231-Id2-sense cells formed small colonies (<25 μm in diameter). These results suggest that Id-2 is able to reduce the overall aggressiveness of breast cancer cells by reducing not only their invasiveness but also their rate of proliferation and their ability to grow in an anchorage-independent manner.

Id-2 Expression in Human Breast Tumor Biopsies. Lastly, we determined the expression of Id-2 protein in a panel of 48 human tumor biopsies from breast cancer patients by immunohistochemistry (Fig. 4; Table 1) using a specific batch of anti-Id-2 antibody, which showed no cross-reactive bands on Western blots (data not shown). Of a total of nine cases of DCIS, one was negative for Id-2, four DCIS were weakly positive, and the other four were clearly positive. Of a total of 14 infiltrating grade I carcinomas of ductal origin, 5 were weakly positive, and 5 were strongly positive. However, of a total of 16 infiltrating grade III carcinomas of ductal origin, only 1 was strongly positive; 8 of them were negative, and 7 were weakly positive. Using the χ^2 test, we determined that the difference in Id-2 expression between DCIS/invasive grade I *versus* invasive grade 2/3 was statistically significant at $P < 0.05$. Therefore, these data indicate an inverse relationship between degree of Id-2 expression and aggressiveness of breast tumors. Id-2, in contrast to Id-1, might represent a marker of good prognosis for breast cancer patients.

DISCUSSION

How mammary epithelial cells receive and integrate external signals leading to tissue-specific expression is far from being fully understood. Among the elements of the ECM signaling pathway responsible for gene regulation during mammary epithelial cell differentiation is the requirement for a laminin-rich basement membrane and the existence of a cooperative signaling pathway between ECM and the lactogenic hormone PRL (29, 30). This cooperative signaling between ECM and PRL may be achieved through integrin- and laminin-directed restructuring of the cytoskeleton, leading to profound changes in cell architecture and gene expression. These changes allow the PRL signal to activate transcription of the milk protein genes (30).

Signals from basement membrane are transduced by β_1 integrins and are required for PRL to activate DNA binding of the milk protein gene transcription factor, Stat5 (30). Thus, basement membrane controls transcription of milk protein genes through the Stat5-mediated PRL signaling pathway. Basement membrane is also necessary for tyrosine phosphorylation of the PRL receptor and thus directly affects cytokine signaling and differentiation (31). Also, in a primary cell culture model, association of a laminin-rich ECM with mammary epithelial cells was required for cell survival and cell differentiation, and it suppressed Brca1 expression in these cells (32). Besides these reports, not much is known about the identity of the transcriptional regulators that integrate the signals from the ECM to trigger differentiation.

Our hypothesis is that Id-2 may represent one of these key transcriptional regulators. Based on our data on Id-2 expression during mammary epithelial cell differentiation *in vitro* and mammary gland development *in vivo*, we predicted that Id-2 may act as an activator, rather than an inhibitor, of mammary differentiation and as an inhibitor of mammary proliferation. We hypothesize that the function of Id-2 as an activator or as an inhibitor is cell-type specific. These variations may be related to tissue function or to specific molecular mechanisms associated with cellular functions such as the levels of Id-2 phosphorylation.

Consistent with a role as an activator of differentiation, Id-2 expression increases not only during mammary epithelial cell differentiation but also during differentiation of myeloid precursors to granulocytes or macrophages (21). Recently, a key role for Id-2 in maintenance of a differentiated phenotype in vascular smooth muscle cells was reported (33). Moreover, overexpression of Id-2 in osteoblastic cells activates bone differentiation in the early stages of development, whereas Id-1 inhibits bone maturation.[4] We hypothesize that, to reach full differentiation, cells such as mammary epithelial cells have to switch off Id-1, resulting in the release of some bHLH transcription factors such as ITF2, which then turn on the expression of Id-2 gene.

Whereas we observed increased Id-2 expression at day 12 of pregnancy during mammary gland development *in vivo*, another group reported that Id-2 mRNA expression reached a maximal level around day 10 of pregnancy (24). One possible explanation is that all our data (*in vitro*, in cells in culture, as well as *in vivo* in mice) have been obtained from BALB/c mice, whereas Mori *et al.* (24) used a different strain (129/Sv) for the Id-2-knockout experiments. Based on the data published by Miyoshi *et al.* (34), it appears that epithelial cells are present in the Id-2-null mice but are unable to form proper alveoli, and therefore mammary epithelial cells are not able to undergo pregnancy-dependent differentiation in the absence of Id-2. This observation is therefore in agreement with our hypothesis that Id-2 plays a key role during the important stages of mammary differentiation. Moreover,

[4] Dr. Carlotta Glackin, personal communication.

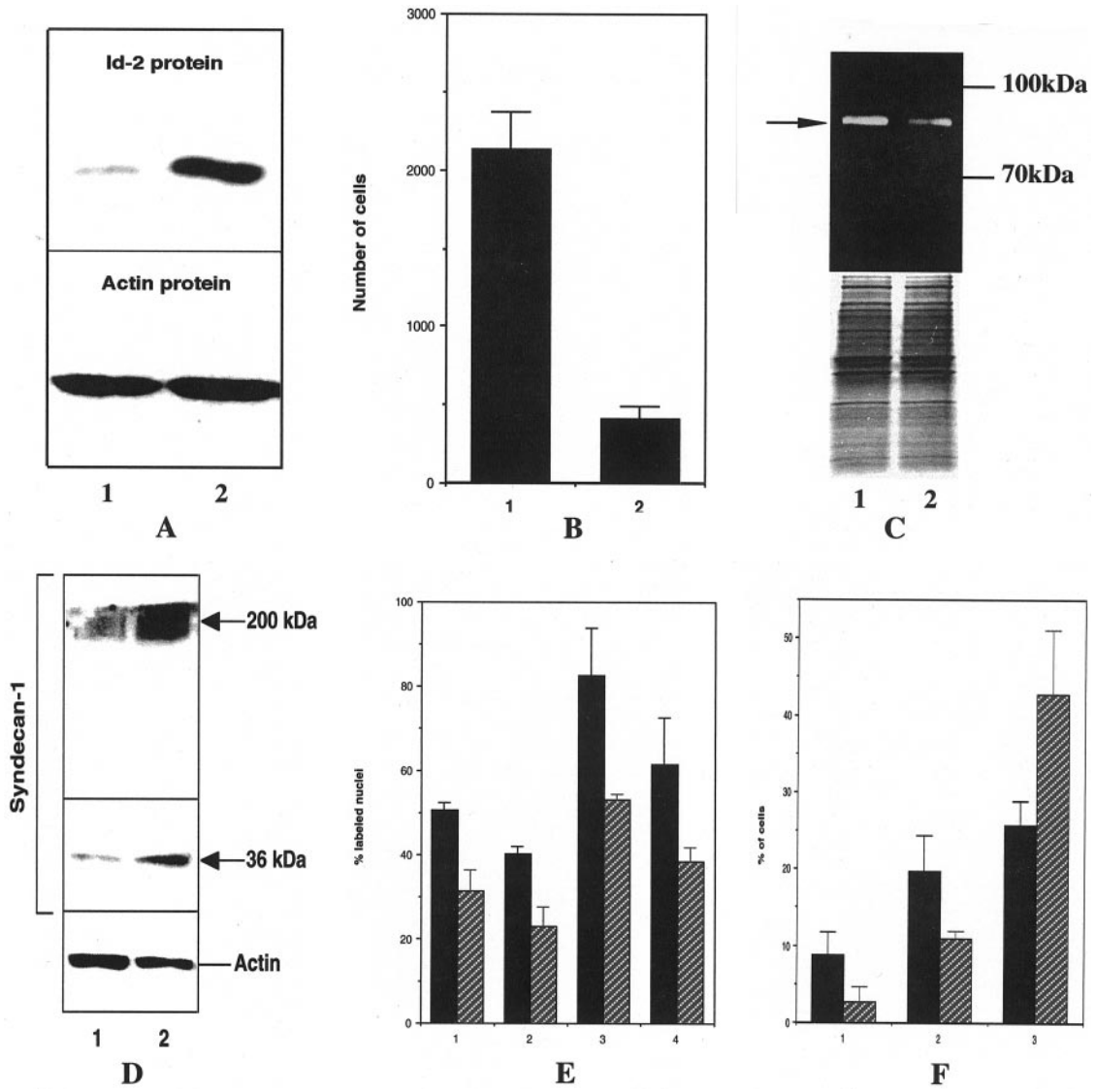

Fig. 3. Id-2 protein reduced the aggressive phenotype of human metastatic breast cancer cells. *A*, Id-2 protein expression in MDA-MB231 breast cancer cells. *Lane 1*, control cells; *Lane 2*, cells infected with Id-2 in sense orientation. *B*, Boyden chamber invasion assay comparing the invasive ability of the infected MDA-MB231 cell populations. *Column 1*, control cells; *column 2*, cells infected with Id-2 in sense orientation. The invasion assay was carried out using 50,000 cells/well. At least 4 wells were used for each cell population. Results represent one experiment of a total of three experiments with similar results. *C*, conditioned medium from MDA-MB231-control (*Lane 1*) as well as MDA-MB231-Id2-sense (*Lane 2*) cells was analyzed on gelatin substrate gels. The expression of gelatinase B (MMP-9 or 92-kDa gelatinase) is indicated by an *arrow*. Coomassie Blue staining is shown as a control of the loading. *D*, Western blot analysis of syndecan-1 expression in MDA-MB231-control (*Lane 1*) compared with MDA-MB231-Id2-sense (*Lane 2*) cells. The *top panel* shows the expression of the major species containing chondroitin sulfate as well as heparan sulfate groups and detected as a high molecular smear around 200 kDa (Ref. 43; using antibody H-174). The *middle panel* shows the expression of the minor species at 36 kDa (Ref. 43; using antibody C-20). *E*, percentage of cells in S phase in MDA-MB231-control (*black bars*) and MDA-MB231-Id-2 sense cells (*hatched bars*). Cells were cultured in medium with 2% (*columns 1* and *3*) or 0.5% serum (*columns 2* and *4*), and proliferation was determined by [^{3}H]thymidine incorporation after 7 h (*columns 1* and *2*) or 16 h (*columns 3* and *4*) of incubation. *F*, anchorage-independent growth assays comparing MDA-MB231-control (*black bars*) and MDA-MB231-Id-2 sense cells (*hatched bars*). Colonies formed were classified as follows: large colonies (>50 μm in diameter), *columns 1*; medium-size colonies (0.25–0.5 μm in diameter), *columns 2*; and small colonies (<25 μm in diameter), *columns 3*.

we further showed that Id-2 gene expression was rapidly up-regulated upon treatment with laminin. These results suggest that the role of Id-2 is to trigger a cascade of events leading to growth arrest, change in the cell shape, and overall the reorganization of the cell structure.

We proposed a similar role of the two Id proteins during breast cancer progression, *i.e.*, the most differentiated breast cancer cells express Id-2 whereas the least differentiated express Id-1. Consistent with this, upon serum withdrawal, only Id-2, and not Id-1, was expressed at a high level in the most differentiated human breast cancer cells T47D and MCF-7. Under the same conditions, Id-2 was expressed at a low level in the metastatic cell lines. Moreover, Id-2 reduced the invasive and migratory phenotypes of MDA-MB231 cells as well as their rate of proliferation and their ability to grow in an anchorage-independent manner. Since cell proliferation is an important aspect of the aggressive phenotype of breast cancer cells, we conclude that Id-2 is able to reduce aggressiveness by reducing not only invasion/migration, but also proliferation.

Interestingly, we found that the reduction in the invasive phenotype might be mediated, at least in part, by a down-regulation in metalloproteinase expression. The level of expression of the gelatinase B (MMP-9 or 92-kDa gelatinase) was reduced in MDA-MB231 cells with ectopic Id-2 expression. Some studies have recently revealed that Id binds to proteins other than bHLH proteins such as the Ets family transcription factors (35). The Ets transcription binding sites are required for the activation of MMP-9 promoter in breast cancer cells (36), and the highly invasive MDA-MB231 cells have high levels of

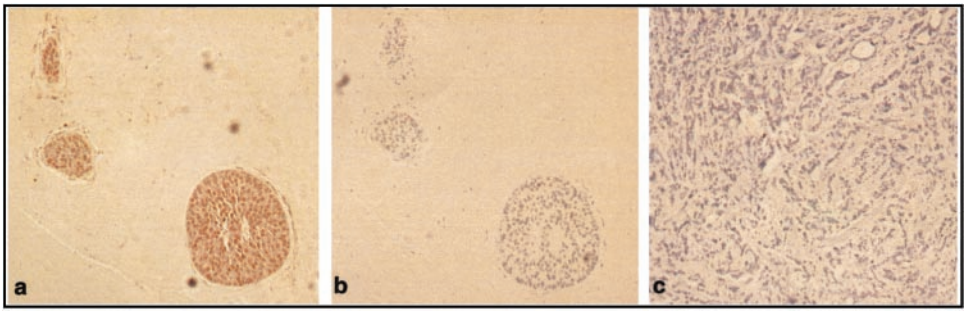

Fig. 4. Id-2 is expressed in the most differentiated human breast cancer biopsies. Representative sections from DCIS (*a* and *b*) and invasive carcinomas (*c*) were analyzed by immunohistochemistry using an antiserum directed against Id-2. The majority of DCIS were positive (as shown in *a*). The control using Id-2 blocking peptide is shown in *b*. The majority of the grade III infiltrating carcinomas showed weak or no Id-2 immunoreactivity (as shown in *c*).

Ets-1 and Ets-2 proteins (37). We therefore hypothesize that this regulation of MMP-9 by Id-2 might occur through Id-2 interaction with the Ets transcription factors.

Id-2 could also indirectly down-regulate MMP-9 expression through the increase in syndecan-1 levels. A suppression of MMP-9 expression by the heparan sulfate proteoglycan syndecan-1 has been reported in human myeloma cell lines (38). Syndecan-1, a membrane proteoglycan, is able to inhibit cell invasion, and the loss of its expression appears to be necessary for the metastatic phenotype of infiltrating ductal breast carcinoma cells (39, 40). Moreover, loss of syndecan-1 causes epithelia to transform into anchorage-independent mesenchyme-like cells, and syndecan-1 is able to maintain normal cell morphology and differentiation as well as reduced proliferation in mouse mammary epithelial cells (41, 42). Because Id-2 up-regulated syndecan-1 expression in MDA-MB231 cells, syndecan-1 could mediate some of the effects of Id-2 on the establishment of a differentiated and noninvasive phenotype in transformed breast epithelial cells.

In summary, we propose that Id-2 protein is an important transcriptional regulator involved in the maintenance of a differentiated phenotype in breast epithelial cells. During specific stages of development (such as the beginning of pregnancy), mammary epithelial cells undergo invasive growth. Cells proliferate and invade the surrounding tissue at the same time. During tumor progression, breast cancer cells also undergo the same type of invasive growth, which is uncontrolled in this case. We determined that Id-1 is highly expressed in all these conditions of invasive growth, whereas Id-2 is expressed at low levels. During the late stages of pregnancy and during lactation, it is necessary for mammary epithelial cells to maintain noninvasive, nonmigratory, and low proliferative phenotypes. Breast tumor cells in nonaggressive DCIS stages often assume phenotypes of noninvasiveness and low proliferation. In both cases (differentiated mammary epithelial cells and nonaggressive *in situ* carcinomas), Id-1 is expressed at low levels, and Id-2 is strongly expressed. Therefore, we suggest that Id-2 is an important protein involved in the maintenance of a noninvasive, nonmigratory, and low proliferative status of both normal mammary epithelial cells and nonaggressive breast cancer cells. Id-2 may also ultimately be useful as an indicator of good prognosis for breast cancer patients.

The molecular mechanisms of Id-2 action as a novel differentiating protein have yet to be elucidated. As suggested above, Id-2 may trigger its effects in breast cells through its binding to non-HLH proteins such as the Ets transcription factors. Alternatively, because the primary function of Id proteins is to dimerize to bHLH proteins, we propose that some bHLH protein complexes involved in the stimulation of cell proliferation, migration, and invasion may be disrupted by the presence of Id-2. We are currently investigating the network of bHLH proteins interacting with Id-1 and Id-2 in breast epithelial cells.

Table 1 *Breast cancer biopsies were obtained and stained for Id-2 protein expression by immunohistochemistry, as described in "Materials and Methods"*

Tumor type	Negative	Weakly positive	Strongly positive
DCIS	1/9 (11%)	4/9 (44.5%)	4/9 (44.5%)
Invasive grade I	4/14 (28.6%)	5/14 (35.7%)	5/14 (35.7%)
Invasive grade II	2/9 (22.3%)	6/9 (66.7%)	1/9 (11%)
Invasive grade III	8/16 (50%)	7/16 (43.7%)	1/16 (6.3%)

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