Helix-Loop-Helix Id-1 and Id-2

in

Mammary Gland Development and Breast Cancer

Yoko Itahana
M. D. Anderson Cancer Center
Department of Molecular and Cellular Oncology

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Introduction

1. HLH family and Id proteins

(a) HLH family

Helix-loop-helix (HLH) transcription factors (1) are important players in the transcriptional network regulating cell growth and differentiation during many essential developmental processes, in both vertebrates and invertebrates (2-5). The 240 known members of the HLH family (2, 4) coordinate cell type-specific gene expression implicated in tissue embryogenesis and cell lineage determination and overall regulate normal or abnormal cell fate within most mammalian tissues (4, 6-16). They are commonly grouped into seven classes (I-VII), based on tissue distribution, dimerization capabilities, and DNA binding specificities (Table 1) (4). The largest HLH subfamilies are class I and class II bHLH. Class I HLH members (E-proteins) are ubiquitous bHLHs, including E2A products (E12 and E47/ITF-1), E2-2/ITF-2, Daughterless, or HEB. Class II HLH members are tissue-specific bHLH, such as MyoD (MyoD1, myogenin), ATONAL (NeuroD/BETA-2, Neurogenin), Achaete-Scute complex (Mash, ac, as), or Twist. Class V HLH includes the four known mammalian Id genes, Id-1 (17, 18), Id-2 (19, 20), Id-3 (21, 22) and Id-4 (23, 24), and their homologue genes in Drosophila (Extramacrochaetae, or Emc (25)), Xenopus (XIdx (26)) and Zebrafish (ZId (27)).

A characteristic feature of HLH proteins essential to their normal function is the ability to form homo- or hetero-dimers through their highly conserved HLH domain (4, 7, 28). Most of these proteins belong to the basic helix-loop-helix (bHLH) family and act as transcriptional enhancers or inhibitors of various genes through direct DNA binding to the canonical E-box sequence (CANNTG) (29). The composite basic domains of bHLH proteins are necessary for DNA binding. E12, E47, myc, MyoD and Daughterless were the first transcription factors identified as recognizing the E-box sites (1, 30). Their discovery led to the identification of common motif in their protein sequence, described as a helix-loop-helix, giving rise to the helix-loop-helix (HLH) protein family.

(b) Id proteins

The four members of the HLH class V subfamily (17, 25), known as Id proteins (inhibitor of differentiation / DNA binding) act as dominant negative regulators of transcriptional factors. While they contain the HLH domain enabling them to dimerize with other members of the family (class I and II) (4), the resulting Id-bHLH heterodimers are unable to bind to DNA because Id HLH lack the basic motif necessary for this (17) (Fig. 1).

Id genes were first identified in murine myoblasts, where they prevented myogenic basic helix-loop-helix (bHLH) transcription factors from binding muscle-specific regulatory elements (17). These transcription factors are key regulators of tissue-specific gene

expression in a number of mammalian and non-mammalian organisms, and constitutive expression of Id proteins has been shown to inhibit the differentiation of various tissues (12). The different family members localize to different chromosomes and show marked differences in their pattern of expression and function (23, 31). Although the family members are similar in the HLH sequence, the regions outside the HLH domain are distinct for each member (Fig. 2), and may determine the tissue-specificity of Id function, as well as the binding specificity for particular bHLH proteins.

(c) Id /bHLH network

The expression, as well as the functionality of Id proteins and their bHLH targets, vary with the cell type and stage of development, differentiation or growth. Eventually, the balance of bHLH/Id activity is critical to specific gene expression. The potential for Id proteins to interfere with bHLHs confers them with central coordinating roles. Ultimately, Id proteins orchestrate in a synergistic manner combinations of protein networks and gene expression; this critical balance is disrupted during tumorigenesis (Fig. 3). Indeed, strong evidence now suggests that Ids behave as pro-oncogenic factors (7, 8, 12, 14, 15, 32, 33). Id proteins are generally overexpressed in tumor cells, correlating with de-differentiated, proliferative and invasive cell phenotypes. Variations in expression levels of Ids have to be integrated into the whole cellular equilibrium, so that the dysregulation of Ids activity can

result in the downstream dysregulation of the expression of diverse genes.

2. Id proteins in breast cells

HLH proteins have been identified as key regulators of mammary epithelial cell differentiation, growth and tumorigenesis *in vitro* as well as *in vivo*. The mammary gland provides a model for studying specific upstream regulation as well as downstream effects of Id proteins and their bHLH partners, through development as well as during tumorigenesis. Indeed, breast tissue is distinct from most other vertebrate tissues in that it continually changes in structure throughout the adult lifetime of reproductively active females (34).

(a) Mammary gland development

The mammary gland undergoes striking changes in morphology and function during development, puberty and adult life. During each menstrual cycle, and particularly during pregnancy, mammary epithelial cells undergo cycles of proliferation, invasion, differentiation, dedifferintiaton, and apoptosis cell death (Fig. 4). 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 (35). During the second half of pregnancy, the levels of casein mRNA increase as mammary epithelial cells cease proliferation. In the lactating gland, the secretory lobules developed from the branching ducts become filled with milk (36). Throughout lactation, epithelial cells remain quiescent but continue to express milk proteins (35). After the weaning, the entire mammary gland undergoes involution, a phase of extensive remodeling characterized by degradation of ECM and epithelial cell death by apoptosis (37, 38).

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 (39). The identification of tissue-specific transcriptional regulators in mammary epithelial cells is crucial, not only for deciphering the regulatory mechanisms of normal growth and differentiation but also for understanding how breast cancer develops.

(b) Id-1

A line of murine mammary epithelial cells, SCp2 cells, was previously developed by Dr. Desprez from mid-pregnant mouse mammary gland (40, 41). A role for HLH Id proteins in

the differentiation of SCp2 cells was suggested by the previous finding that Id-1 expression declined to undetectable levels when the cells were induced to differentiate in culture on treatment with extracellular matrix and lactogenic hormones (42). Conversely, when SCp2 cells were transfected with constitutively expressed Id-1 genes, the transfected cells failed to differentiate, even in the presence of extracellular matrix and lactogenic hormones, and then proliferated and became invasive (43). Additionally, the expression of Id-1 *in vitro* was reported to be linked to apoptosis (44). Here, I show that Id-1 expression in the mammary gland followed a pattern expected from the cell cultured studies.

Moreover, high Id-1 levels correlated with invasiveness in breast cancer cells in culture, and when a noninvasive breast cancer cell line was transfected with Id-1, it became invasive (45, 46). Using immunohistochemistry, I examined Id-1 expression in human breast cancer biopsies (46, 47). Almost all of the ductal carcinomas *in situ* examined were negative or weakly positive for Id-1 staining, whereas the majority of infiltrating grade III carcinomas of ductal origin were strongly positive. These findings suggest that Id-1 might serve as a reliable marker for breast cancer progression, invasion, and metastasis. Furthermore, I demonstrated that using antisense technology to target endogenous Id-1 gene expression can reduce breast cancer cell metastasis to the lungs (47). These results suggest that Id-1 could be a potential molecular target for breast cancer therapy.

(c) Id-2

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. 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 (12, 48). 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 (49). Id-2 gene expression was also maintained during embryonic stem cell-derived hematopoietic differentiation (50). 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 (51). These results suggest that in some tissues, Id-2, unlike Id-1, may promote differentiation.

Id-2, as well as Id-1, also plays an important role during mammary gland development. In addition to the above-mentioned phenotypes, Id-2-deficient female mice exhibited a lactation defect (52), which could be indicative of a positive role of Id-2 during the differentiation process. Consistent with this finding, I report some results using murine mammary epithelial cells in culture and *in vivo* (44) which suggest that Id-2 may promote cell differentiation in the 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*, I propose that Id-2 is acting as an activator, rather than an inhibitor, of mammary gland differentiation (44).

Moreover, I show that the extracellular matrix component laminin, and not the lactogenic hormones, is responsible for the increase in Id-2 expression during differentiation. I also report that Id-2 expression was inversely correlated with the rate of proliferation in murine mammary epithelial cells. Furthermore, I show that Id-2 was expressed at a high level in the more differentiated human breast cancer cells. On the other hand, its level of expression was very low in the more aggressive and metastatic breast cancer cells. However, when Id-2 was constitutively expressed in these aggressive breast cancer cells, their proliferative and invasive abilities were significantly reduced. Likewise, Id-2 protein expression was low or undetectable in human biopsies from grade III invasive carcinomas. However, Id-2 was detectable in most of the *in situ* carcinomas as well as in the least aggressive invasive carcinomas investigated (such as the grade I invasive carcinomas) (53). These results suggest that Id-2 might serve as a good prognostic marker in patients with breast cancer.

Materials and Methods

Mammary Gland Extracts

Mammary tissues were obtained from BALB/c female mice, purchased either from Simonsen Laboratories, Inc. (Gilroy, CA) or Harlan (San Diego, CA). To obtain mammary tissue during pregnancy, lactation and involution, 12 wk old virgin females were mated. Mammary gland involution was induced by removing the pups 20 d after birth. In all cases, the two fourth inguinal mammary glands were harvested. Virgin animals were sacrified at 5, 7 and 12 wks of age; pregnant animals at 2, 5, 12 and 18 d after onset of pregnancy; lactating animals at 2, 7, 12, 20 d after onset of lactation; and involuting animals 2, 3 and 5 d after weaning. Mammary glands were immediately frozen at -70^{o}C. RNA and proteins were extracted using TriPure Isolation Reagents (Roche Applied Science).

Cell Culture

The derivation of SCp2 cells (murine mammary gland epithelial cells) has been described (40). SCp2-Id-1 cells were generated by transfecting SCp2 cells with the MMTV-Id-1 expression vector, as described (42). Cells were grown in Dulbecco's modified Eagle's and Ham's F12 media (DME-F12 1:1) supplemented with 5% FBS, insulin (5µg/ml, Sigma) and gentamicin (50µg/ml, Invitrogen) at 37^{o}C in a humidified CO_2 5% atmosphere.

Human breast cancer cell lines T47D, MCF-7, MDA-MB231 and MDA-MB-436 were purchased from the American Type Culture Collection. Murine breast cancer cell line 4T1 was obtained from Dr. S. Ostrand-Rosenberg (University of Mary land, Baltimore). All breast cancer cell lines were grown in RPMI 1640 medium containing 10% FBS and insulin (5μg/ml). For experiments using serum-free medium, FBS was omitted from medium. To induce cell differentiation, cells were cultured in serum-free medium for 2 days, and then added either 1.0 % extracelullar matrix (Matrigel, Collaborative research) or 50μg/ml laminin (Sigma) in medium lacking serum but containing lactogenic hormones (insulin, 5μg/ml; hydrocortisone, $1.4x10^{-6}$M; prolactin, 5μg/ml).

Retrovirus Production and Infection

The full-length mouse Id-2 cDNA was cloned in a sense as well as an antisense orientation in a pLXSN retroviral expression vector, and constructs were used to infect SCp2 cells. The full length human ITF-2 cDNA (obtained from Dr. T. Kadesch) was cloned in the same pLXSN vector. pLXSN-control (LXSN-ctl) and pLXSN-ITF-2 vectors were used to infect SCp2-Id1 cells. The full-length human Id-2 cDNA, a kind gift from Dr. Eiji Hara (Manchester, United Kingdom), was cloned into a pLXSN vector in a sense orientation. The full-length human Id-1 was also cloned in a pLXSN vector in an antisense orientation. Human Id-1 and Id-2-pLXSN constructs were used to infect human breast cancer cell lines.

These viral vectors were then packaged in TSA-54 cells (Cell Genesis, Foster City, CA) using calcium phosphate. Twenty-four hr after transfection, culture medium (containing infectious virus) was harvested twice at 4 hour intervals and frozen at -80 ^{o}C. Retrovirus were mixed with 5ml of medium containing 4mg/ml Polybrene and added to the 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 (54). PolyA+ mRNA was prepared according to the protocol published by New England BioLabs Inc. Samples (15µg for total RNA and 4µg for polyA+ mRNA) were size fractionated by electrophoresis through denaturing formaldehyde-agarose gels and transferred to Nylon membrane (Hybond-N, Amersham Corporation). The blots were hybridized with ^{32}P-labeled probes prepared by random oligonucleotide priming (Amersham Corporation), washed and exposed to Kodak XAR-5 film for autoradiography. The ß-casein probe was the 540-bp mouse cDNA (from J. Rosen, Baylor College of Medicine, Houston, TX). The Id-1 and Id-2 probes were respectively cut out from pLXSN-Id-1 and pLXSN-Id-2 vectors. The mouse ITF-2 cDNA probe was obtained by screening the SCp2 library. β-actin probe was obtained from Clontech. 28 and 18S ribosomal RNA are shown as controls for RNA integrity and quantitation.

Western Analysis

Proteins were extracted from cells using cell lysis buffer [50mM Tris-HCl (pH 7.4), 1% (wt/v) sodium deoxycholate, 1% (v/v) NP-40, 0.1% (wt/v) SDS, 150mM NaCl], and protease inhibitor cocktail (Calbiochem, CA). Insoluble material was removed by centrifugation at 12,000Xg for 15 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to Immobilion-P membranes (Millipore). For immunodetection of proteins, membranes were blocked in 5% nonfat dried milk in TBST buffer [20mM Tris-HCl (pH 7.5), 100mM NaCl, 0.1% (v/v) Tween-20], and then incubated with primary antibodies diluted in the blocking buffer. Blots were probed with anti-ß-casein (from Dr. Mina J. Bissell, Lawrence Berkeley Laboratory, Berkeley, CA), anti-Id-1 (Santa Cruz Biotechnology), anti-Id-2 (Santa Cruz Biotechnology), anti-syndecan-1 (H-174 and C-20 from Santa Cruz Biotechnology), anti-membrane-type matrix metalloproteinase 1 (MT1-MMP) (Chemicon), or anti-actin (Chemicon) antibodies. Membranes were washed 3 times in TBST buffer after incubation for 1hr in HRP-labeled secondary antibodies (Santa Cruz Biotechnology). Detection for the bound antibodies was done with either ECL-Plus or ECL chemiluminescence substrate according to manufacturer's instructions (Amersham Corporation).

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). Cells (50,000 per well for the MDA-MB231 cells and 100,000 per well for the MDA-MB436 cells) were added to the upper chamber in 200 µl of serum-free medium (containing 0, 0.3, 3 or 30 µM mitomycin C (Sigma)). The lower chamber was filled with 300 µl of conditioned medium from fibroblasts (55). After 20 hr 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 by using light microscopy. Cells were assayed in triplicate or quadruplicate, and the results averaged.

Anchorage-dependent Growth Assays

Liquefied 2 % agarose was mixed with an equal volume of 2X DMEM/F12 growth medium lacking serum and supplemented with insulin (10µg/ml) and gentamicin (100µg/ml) (2X 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 2X medium, and 10 ml of this solution was mixed with 1 ml of growth medium containing $1x10^5$

MDA-MB231 cells to yield $1x10^4$ cells/ml in 0.27 % agarose; one ml of this cell suspension was layered on top of the 1 % agarose base and 1 ml of DMEM/F12 containing 10 % FBS was added. The cells were incubated for 2 to 3 weeks. Counts were performed according to the size of the colonies.

Zymography

Proliferating MDA-MB231 cells ($1x10^6$ in 100 mm dishes) were shifted to serum-free medium for 2 days, at which time they were given 10 ml fresh serum-free medium. Forty eight hr later, the conditioned medium was collected and concentrated 10- to 15- fold using 10 kDa cutoff filters (Millipore). The concentrated medium was analyzed on gelatin substrate gels, as described by Fisher and Werb (56). Briefly, gels consisted of 8-10% polyacrylamide and 1 mg/ml gelatin (Sigma). Concentrated conditioned medium was mixed with non-reducing Laemmli sample buffer [0.25 M Tris-HCl (pH 6.8), 10% SDS, 4% sucrose, 1mg/ml BPB] and incubated at 37^oC for 15 min. After electrophoresis, the gels were incubated for 1 hr in 2.5% Triton-X100 at room temperature, followed by overnight incubation in substrate buffer [100 mM Tris-HCl (pH 7.4), 15 mM $CaCl_2$] at 37^oC. The gels were stained with Coomassie Blue for 30 min and destained with 30% methanol/10% acetic acid. Gelatinase activity was visible as clear bands, indicative of proteolysis of the substrate protein.

CellStat System to Monitor Cell Proliferation

In order to monitor the cellular proliferation of mouse mammary epithelial SCp2 cells, we used the apparatus kindly provided by CellStat Technologies Inc (Belmont, CA) (57). This non-invasive, non-destructive 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, the intensity of the signal being 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 as well as Id-2 antisense mouse mammary SCp2 cells) was plated in six wells. Cell growth was measured every 60 min and the readings from the six wells per 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 7h or 16 h of the experiments whereupon they were fixed with methanol/acetone (1:1) for 5 min at -20^{o}C and stained with 4'6-diamidino-2-phenylindole (DAPI). The coverslips were air dried, coated with Kodak NTB2 emulsion (1:2 dilution), and exposed for 16 to 24 hr. The coverslips were developed

with Kodak D-19, fixed with Kodak Rapid-Fix, and view by phase-contrast microscopy. The percentage of labeled nuclei was calculated by comparing the number of ^{3}H-thymidine-labeled nuclei with the number of DAPI-stained nuclei in a given field, using phase contrast and fluorescence microscopy.

Analysis of Lung Metastasis in Nude Mice

To produce experimental lung metastasis, groups of 10 five- to six-week-old female athymic BALB/c nude mice (Simenson Laboratories, Gilroy, CA) were injected with either MDA-MB231 infected with pLXSN control vector, MDA-MB231 infected with Id-1 in an antisense orientation, or parental MDA-MB231 cells. The cells were trypsinized and resuspended in culture media at a density of $2x10^{6}$ per 200 µl and injected into the lateral tail vein of each mouse. All mice were sacrificed 7 weeks after injection. The lungs were dissected out, infused with 15% India ink intratracheally, and fixed in Fekete's solution. Visible lung metastases were counted using a dissecting microscope.

Plasmid for the Non-viral Systemic Gene Delivery Experiments

Vector control plasmid p4694 (59), Luciferase control plasmid p4726 (59), the metastasis suppressor gene CC3 containing plasmid (60), as well as mouse antisense Id-1 targeting plasmid were kind gifts from Dr. Robert J. Debs (California Pacific Medical Center,

San Francisco). The mouse antisense Id-1 targeting plasmid was constructed by ligating full-length mouse Id-1 cDNA (~1.2 kb) in an antisense orientation into p4694.

In vivo Gene Delivery and Analysis of Anti-metastatic Activities

Murine 4T1 breast cancer cells were freshly thawed and grown in 5% FBS in MEM media for 48 hr. On day zero, groups of 10 mice were injected with 50,000 4T1 cells in 200 µl of culture media into the tail vein of each syngeneic 8 week-old female BALB/c mice (Simenson Laboratories, Gilroy, CA). On day 3 after tumor cell injection, each mouse was injected with 25 µg of plasmid DNA complexed with the cationic liposome *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N* -trimethyl-ammonium chloride at 1 µg DNA per 24 nmol of liposome prepared as previously described (60). Mice were sacrificed 21-25 days after tumor injection. The lungs were dissected out, weighed, and infused transtracheally as previously described (61). The potential statistical significance of differences of total lung tumor numbers between various groups was analyzed using the unpaired two-tailed Student's *t*-test.

Immunohistochemistry

Formalin-fixed paraffin-embedded murine mammary gland tissue sections, formalin-fixed paraffin-embedded murine lung tumor sections from CLDC-injected mice, as

well as paraffin-embedded human tumor tissue sections (obtained from California Pacific Medical Center) were used to determine Id-1, Id-2 and MT1-MMP protein expression. Slides were dewaxed, rehydrated and placed in a container containing 1 liter of 0.01 M citrate buffer (pH6.0), 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 TBS (20mM Tris-HCl (pH 7.5), 100mM NaCl), and then incubated in 10% non fat dry milk for 30 min at room temperature. Subsequently, they were incubated with 1 µg/ml of anti Id-1 antibody or anti Id-2 antibody (Santa Cruz Biotechnology) overnight at 4 ^{o}C. The slides were washed in TBS, incubated with biotinylated swine anti-rabbit F(ab)'2 (1:400) (DAKO) for 30 min, and then incubated with 1:500 streptavidin-horse radish peroxidase (DAKO) for 30 min. After washing in TBS, peroxidase was visualized by incubating in 0.5 mg/ml diaminobenzidine-4HCl (DAB) and 0.03 % hydrogen peroxide in TBS. Finally, the slides were slightly counterstained with hematoxyline. Control slides were incubated with rabbit Igs, with Id-1 or with Id-2 blocking peptide. To determine Id-1 and Id-2 expression in breast cancer biopsies, I semiquantitatively assessed the percentage of positive tumor cells (score 2-4 points) and the staining intensity (score 1-3 points). Points for percentage of positive cells and the staining intensity (levels of expression) were added, and specimens were attributed to the three groups according to their overall scores (negative if <2 points; weak if 2-4 points; strong positive if 5-7 points). For the experiments using sections from mouse

mammary glands, we also used antibodies against keratin 8/18 (Sigma C-2931), keratin 14 (Convance/Babco PRB-155P) as well as α-smooth muscle actin (Sigma A-2547).

Immunofluorescence Analyses

$2x10^5$ cells (MDA-MB231 or MDA-MB-436) infected either with pLXSN control or with pLXSN-Id-1 antisense vector were plated on coverslips. The coverslips were fixed in 1 % paraformaldehyde and incubated with 1 μg/ml of anti Id-1 antibody (same batch of antibody used for the immunohistochemistry experiments), followed by incubation with FITC-conjugated secondary antibody (Santa Cruz Biotechnology). The coverslips were counterstained with 4'6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

***In situ* Hybridization**

(Preparation of Riboprobes) pBluescriptsKS-mouse Id-1 was kindly gifted from Dr. M.W. Kelley (National Institutes of Health, Bethesda). Mouse Id-1-antisense and sense riboporobes labeled with digoxigenin were prepared by using a DIG RNA labeling kit (T7/T3) (Roche Applied Science) according to the manufacture's instructions. Dig-labeled probes were quantified by the dot blot method described in the kit's protocols and the equal amount of antisense and sense probes were used for the hybridization. Sense probe was used as a negative control.

(Hybridization and Detection) Murine mammary gland tissue sections were deparaffinized, rehydrated and fixed with 4 % paraformaldehyde for 5 min at room temperature. The slides were then washed and treated with proteinase K (20 μg/ml) at 37^{o}C for 15 min, immersed in 0.2N HCl for 10 min at room temperature, and acetylated for 5 min with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (TEA) at room temperature. The slides were washed, dehydrated, air-dried and hybridized with DIG-labeled probes (100ng/ml) in hybridization buffer [50% formamide, 10mM Tris-HCl (pH 7.4), 600mM NaCl, 1mM EDTA, 0.25% SDS, 10% Dextransulfate, 1x Denhardt's, 200μg/ml of tRNA from E. coli] overnight at 52^{o}C in a humid chamber. After hybridization, the slides were briefly washed in 5x SSC at 55^{o}C, and sequentially washed with 50 % (v/v) formamide/2x SSC at 55^{o}C for 30 min and TNE [10mM Tris-HCl (pH 7.4), 500mM NaCl, 1mM EDTA] at 37^{o}C for 15 min twice. Then, the slides were treated with RNase A (20 μg/ml) in TNE at 37^{o}C for 30 min, followed by washing with TNE (37^{o}C for 10 min), 2x SSC (42^{o}C for 20 min) and 0.2x SSC (42^{o}C for 20 min). After washing with buffer 1 [100mM Tris-HCl (pH 7.5), 150 mM NaCl] for 5 min at room temperature, the slides were incubated with 1.5 % (w/v) blocking reagent (Roche Applied Science) for 1 hour at room temperature, followed by overnight incubation with anti-Digoxigenin-AP antibody (Roche Applied Science) at 4^{o}C. The slides were then washed with buffer 1 and buffer 3 [100mM Tris-HCl (pH 9.5), 100 mM NaCl, 50mM $MgCl_2$]. The color was developed in

buffer 3 with nitroblue tetrazolium (NTB) and 5-bromo- 4-chloro-3-indolyl phosphate (BCIP) (Roche Applied Science) for 4-5 hr at room temperature. The slides were counterstained with Nuclear Fast Red (Vector) to visualize the nuclei.

The abbreviations used are : bHLH, basic helix-loop-helix; MT1-MMP, membrane-type matrix metalloproteinase1; MMP-9, matrix metalloproteinase 9; ECM, extracellular matrix; Lm, laminin; LH, lactogenic hormones; PRL, prolactin; Hyd, hydrocortisone; FBS, fetal bovine serum; CLDC, cationic liposome-DNA complex; DCIS, ductal carcinomas *in situ*; ctl, control.

Results

Id-1 expression during murine mammary gland development

Id-1 expression was examined during normal murine mammary gland development *in vivo*, using Northern analysis (Fig. 5A). The expression of β-casein, a marker of mammary epithelial cell differentiation, is inversely correlated with Id-1 expression, suggesting a role for Id-1 *in vivo* similar to that observed in murine mammary epithelial cells SCp2 in culture (42, 43). During the early stages of pregnancy, when epithelial cells of ductal trees proliferate and invade the stroma, Id-1 is highly expressed. Since RNA was isolated from a whole tissue, it was important to confirm that Id-1 was expressed by epithelial cells. Therefore, immunohistochemistry was performed using an Id-1-specific antibody (Fig. 5B). Id-1 was only expressed in keratin 8/18-positive mammary epithelial cells (and not in myoepithelial or stroma cells). Id-1 expression was also confirmed at the mRNA levels using the technique of *in situ* hybridization (Fig. 5C). A strong signal was detected only in the epithelial cells at the end buds of growing ductal structures, confirming that proliferating and invading mammary epithelial cells express high levels of Id-1. During mid-pregnancy, Id-1 is down-regulated and remains undetectable through much of the lactating period, corresponding to a highly differentiated stage. Surprisingly, Id-1 was re-expressed during *in vivo* mammary gland involution, a time when mammary epithelial cells undergo extensive

apoptosis (37). This re-expression raised the possibility that Id-1 may also regulate apoptosis in mammary epithelial cells *in vivo*.

Id-2 expression during murine mammary gland development

Id-2 expression was examined during normal murine mammary gland development *in vivo*, using Northern analysis (Fig. 6A) and Western analysis (Fig. 6B) (44). Id-2 expression was correlated with β-casein and was inversely correlated with Id-1 expression, i.e. Id-2 was expressed when cells differentiated during the second part of pregnancy and during lactation. To confirm that Id-2 was specifically expressed by epithelial cells *in vivo*, immunohistochemisty was performed using an Id-2-specific antibody as well as Id-1. A strong signal corresponding to Id-2 protein was detected at day 12 of lactation in the nuclei of differentiated epithelial cells (Fig. 6C, *panel* c). A signal was already detected at day 18 of pregnancy (*panel* b) and no signal was detected from proliferating and invasive cells from 7 weeks-old virgin mice (*panel* a). The increase in both Id-2 mRNA and protein expression during mid-pregnancy corresponded to a time when differentiation occurs and milk proteins such as β-casein begin to be expressed.

Id-2 expression in murine mammary epithelial SCp2 cells *in vitro*

Id-2 expression was examined in proliferating and differentiating SCp2 cells treated

with extracellular matrix (ECM) and lactogenic hormones. Western analysis showed that differentiated SCp2 cells expressed high levels of Id-2, whereas Id-2 was not detected in proliferating cells (Fig. 7A). In comparison, Id-1 was detectable only in proliferating cells as reported previously (Fig. 7A) (42). I confirmed this inverse expression pattern of Id-1 and Id-2 by Northern analysis of mRNA isolated from cells proliferating or treated with laminin (the major component of ECM required for mammary epithelial differentiation) and lactogenic hormones for 24 and 48 hr (Fig. 7B). Thus, in contrast to Id-1, Id-2 appeared to be associated with differentiation in mammary epithelial cells *in vitro*, as well as *in vivo*.

Regulation of Id-2 expression by laminin

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 extracellular matrix (ECM) (62). SCp2 cells stop proliferation and differentiate when treated by hormones and the ECM component laminin, and Id-2 is up-regulated during this process (Fig. 7A and 7B).

To find out the crucial component responsible for the increase of Id-2 expression in differentiation process, 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 prolactin or

hydrocortisone. RNA was isolated and analyzed for Id-2 and β-casein expression (Fig. 8A). Upon addition of laminin, Id-2 mRNA was clearly up-regulated independently of absence or presence of lactogenic hormones (*lanes* 2, 4 and 6). This result suggests 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.

However, there were some basal levels of Id-2 mRNA in cells cultured in plain medium without any lactogenic hormones, with hydrocortisone only, or with both hydrocortisone and prolactin (*lane* 1, 3 and 5 respectively). I show in Figure 7B, 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, I proposed that some bHLH proteins, previously sequestered by Id-1, became available to up-regulated Id-2 expression. In order to confirm this hypothesis, I compared Id-2 expression (Fig. 8B) in SCp2 cells which constitutively express Id-1 (SCp2-Id-1 cells) and which were subsequently infected with either control pLXSN (*lane* 1) or pLSXN-ITF2 (*lane* 2) vector. ITF2 is an Id-1 interacting bHLH transcription factor, and it was previously determined that its overexpression could reverse the phenotypes induced by constitutive Id-1 expression, i.e. reduction of proliferation as well as re-establishment of milk secretion in SCp2-Id-1-LXSN-ITF2 cells (44). Here I show that the effects of ITF2 overexpression on the re-establishment of a more normal phenotype are correlated with the up-regulation of Id-2 expression in SCp2-Id-1-LXSN-ITF2

cells (Fig. 8, *lane* 2).

Determination of Id-2 up-regulation as a necessary step for differentiation

To determine whether Id-2 was essential for mammary epithelial cell differentiation and milk production, SCp2 cells were infected with pLXSN-control, pLXSN-Id-2-sense, or pLXSN-Id-2-antisense retroviruses (Fig. 9). These cells were induced to differentiate using laminin and lactogenic hormones. β-casein expression was significantly higher in SCp2-LXSN-Id-2-sense cells compared with control cells. Moreover, β-casein expression was almost undetectable in SCp2-LXSN-Id-2-antisense cells, which expressed much lower levels of Id-2 protein compared with control cells (Fig. 9). These results suggest that ectopic expression of Id-2 accelerates differentiation, whereas depletion of Id-2 protein inhibits differentiation.

Effect of Id-2 expression on cell proliferation

Using the same three populations of infected cells mentioned above (control, Id-2-sense and Id-2-antisense cells), I show next that Id-2 is not only necessary for differentiation, but also that Id-2 acts as a strong regulator of the cell cycle. Cell proliferation was measured using Cell Stat system. Cells infected with Id-2 sense constructs grew more slowly than control cells (Fig. 10). 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 epithelial cells. Since Id-2, when overexpressed in mammary epithelial cells, triggers a high level of differentiation and milk secretion (Fig. 9), it is unlikely that this reduction of proliferation could be explained by an induction apoptosis. Moreover, Id-2 overexpressing cells maintained a high level of viability using the technique of trypane blue exclusion (data not shown).

Id-1 and Id-2 expression in human breast cancer cell lines

Since the loss of proper regulation of proliferation and differentiation is an important aspect of caner progression, Id-1 and Id-2 expression were examined in human breast cancer cell lines with varying degrees of invasiveness in culture; T47D and MCF-7 cell lines which display non-aggressive and differentiated characteristics in culture, and the two aggressive and metastatic MDA-MB231 and MDA-MB436 cell lines (Fig. 11). Id-1 expression was undetectable in T47D cultured in serum-free medium (Fig. 11A). Under the same condition, however, Id-1 was highly expressed in MDA-MB231 and MDA-MB436 as previously shown (Fig. 11A) (43). In contrast, Id-2 mRNA was detectable in T47D cultured in serum-free conditions and undetectable in two aggressive cell lines MDA-MB231 and MDA-MB436 (Fig. 11A). These results therefore confirmed the inverse correlation between the expression of Id-1 and Id-2 in human breast cancer cells that was also detected both in normal murine

mammary epithelial cells in culture and *in vivo* (Fig. 5A, 6A and 7). This inverse relationship is particularly clear in Figure 11B, where the levels of Id-2 mRNA increased in MCF-7 cells upon serum-withdrawal whereas the levels of Id-1 mRNA decreased. Therefore, I hypothesized that Id-2 can only be expressed when Id-1 is down-regulated. As a potential explanation, I propose that some bHLH protein such as ITF2, previously sequestered by Id-1, up-regulates Id-2 expression at the promoter level through their binding to E-boxes.

Targeting Id-1 reduces human breast cancer cell invasion *in vitro* and *in vivo*

Id-1 is highly expressed in aggressive and metastatic MDA-MB231 and MDA-MB436 cell lines. I therefore investigated whether targeting Id-1 gene expression in breast cancer tumor cells would reduce the invasive phenotype of metastatic cells *in vitro*. The full length human Id-1 cDNA was expressed in an antisense orientation in MDA-MB231 and MDA-MB436 cells. Using immunofluorescence as well as Western blotting, I determined that levels of Id-1 protein were substantially reduced in MDA-MB436 (Fig. 12A and B) and in MDA-MB231 (Fig. 12A and 13A) cells infected with pLXSN-Id-1 antisense construct compared to pLXSN-control infected cells. I then assessed the ability of the different populations of stably infected cells expressing various levels of Id-1 to migrate and invade a reconstituted basement membrane in Boyden Chamber (58). The invasive activity of each

cell population directly correlated with the level of its Id-1 protein expression (Fig. 12C and 13B). Specifically, the populations of MDA-MB436 and MDA-MB231 that expressed high level of Id-1 (pLXSN-conrtol) were significantly more invasive than the Id-1 antisense infected cells that expressed low level of Id-1 (pLXSN-Id1 antisense cells). In order to study the effect of Id-1 targeting on invasion/migration only, and not on proliferation, the invasion assays were performed in the presence of three different biologically active concentration of mitomycin C (0.3, 3 and 30μM) (Fig. 12C and 13B). The same pattern of reduction in invasiveness was documented, suggesting that the effect of Id-1 targeting on invasion/migration is independent of its effect on cell proliferation.

I also performed experiments to determine the effects of Id-1 targeting on the proliferation/survival rate of these cell populations. It was determined that the effects of the Id-1 antisense construct on MDA-MB231 cell proliferation were modest but significant ($p<0.003$) (approximately 10-15% decrease of the labeling index of pLXSN-Id1 antisense cells compared to pLXSN-control) when cells cultured in serum-free medium. There was no statistical difference in the apoptotic rate among these populations with varying Id-1 levels (data not shown). On the based of these results obtained by using cultured cells, I then tested whether stable reduction of Id-1 expression in MDA-MB231 tumor cells could reduce their metastatic spread. I have chosen MDA-MB231 cell line as a model for *in vivo* experiment for the reasons that (*i*) this aggressive cell line is among the few that metastasize

in nude mice (63), and (*ii*) the effect of Id-1 targeting on cancer cells alone can be tested only on cell lines that express constitutively high levels of Id-1 protein independent of the nature of the microenvironment surrounding the tumor cells (i.e. serum, tissue, or extracellular matrix). Nude mice injected with MDA-MB231 cells expressing antisense Id-1 exhibited significantly fewer lung tumors ($p < 0.0005$) than mice injected with the MDA-MB231 control or the parental MDA-MB231 (Fig. 13C). These results further support the hypothesis that the invasive/growth and metastatic phenotypes in human breast cancer cells are directly related to the levels of expression of Id-1 gene.

Targeting Id-1 decreases the levels of expression of the matrix metalloproteinase MT1-MMP

Using the techniques of differential display and subtractive hybridization, a number of genes regulated by Id-1 were isolated in murine mammary epithelial cells. Amongst these were genes controlling cell cycle progression as well as cell-cell interaction (64). One gene encoding for the matrix metalloproteinase MT1-MMP, which was strongly up-regulated in cells transfected with Id-1, was repeatedly pulled out (unpublished data). Several groups have reported the role of this ECM-remodeling enzyme in increasing tumor invasion (65). Therefore, it was investigated whether MT1-MMP was down-regulated in MDA-MB231 cells infected with an antisense Id-1 construct. I used six different sub-populations isolated from

the pool MDA-MB231 antisense Id-1 cells that expressed different levels of Id-1 protein. I found that decreased levels of expression of the matrix metalloproteinase MT1-MMP was highly correlated with decreased Id-1 protein expression and decreased invasion (Fig. 14).

Constitutive expression of Id-2 reduces the invasive phenotype of human metastatic breast cancer cells

I hypothesize that aggressive breast cancer cells have acquired the ability to constitutively express Id-1 gene (46) and therefore have lost the ability to 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. I therefore chose to focus on the MDA-MB231 cells to determine the effects of Id-2 overexpression on the reduction of aggressiveness. I 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 than control cells (Fig. 15A). It was then quantified the levels of invasiveness and migration of MDA-MB231-control cells compared to MDA-MB231-Id2 sense cells (Fig. 15B). Cells expressing high levels of Id-2 were four-fold less invasive than the control cells.

I used Zymography to further characterize further the mechanisms by which Id-2 overexpression reduced the invasive ability of metastatic breast cancer cells. I show that the levels of expression of the matrix metalloproteinase MMP-9 (92kDa gelatinase) were reduced in cells expressing Id-2 proteins (Fig. 15C), representing a potential explanation for the reduction in the invasive phenotype of MDA-MB231 cells. I also compared the levels of syndecan-1 protein expression between MDA-MB231-control and MDA-MB231-Id2 sense cells. As described further in the 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. I determined that syndican-1, which was expressed at a low level in MDA-MB231-control cells, was significantly up-regulated in MDA-MB231-Id2 sense cells (Fig. 15D).

Constitutive expression of Id-2 reduces the proliferation capacity of human metastatic breast cancer cells

Next, I 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, labeled with ^{3}H-methyl-thymidine for 7or 16 hr and the labeling index of the two cell populations was determined. MDA-MB231-Id2 sense cells

consistently showed a significant reduction of their capacity for proliferation compared to control cells (Fig. 16A). Finally, I also determined that the ability of the MDA-MB231-Id2 sense cells to grow in an anchorage-independent growth assay was strongly reduced (Fig. 16B). The majority of the MDA-MB231-control cells formed large colonies (>50μm diameter) whereas the majority of the MDA-MB231-Id2 sense cells formed small colonies (<25μm 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-1 expression in human breast tumor biopsies

Id-1 protein expression was also examined in breast cancer biopsies. Immunohistochemistry was performed by using a specific anti-Id-1 antibody. Multiple parallel assays including Western blotting, immunofluorescence (Fig. 12A), and *in situ* hybridization (Fig. 5C) were performed to verify to the specificity and accuracy of this antibody. Using a limited number of biopsies as a first step, I showed that almost all DCIS (ductal carcinoma *in situ*) were negative for Id-1 staining (8 out of 10 cases), whereas majority of the infiltrating carcinomas showed strong Id-1 staining (7 out of 12 cases) (Fig. 17) (46). Further, I analyzed a total of 58 cases of infiltrating carcinomas for Id-1 expression (Fig. 18) (47). The majority of the Id-1 positive cells showed prominent cytoplasmic

staining. Only about 20% of the grade I invasive carcinomas biopsies showed strong Id-1 staining, whereas more than 60% of the grade III invasive carcinomas investigated showed strong Id-1 expression (Fig. 18). These data further support my hypothesis that Id-1 not only represents a significant marker for human breast tumor progression, but also a potent target for anti-breast cancer therapy, because Id-1 is overexpressed in a high proportion of aggressive breast carcinomas.

Id-2 expression in human breast tumor biopsies

I also determined the expression of Id-2 protein in a panel of 48 human tumor biopsies from breast cancer patients by immunohistochemistry (Fig. 19) using a high quality batch of anti-Id-2 antibody. Out of a total 9 ductal carcinomas *in situ* (DCIS), one was negative for Id-2, 4 DCIS were weakly positive, and the other 4 were clearly positive. Out of a total of 14 infiltrating grade I carcinomas of ductal origin, 5 were weakly positive, and 5 were strongly positive. However, out of a total 16 infiltrating grade III carcinomas of ductal origin, only one was strongly positive, 8 of them being negative and 7 being weakly positive. Using the Chi-square test, I determined that the difference in Id-2 expression between DCIS/Invasive Grade I versus 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.

Systemically targeting Id-1 reduces murine breast cancer cell metastasis in tumor-bearing mice.

Further studies were undertaken to determine whether systemically targeting Id-1 expression *in vivo* could reduce the metastatic phenotype of breast cancer cells. Cationic liposome-DNA complex (CLDC)-based nonviral systemic gene transfer was used to assess the effects of Id-1 gene down-regulation in immunocompetent tumor-bearing mice. It is known that metastatic cancer is a result of not only alterations of gene expression within tumor cells but also from the complex interplay of the host environment with the tumor cells (66, 67). Thus, the systemic approach more accurately recapitulates the biologic as well as potential therapeutic settings of metastatic cancer. As my model, the 4T1 murine metastatic breast cancer cells, which primarily metastasize to the lungs of syngeneic BALB/c mice, was used. 4T1 cells, like the human MDA-MB436 and MDA-MB231 cells, express high levels of Id-1 mRNA (data not shown) and protein (data not shown) (47). These 4T1 mammary carcinoma cells are poorly immunogenic and have growth characteristics that resemble highly invasive human metastatic mammary carcinoma, and the extent of disease is comparable to human stage IV breast cancer (68). Moreover, breast cancer metastasizes more to the lungs (70.3%) of human patients than to the liver (58.4%), bone marrow (53.1%), or brain (14.3%)

(69).

To reduce Id-1 expression in tumor-bearing mice, mice were intravenously (i.v.) injected with CLDC containing the full-length antisense Id-1 cDNA in the long-expressing human ctytomegalovirus-driven vector (59). The CLDC-based antisense construct significantly reduced the metastatic spread of 4T1 breast cancer cells in syngeneic BALB/c mice (Fig. 20A). Specifically, a single injection of CLDC containing the antisense Id-1 plasmid, 3 days after i.v. inoculation of 50,000 4T1 cells, significantly reduced the total number of lung metastases compared with tumor-bearing mice treated with CLDC containing the luciferase gene (mock-treated control group). Interestingly, this reduction was comparable to that achieved by injection of the potent metastasis suppressor gene, CC3 (60, 70). To determine the effect of systemic delivery of the antisense Id-1 plasmid on Id-1 protein levels in 4T1 lung tumors, tumor-bearing mice were injected with CLDC containing either the antisense Id-1 or control luciferase cDNAs 2 days before killing. By using immunohistochemistry, I found that a high level of Id-1 protein was detected in 4T1 cells from CLDC-luciferase control treated mice (Fig. 20B, *panel* a), whereas significantly lower levels of Id-1 protein were detected in 4T1 cells from CLDC-Id-1 antisense treated mice (Fig. 20B, *panel* b). The significant and rapid reduction of Id-1 protein level presumably was a result of the fast turn over rate of Id-1 mRNA (46). Moreover, and in agreement with the *in vitro* data presented in Figure 14, MT1-MMP levels were significantly reduced in 4T1 cells

from CLDC-Id1 antisense treated mice (Fig. 20B, *panel* d) in comparison to CLDC-luciferase control treated mice (Fig. 20B, *panel* c). High levels of MT1-MMP staining were observed in the cytoplasm of the cancerous 4T1 cells from the control treated mice, as previously reported for colon cancer cells (71).

Discussion

In vivo, the growth and differentiation of the mammary gland progress through several stages during puberty. Ultimate function (expression and secretion of milk proteins) is achieved by the epithelial cells during lactation. Mammary epithelial cells lose their ability to proliferate as they progress toward functional differentiation. Once lactation terminates, most of the epithelial cells undergo apoptosis. Very little is known about the molecular mechanisms that coordinate growth, differentiation and apoptosis during mammary gland development and remodeling (39).

Both Id-1 and Id-2 appear to play an essential role in the regulation of breast epithelial cell differentiation and proliferation. The coordination of the Id-1/Id-2 balance may be important for normal mammary gland development and the dysregulation in this balance could be responsible for the appearance of an invasive and metastatic phenotype in breast cancer cells. The expressions and the functions of Id-1 and Id-2 in breast epithelial cells are summarized in Table 2.

Id-1 expression in normal breast cells

I determined Id-1 expression in the mammary gland using Northern analysis and immunohistochemistry as well as the technique of *in situ* hybridization (Fig. 5). Id-1

expression follows a pattern expected from the cell culture studies. In the mammary gland, Id-1 expression was inversely correlated with that of β-casein mRNA. β-casein was used as a marker of differentiation in mammary gland. Id-1 mRNA was expressed highly in the developing gland but was reduced during pregnancy and completely abolished during lactation. Interestingly, Id-1 was also expressed highly during involution. This expression corresponds to the onset of mammary epithelial apoptosis (37). Thus, Id-1 may not only be important for the proliferation of mammary epithelial cells in virgin and early pregnant mice but also for the onset of programmed cell death when the gland involutes. In agreement with this, it has been reported that Id-1 expression stimulated proliferation in SCp2 mammary epithelial cells when the cells were cultured at subconfluence in a serum-free condition, whereas the same cells underwent apoptosis when cultured at high density in a serum-free condition (44). Thus, depending on extracellular signals and cell-cell interactions, Id-1 protein can promote either growth or apoptosis.

Recently, another research group used immunohistochemistry to determine the distribution of Id-1 expression in the mammary gland (72). Immunoreactivity was detected in the cytoplasm of myoepithelial cells and in vascular endothelial cells. This report claimed that Id-1 was not expressed in luminal epithelial cells in murine mammary gland. This result is in disagreement with mine. However, the staining observed in the myoepithelial cells did not correspond to Id-1, because it was also observed in myoepithelial cells of mammary gland

tissue from Id-1 null mice. This report therefore exemplifies the complications resulting from immunohistochemistry using antibodies that give non-specific signals on western blots, and highlights the urgent need for reliable antibodies which work on tissue sections.

Id-2 expression in normal breast cells

Unlike Id-1, which is expressed during proliferation and is able to suppress differentiation in all cell types examined, the data reported on Id-2 are much less consistent. The Id-2 protein contains a HLH motif similar to that of Id-1 (90% identity (19, 20)), but the two proteins differ markedly in the rest of their sequence. Id-1 and Id-2 are encoded by unlinked genes. 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 (12, 48). In addition, overexpression of Id-2 inhibited the differentiation of myoblasts (73) and led to a stage-specific developmental block early in thymopoiesis (74). However, there are also other reports inconsistent with a role for Id-2 as an inhibitor of differentiation.

Based on my data on Id-2 expression during mammary epithelial cell differentiation *in vitro* (Fig. 7-10) and mammary gland development *in vivo* (Fig. 6), I propose that Id-2 acts as an activator, rather than an inhibitor, of mammary differentiation and as an inhibitor of mammary proliferation. I 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 (49). Recently, a key role for Id-2 in maintenance of a differentiated phenotype in vascular smooth muscle cells was also reported (75). Moreover, overexpression of Id-2 in osteoblastic cells activates bone differentiation in the early stages of development, whereas Id-1 inhibits bone maturation (Dr. Carlotta Glackin, personal communication). I 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 transcriptional factors such as ITF2, which then turn on the expression of Id-2 gene (Fig. 21). ITF-2 is a member of the ubiquitous bHLH protein family (class I). By the screening of a yeast-hybrid library from mammary epithelial cells, it has been reported that Id-1 interacts with ITF-2 (44). There was no change in its level of expression in SCp2 cells, whether growing or differentiated (44). It has also been reported that ectopic ITF-2 overexpression counterbalanced the effects of Id-1 on differentiation, proliferation, and apoptosis in SCp2 mammary epithelial cells (44). Neuman *et al*. reported that Id-2 gene contains several E-boxes within its promoter, and bHLH factors regulate Id-2 expression in F9 cells (76). Since Id-2 appears to be involved in the normal process of mammary epithelial cell

differentiation and the expression of Id-2 is up-regulated when Id-1 is absent, Id-1 could be the inhibitor of Id-2 gene expression through interaction with a specific bHLH. Therefore, Id-1 Id-2 and ITF-2 might be acting within a network governing mammary epithelial cell phenotype and potentially acting as molecular switches of the differentiation (Fig. 21).

Whereas I 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 (52). One possible explanation is that all my data (*in vitro* and *in vivo* in mice) have been obtained from BALB/c mice, whereas Mori *et al*. (52) used a different strain (129/Sv) for the Id-2 knock out experiments. Based on the data published by Miyoshi *et al*. (77), 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 my hypothesis that Id-2 plays a key role during the important stages of mammary differentiation. Moreover, I 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.

Id-1 and Id-2 expression in breast cancer cell lines

I showed that 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 (Fig. 11). This inverse relationship is particularly clear in cells cultured in serum-free conditions. 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, whereas Id-1 was highly expressed. Therefore, I hypothesized that Id-2 can only be expressed when Id-1 is down-regulated. As a potential explanation, I propose that some bHLH protein such as ITF-2, previously sequestered by Id-1, up-regulated Id-2 expression at the promoter level through their binding to E-boxes (Fig. 21).

Moreover, Id-1 positively regulates the invasive phenotype of breast cancer cells. Targeting Id-1 expression in human breast cancer MDA-MB231 and MDA-MB436 cells reduced their invasiveness (Fig. 12 and 13). I also identified the MT1-MMP gene to be significantly down-regulated by targeting Id-1 in MDA-MB231 cells (Fig. 14). MT1-MMP is a major MMP because it can degrade extracellular matrix components directly as well as indirectly by activating MMPs. The precise localization of MT1-MMP between cancer cells and surrounding stroma cells has been the subject of controversy. Recently, Dalberg *et al*. (78) detected MT1-MMP mRNA expression in all invasive breast cancer tumor biopsies investigated and found that it was mainly localized in the tumor cells. Mimori *et al*. (79)

also determined that the highest expression of MT1-MMP mRNA was found in breast cancer specimens showing lymph-node metastasis and/or lymph-vessel invasion. All these data on MT1-MMP indicate that it plays a crucial role during breast cancer progression. However, very little has been known about the regulation of this very important enzyme until recently. Studies on the MT1-MMP promoter indicated that that it contains several regulatory sequences, including some E-boxes that were recognized by bHLH transcription factors (80). Therefore, I hypothesize that MT1-MMP expression in regulated by the Id-1-interacting bHLH transcription factors and that MT1-MMP might be a key mediator of the anti-invasion and anti-metastasis effects of Id-1 down-regulation in breast cancer cells.

In contrast with Id-1, 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 (Fig. 15 and 16). Since cell proliferation in an important aspect of the aggressive phenotype of breast cancer cells, I conclude that Id-2 is able to reduce aggressiveness by reducing not only invasion/migration, but also proliferation. Interestingly, I found that the reduction in the invasive phenotype might be mediated, at least in part, by 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 (Fig. 15C). Some studies have recently revealed that Id binds to proteins other than bHLH proteins such as the Ets family transcription factor (15). The Ets transcription

binding sites are required for the activation of MMP-9 promoter in breast cancer cells (81), and the highly invasive MDA-MB231 cells have high levels of Ets-1 and Ets-2 proteins (82). I therefore hypothesize that this regulation of MMP-9 by Id-2 might occur through Id-2 interaction with Ets transcriptional factors.

Id-2 could also indirectly down-regulate MMP-9 expression through the increase in syndecan-1 levels. As suppression of MMP-9 expression by the heparan sulfate proteoglycan syndecan-1 has been reported in human myeloma cell lines (83). 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 (84, 85). Moreover, loss of syndecan-1 causes epithelial 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 (86, 87). Because Id-2 up-regulated syndecan-1 expression in MDA-MB231 cells (Fig. 15D), 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.

Id-1 and Id-2 expression in human breast tumor biopsies

The studies of Id-1 expression using breast cancer biopsies (Fig. 17 and 18) indicated that Id-1 expression might be a reliable prognostic marker for breast cancer invasion and

metastasis. This hypothesis was confirmed recently by another laboratory. Id-1 protein expression was investigated in 191 patients with lymph-node negative breast cancer (88). Patients with strong or moderate Id-1 expression had a significant shorter overall (p=0.003) and disease-free survival (p=0.01) compared to those with absent or low expression. Thus, aberrant expression of Id-1 protein represents a strong independent prognostic marker in node-negative breast cancer. In addition, it has been proposed that Id-1 could be a promising candidate for future therapy and that inhibiting Id-1 expression might be of benefit for patients with breast cancer. In agreement with this hypothesis, I showed that human metastatic breast cancer cells became significantly less invasive *in vitro* and less metastatic *in vivo* when Id-1 is down-regulated by stable transduction with Id-1 antisense (Fig. 12-14) (47).

In contrast to Id-1, little Id-2 protein expression was detectable in human biopsies from aggressive and invasive carcinomas (Fig. 19). Moreover, Id-2 was strongly expressed in non-aggressive DCIS stages, where Id-1 was expressed at low levels (Fig. 19). DCIS stages often assume phenotypes of noninvasive and low proliferation. Therefore I suggest that Id-2 is an important protein involved in the maintenance of a noninvasive, non-migratory, and low proliferative status of non-aggressive breast cancer cells as well as normal mammary epithelial cells. Id-2 may also ultimately be useful as an indicator of good prognosis for breast cancer patients.

Id-1 as a potential target in breast cancer therapy

I demonstrated that using antisense technology to target endogenous Id-1 gene expression can reduce breast cancer cell metastasis to the lungs (Fig. 20). Stable infection of human breast cancer cells with antisense Id-1 significantly reduced lung metastasis after their inoculation into nude mice. Therefore, specifically targeting Id-1 in cancer cells alone was sufficient to reduce metastatic spread in mice, when MDA-MB231 was used as the tumor model. Furthermore, systemic CLDC-based injection of an Id-1 antisense plasmid also significantly reduced lung metastasis. In these experiments performed by using syngeneic BALB/c mice, I can not rule out the possibility that the Id-1 antisense construct may also be delivered to other cell types, including vascular endothelial cells (60). Because expression of Id genes is required also for angiogenesis and neo-vascularization (10), targeting Id-1 expression in tumor cells as well as endothelial cells from the tumor blood vessels might produce additive or even synergistic synergistic anti-tumor effects.

Taken together, these results indicate that Id-1 protein is a molecular target for blocking breast tumor metastasis. I hypothesize that Id-1 would be a highly effective and selective target for breast cancer therapy. First, Id-1 is not a transcription factor *per se*. Id-1 has been shown to regulate the activity of several important regulatory proteins involved in transcriptional regulation. Amongst these proteins are the basic helix-loop-helix transcription factors, Rb, and the Ets family members (12). Through these different

interactions, Id-1 protein is central to the pathways regulating proliferation, differentiation, migration, invasion, and cell-cell interaction. Therefore, using Id-1 as a target could affect various aspects of breast carcinogenesis and progression. Second, even though Id-1 is widely expressed during development and tumorigenesis, it is not expressed in most of the mature adult tissues (9). The scarcity of Id-1 expression in adult tissues could be an advantage for systemic therapy because the majority of the normal cells will not be affected. Third, as demonstrated in my studies, as well as from the knockout animal model (10), an only partial reduction of Id-1 levels can profoundly affect tumor behavior. Therefore, Id-1 protein is a promising molecular target for breast cancer therapy.

In addition to breast cancer therapy, I hypothesize that Id-1 targeting could be useful in other types of cancer therapy. Higher levels of Id-1 gene expression have also been detected in tumor cells from other tissue types, compared with normal cells of the same tissue origin (89-94). Moreover, Id-1 expression has been shown to be an unfavorable prognostic marker in early-stage cervical cancer (95), and stronger Id-1 expression was associated with poor differentiation and more aggressive behavior of ovarian tumor cells (96).

As mentioned above, in contrast with Id-1, Id-2 appears to act as an important protein for the maintenance of differentiated and non-invasive phenotype in breast cancer cells. Due to the availability of a new vector capable of delivering two different cDNAs at the same time (Dr. Rober J. Debs, personal communication), I propose that Id-2 sense delivery, in

combination with Id-1 antisense delivery, could be more effective in inhibition of metastasis when delivered in invasive and aggressive breast cancer.

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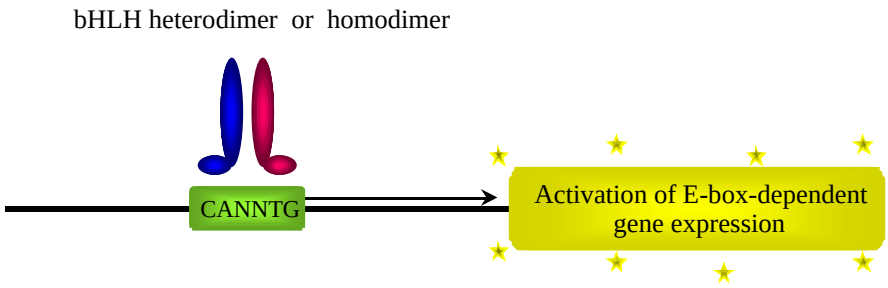
Table 1: Classification of the HLH transcriptional regulators

Class	Protein families	Function	Tissue distribution, dimerization, DNA-binding and other features
Class I	E12, E47, HEB, E2-2 / ITF-2 (E proteins)	Myogenesis, lymphogenesis, neurogenesis, sex determination, lymphomagenesis, etc.	Ubiquitous pattern of expression and capable of forming either homo- or heterodimer
Class II	MyoD, Myogenin NeuroD/BETA2 dHAND, eHAND Mash Twist Mist SCL/Tal1	Myogenesis Neurogenic differentiation, pancreatic development Cardiac morphogenesis Positive regulator of neurogenesis Inhibition of myogenic and neurogenic differentiation Exocytosis of serous secretions Hematopoiesis	Tissue-restricted pattern of expression and incapable of forming homodimers and preferentially heterodimerize with the E proteins; Class I and Class II heterodimers can bind both canonical and noncanonical E-box sites
Class III	Myc TFE SREBP (bHLH-Zip)	Cell proliferation, differentiation, oncogenesis, apoptosis, etc. Transcription in immunoglobulin heavy chain enhancer Sterol synthesis, adipocyte determination	Presence of leucine zipper adjacent to the HLH motif
Class IV	Mad, Max, Mxi (bHLH-Zip)	Interaction with Myc family proteins and regulation of cell proliferation	Dimerization with the Myc protein or with each other
Class V	Id	Inhibition of DNA binding, cell proliferation, differentiation, etc.	Lack the basic DNA-binding domain and act as dominant negative regulators of Class I and Class II bHLH proteins
Class VI	HES, HESR1	Notch signaling pathway, cell proliferation	Presence of proline in their basic region
Class VII	AHR, ARNT, Sim HIF	Biological responses to planar aromatic hydrocarbons Regulator of O_2 homeostasis	Presence of a bHLH-PAS domain

(A) Lack of Id protein



(B) Sufficient Id protein

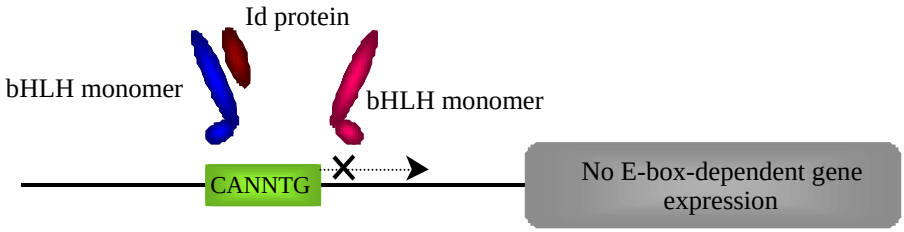


Fig. 1: Id proteins as dominant negative regulators of bHLH transcription factos
The basic helix-loop-helix (bHLH) proteins are transcription factors acting through direct DNA binding, and Id proteins are dominant negative regulators of these bHLH factors.

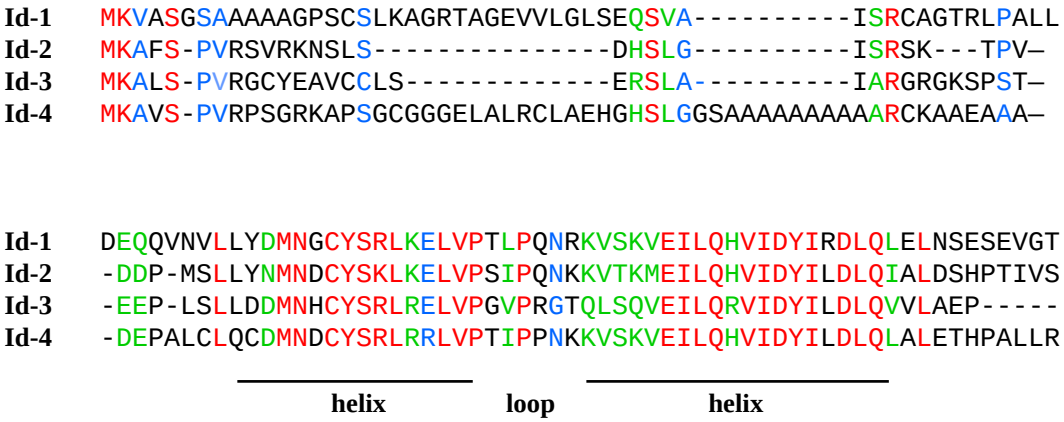


Fig. 2: Amino Acid sequence alignment of Id proteins

The amino acid sequence of mouse Id-1, Id-2 Id-3 and Id-4 are shown. They are all of similar size (13-20kDa). The regions outside of the highly conserved HLH domain are largely divergent, and may determine the tissue-specificity of Id function, as well as the binding specificity for particular bHLH proteins.

Red; fully conserved residue

Green; strongly conserved residue

Blue; weakly conserved residue

Black; no consensus

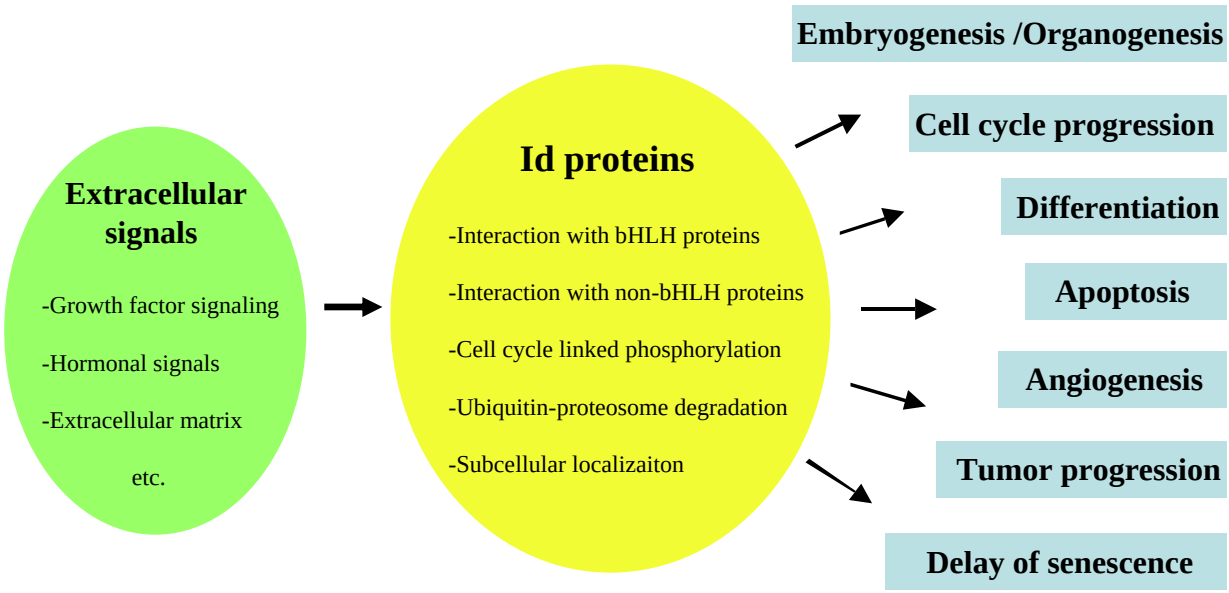


Fig. 3: The implication of Id proteins as regulators of various biological events

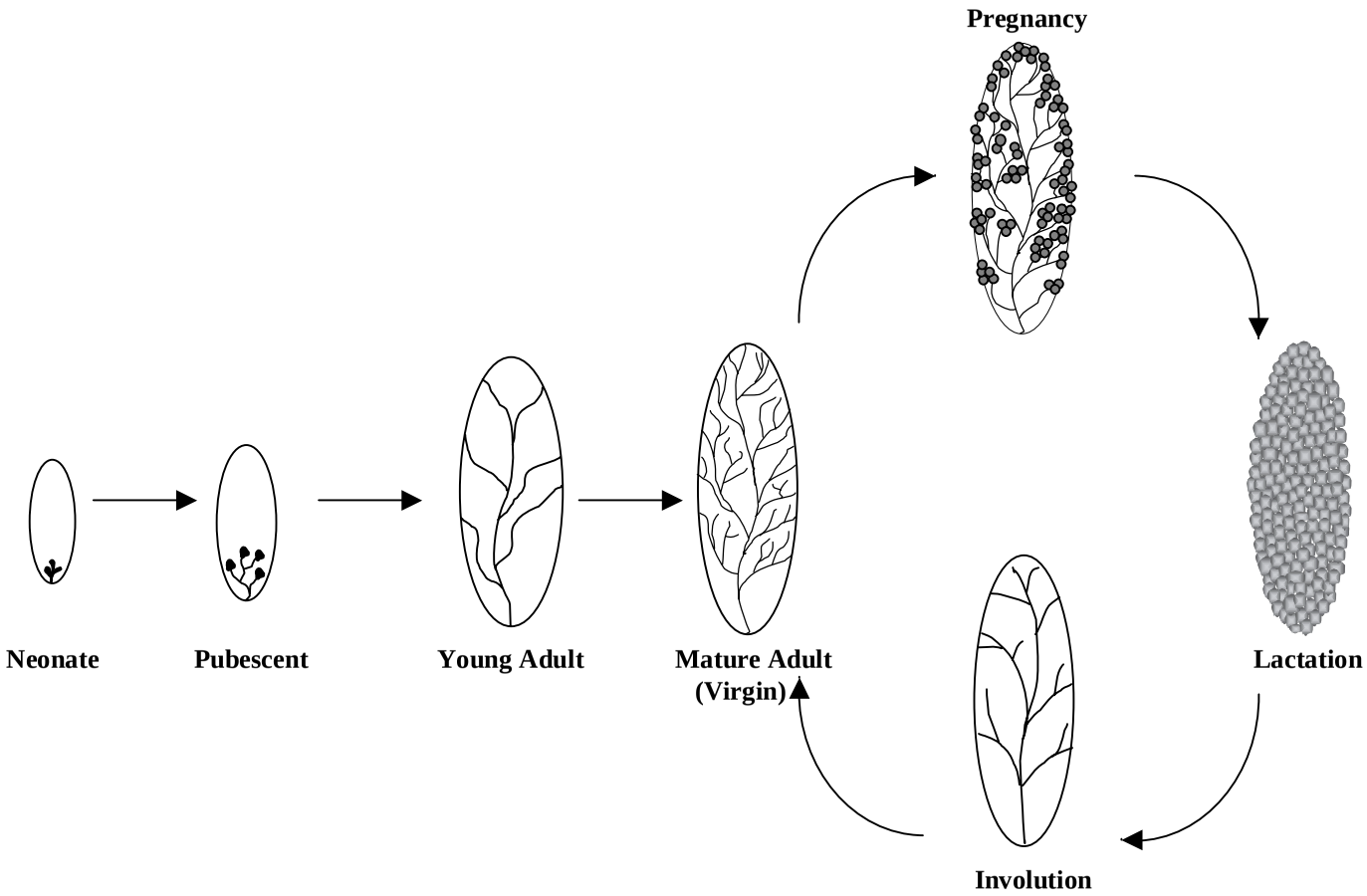


Fig. 4: Mammary gland development

The mammary gland undergoes extensive proliferation, differentiation and remodeling during normal development. At puberty, epithelial ducts infiltrate the stromal tissue and create branching networks. In pregnancy, the luminal epithelial cells of these ducts change their fate and proliferate rapidly to form spherical alveolar structures that are able to synthesize and secrete milk proteins. In the lactating gland, the secretory lobules developed from the branching ducts become filled with milk. Following a lactation phase, the secretory epithelial cells die by apoptosis and the gland rapidly regress to the virgin-like state in a process known as involution.

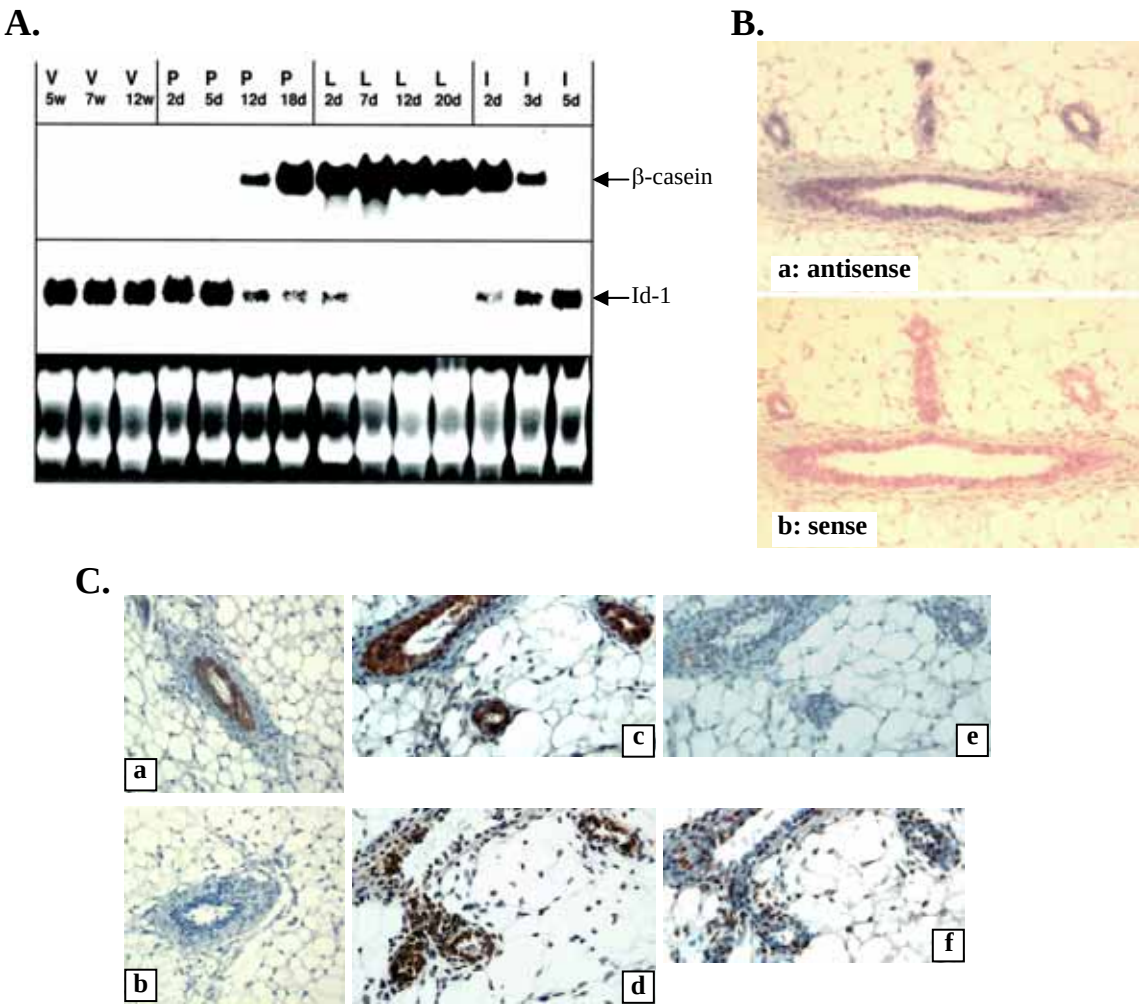


Fig. 5: Id-1 expression during murine mammary gland development

A, total RNA was extracted from glands at different stages of development and Northern analyses using cDNA probes for mouse β-casein and Id-1 were performed.
(V, virgin 5 ,7 and 12 weeks; P, pregnant 2, 5, 12 and 18 days; L, lactation 7, 12, 20 days; I, involution 2, 3 and 5 days)

B, Id-1 protein expression was detected by immunohistochemistry (*panel* a and c). The negative control using Id-1 blocking peptide is shown in *panel* b. *Panel* c, d, e and f are adjacents sections. Keratin-8/18 staining (*panel* d) as a marker for epithelial cells, keratin-14 staining as a marker for myoepithelial cells (*panel* e) and α-smooth-muscle actin staining as a marker for stroma cells (*panel* f) were performed. All sections are from pregnant 5 days. The slides were counterstained with hematoxyline. Id-1 protein was only expressed in keratin 8/18-positive mammary epithelial cells.

C, Id-1expression at the mRNA levels was detected by *in situ* hybridization using the section from pregnant 5 days (*panel* a). Id-1 sense probe was used as a negative control (*panel* b). The slides were counterstainded with Nuclear Fast Red. A strong signal of Id-1 was detected only in the epithelial cells.

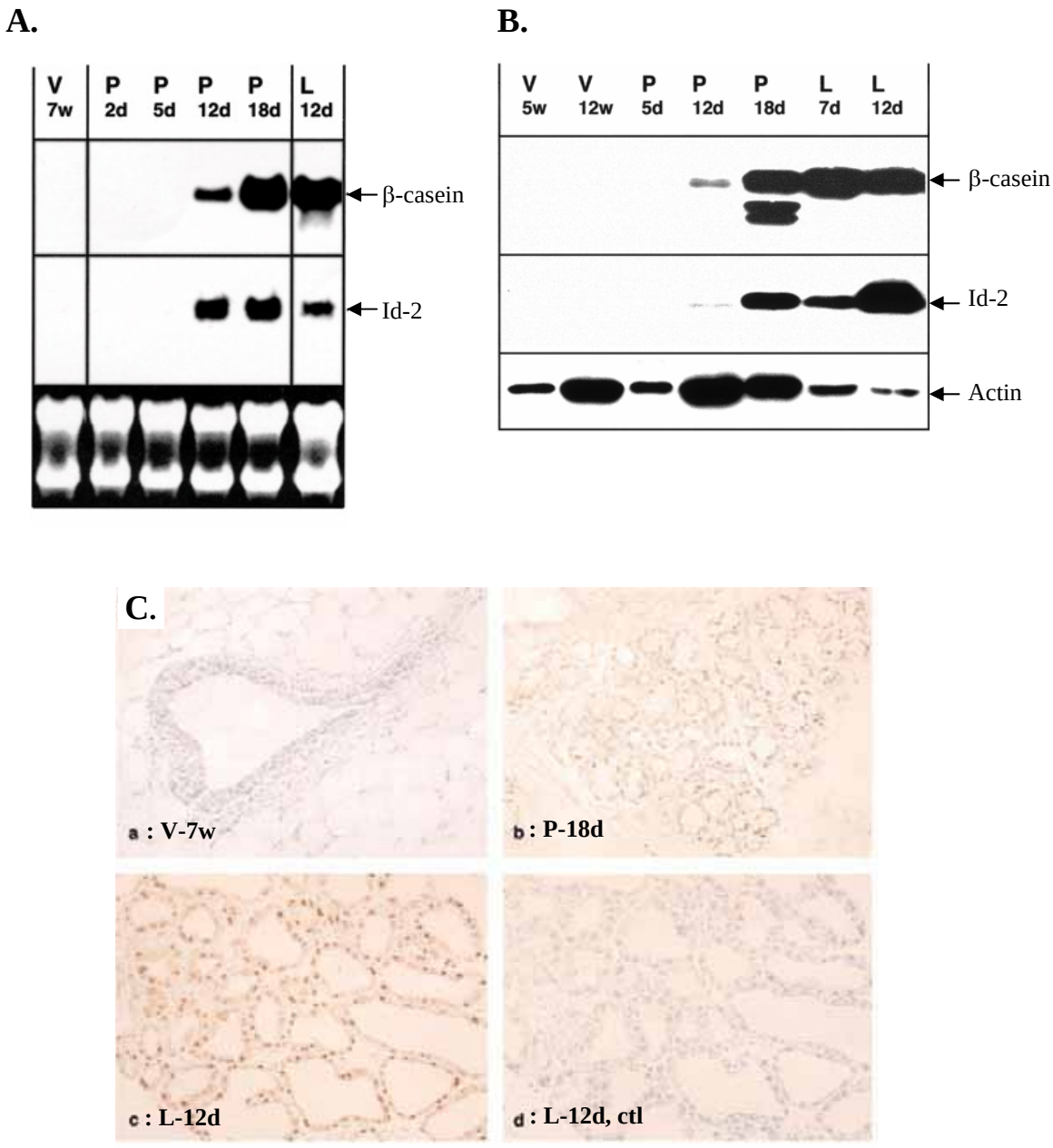


Fig. 6: Id-2 expression during murine mammary gland development
A, total RNA was extracted from glands at different stages of development and Northern analyses using cDNA probes for mouse β-casein and Id-2 were performed.
B, protein was extracted from glands at different stages of development and Western analyses were performed using antibodies specific for β-casein, Id-2 and actin.
C, Id-2 protein expression was detected by immunohistochemistry. The negative control using Id-2 blocking peptide is shown in *panel* d. The slides were counterstained with hematoxyline.
(V, virgin 5 ,7 and 12 weeks; P, pregnant 2, 5, 12 and 18 days; L, lactation 7, 12 days)

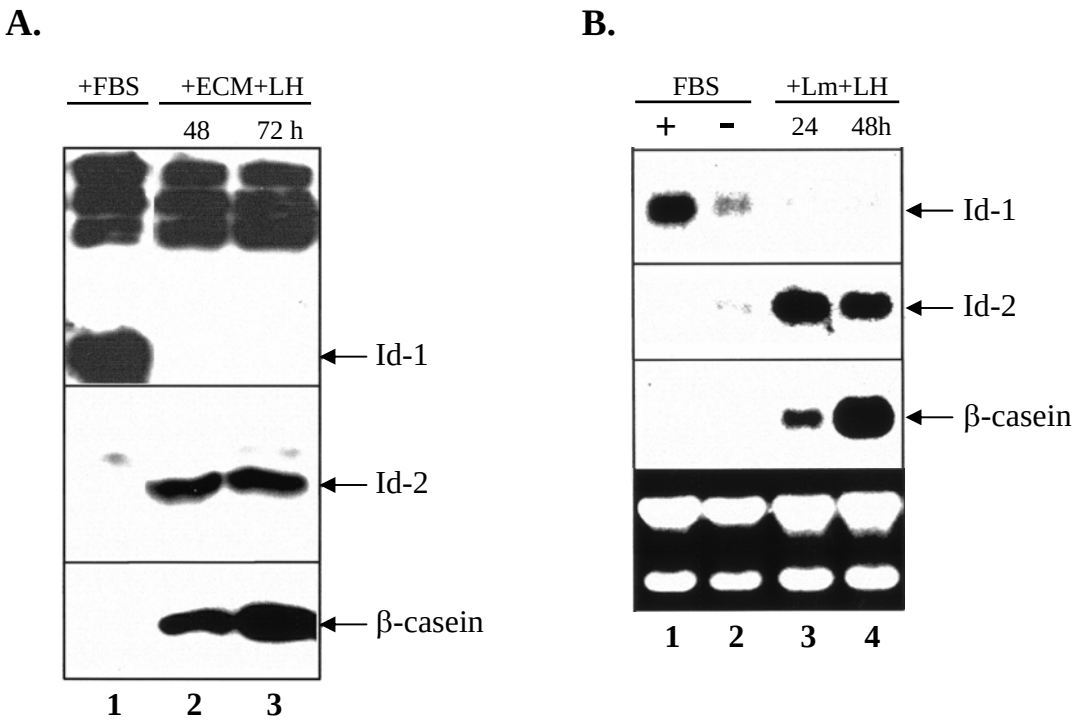


Fig. 7: Inverse correlation between Id-1 and Id-2 expression in SCp2 cells
Id-1 was strongly expressed only in proliferating cells. In comparison, Id-2 was highly expressed in differentiating cell and not in proliferating cells.
A, SCp2 cells were cultures in growing (*lane* 1) and differentiating conditions treated with ECM + LH for 48 (*lane* 2) and 72 h (*lane* 3). Protein was extracted and Western analysis was performed using antibodies specific for Id-1, Id-2, and β-casein.
B, SCp2 cells were cultured in growing (*lane* 1), serum-starved (*lane* 2), and differentiating conditions treated with laminin +LH for 24 (*lane* 3) and 48 h (*lane* 4). Total RNA was extracted and analyzed using probes specific for Id-1, Id-2, and β-casein by Northern blot.
ECM, extracellular matrix; LH, Lactogenic hormones; Lm, laminin

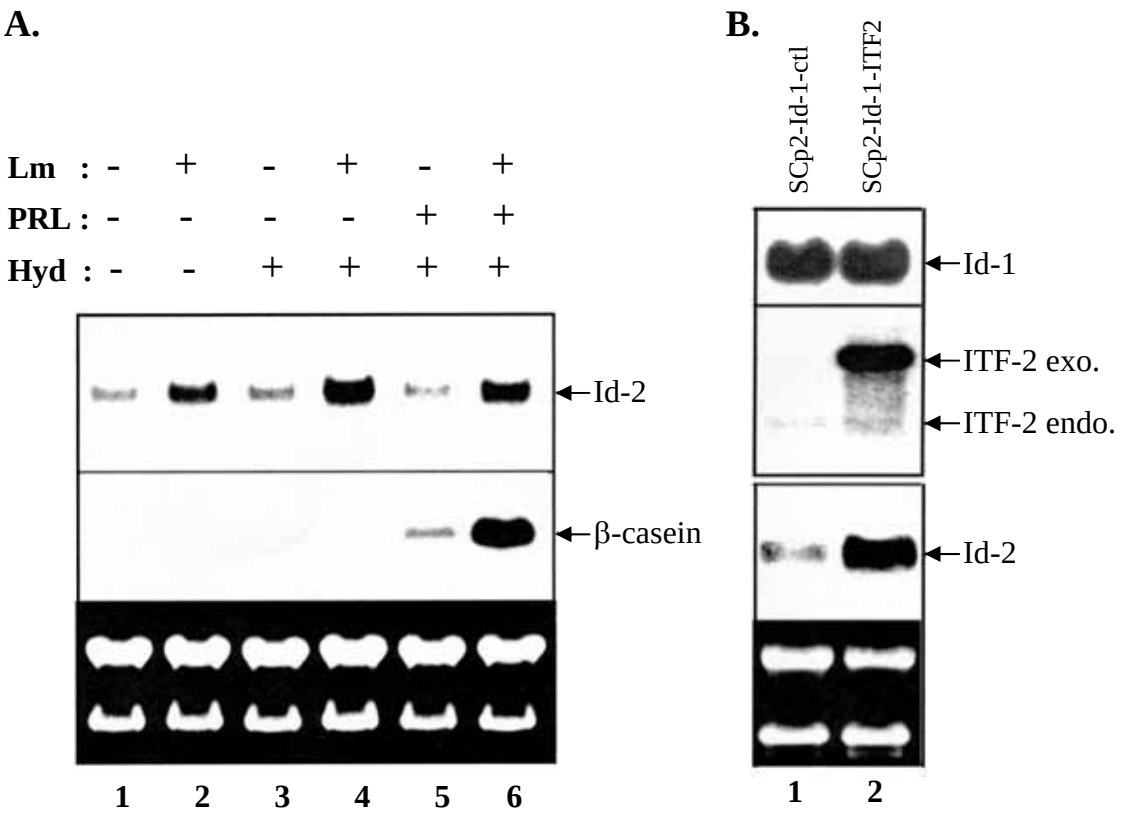


Fig. 8: Regulation of Id-2 expression by laminin in SCp2 cells

A, Id-2 expression was up-regulated by laminin. SCp2 cells were cultures in serum-free medium or treated with laminin and /or lactogenic hormones (hydrocortisone and/ or prolactin) for 2 days.

Total RNA was extracted and analyzed by Northern blot for Id-2 and β-casein.

Lm, laminin; PRL, prolactin; Hyd, hydrocortisone.

B, Ectopic ITF-2 overexpression induced Id-2 expression despite in the presence of Id-1. SCp2 cells transfected with Id-1 were subsequently infected with pLXSN-control vector (SCp2-Id-1-LXSN-ctl in *lane* 1) or pLXSN-ITF2 vector (SCp2-Id-1-LXSN-ITF2 in lane 2).

Northern analyses were performed using probes for Id-1, ITF-2 and Id-2.

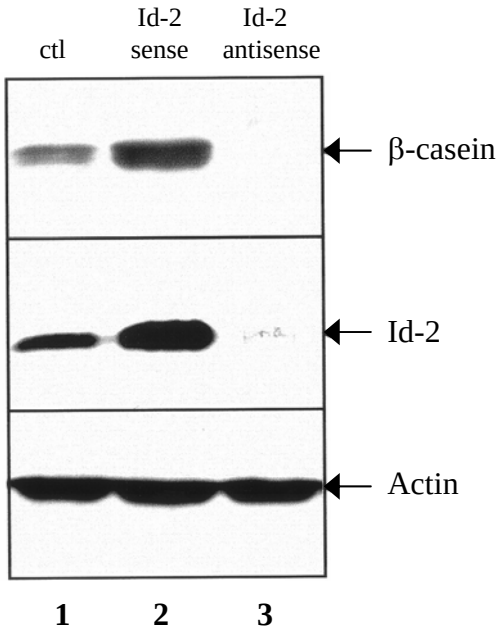


Fig. 9: Effect of Id-2 expression on mammary epithelial cell differentiation and milk production

lane 1; SCp2 cells infected with pLXSN-control vector

lane 2; SCp2 cells infected with pLXSN-Id-2-sense expression vector

lane 3; SCp2 cells infected with pLXSN-Id-2-antisense expression vector

All cell populations were treated with laminin + LH (lactogenic hormones) for 48h. Proteins was extracted and Western analysis was performed. Actin protein was used as a control for loading.

β-casein expression was increased in SCp2 cells infected with pLXSN-Id-2-sense expression vector (*lane 2*) and inhibited in the cells infected with pLXSN-Id-2-antisense expression vector (*lane 3*).

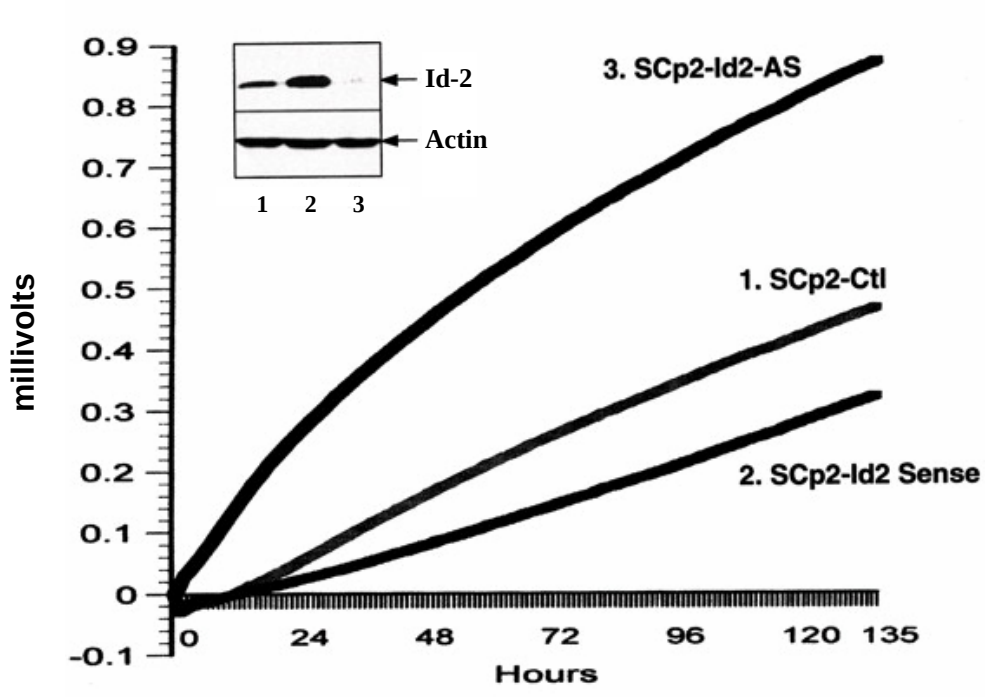


Fig. 10: Effect of Id-2 expression on cell proliferation
1, SCp2 cells infected with pLXSN-control vector
2, SCp2 cells infected with pLXSN-Id-2-sense expression vector
3, SCp2 cells infected with pLXSN-Id-2-antisense expression vector
Cell proliferation was reduced in SCp2-Id2-sense cells and increased in SCp2-Id2-antisense cells.
Each cell population was plated at 15,000 cells/well in 24-wells. Six wells were prepared for each population. Cell growth was measured every 60 min, and the readings from the 6-wells/cell population were averaged.
Insert, a Western blot comparing the levels of Id-2 protein in the three populations.

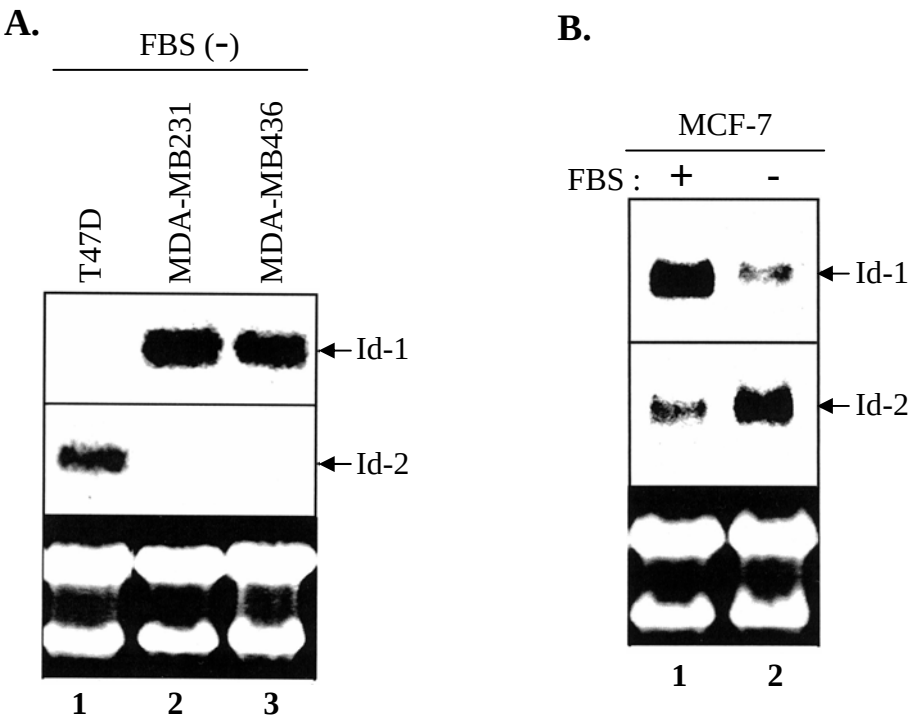


Fig. 11: Id-1 and Id-2 expression in human breast cancer cell lines
A, Id-1 expression was high in aggressive breast cancer cell lines, whereas Id-2 expression was high in non-aggressive breast cancer cell lines under serum-free conditions.
lane 1; non-aggressive T47D cells
lane 2; highly aggressive and metastatic MDA-MB231 cells
lane 3; aggressive and metastatic MDA-MB436 cells
Cells were cultured in serum-free medium for 48 h before total RNA was extracted and subjected to Northern blotting.
B, the inverse relationship was observed between the expression of Id-1 and Id-2 in MCF-7 cells.
Non-aggressive MCF-7 cells were cultured in serum containing medium (*lane 1*) or serum-free medium for 24 h (*lane* 2) before total RNA was extracted and subjected to Northern blotting.

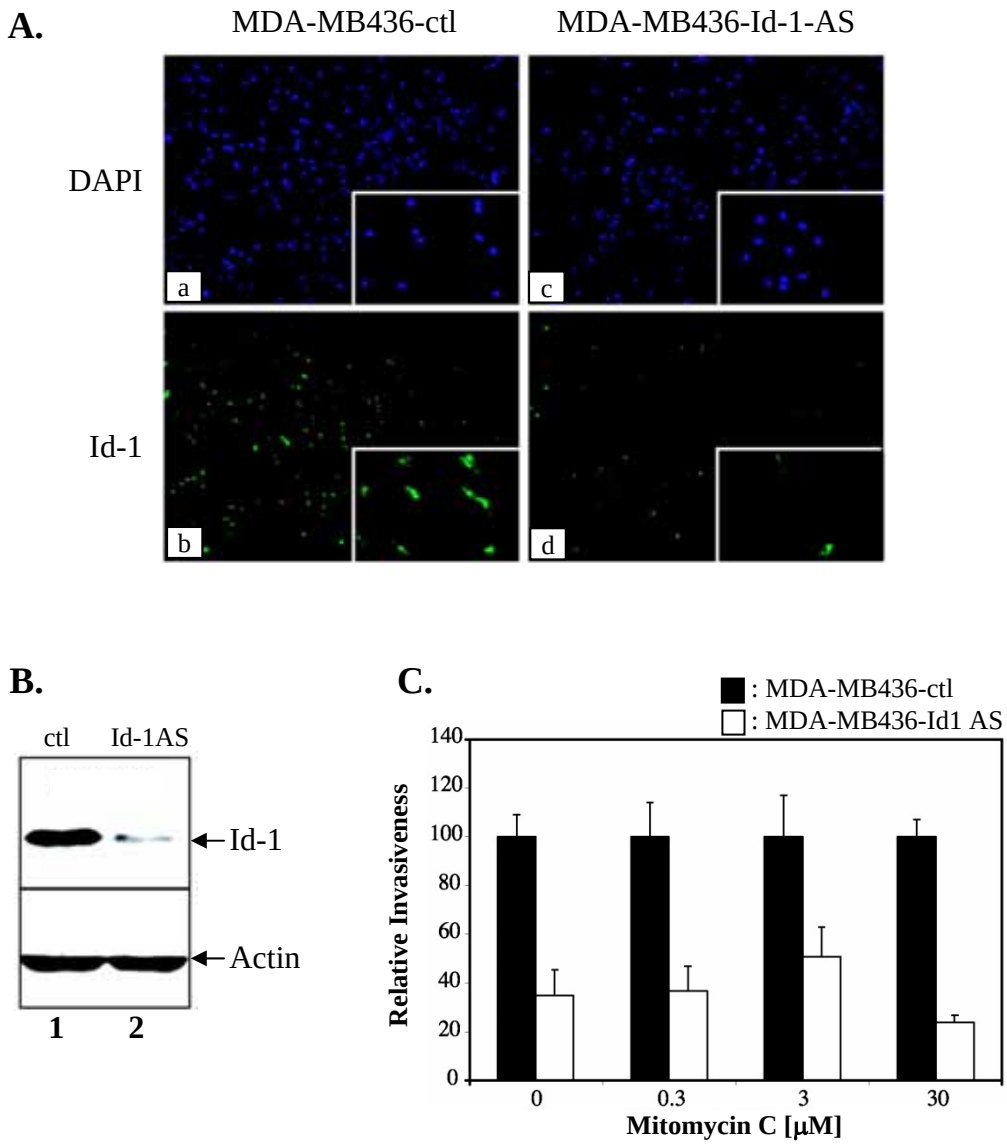


Fig. 12: Targeting Id-1 expression in human MDA-MB436 breast caner cells reduced their invasiveness *in vitro*.

A, Id-1 expression was detected by immunofluorescence.
panel a and b; MDA-MB436 infected with pLXSN control vector
panel c and d; MDA-MB436 infected with pLXSN-Id-1-antisense vector
Nuclei staining (4'6-diamino-2-phenylindole; DAPI) (*panel* a and c) and Id-1 staining (*panel* b and d) were performed.
B, Western blot analysis of Id-1 expression in MDA-MB436 cells
lane 1; MDA-MB436 infected with pLXSN control vector
lane 2; MDA-MB436 infected with pLXSN-Id-1-antisense vector
C; Boyden chamber invasion assay comparing the invasive ability of the infected MDA-MB436 cell populations in the absence or presence of mitomycin C (0, 0.3, 3, 30 μM). Shown are MDA-MB436-control (■) and MDA-MB436-Id-1-antisense (□). Data are presented as relative invasiveness, where the respective control are set as 100%. The cell populations infected with Id-1 antisense construct had significantly lower invasive potential than the control populations ($p<0.05$).

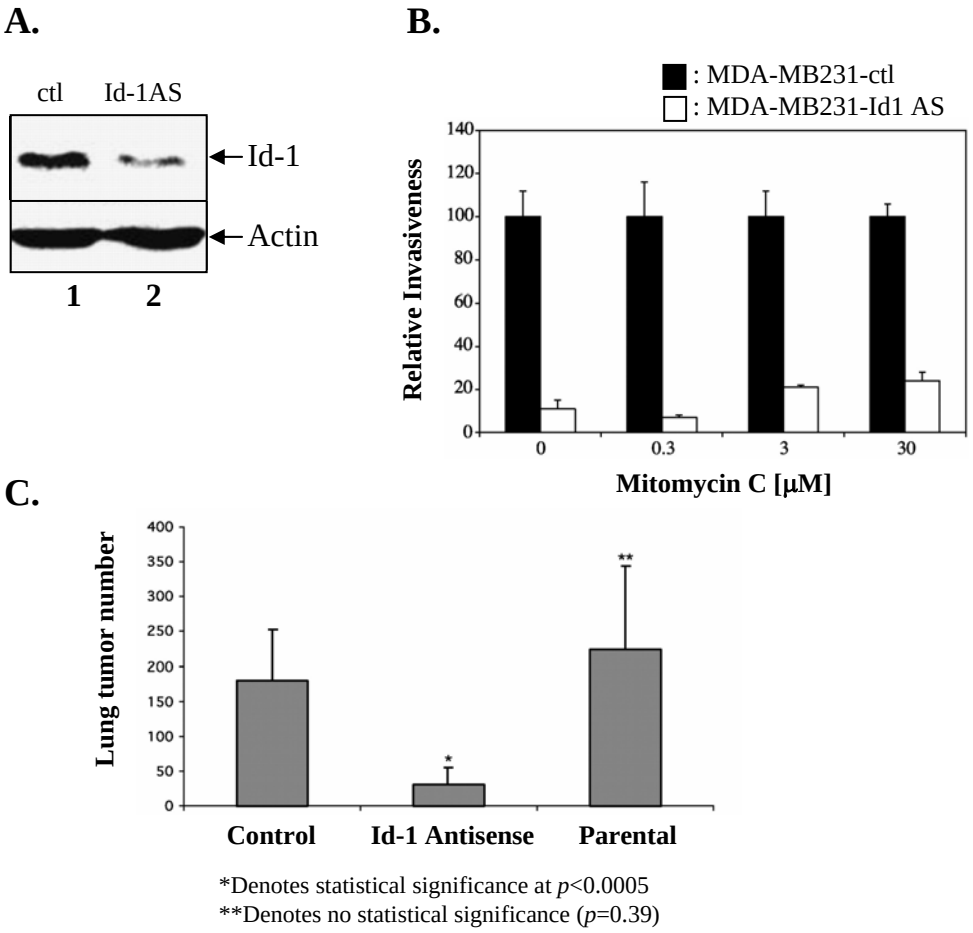


Fig. 13: Targeting Id-1 expression in human MDA-MB231 breast caner cells reduced their invasiveness *in vitro* and their metastatic spread *in vivo*.
A, Western blot analysis of Id-1 expression in MDA-MB231 cells
lane 1; MDA-MB231 infected with pLXSN control vector
lane 2; MDA-MB231 infected with pLXSN-Id-1-antisense vector
B, Boyden chamber invasion assay comparing the invasive ability of the infected MDA-MB231 cell populations in the absence or presence of mitomycin C (0, 0.3, 3, 30 μM). Shown are MDA-MB231-control (■) and MDA-MB231-Id-1-antisense (□). Data are presented as relative invasiveness, where the respective control are set as 100%. The cell populations infected with Id-1 antisense construct had significantly lower invasive potential than the control populations ($p<0.001$).
C, Targeting Id-1 expression in MDA-MB231 cells reduced not only their invasiveness *in vitro* but also their metastatic spread *in vivo*. The graph shows total lung tumor numbers in nude mice that were injected systemically with MDA-MB231 infected with pLXSN vector (Control), MDA-MB231 infected with pLXSN-Id-1 antisense construct (Id-1 Antisense), or parental MDA-MB231(Parental) cells. Compared with control: *, $p<0.0005$; **, $p<0.39$(not significant).

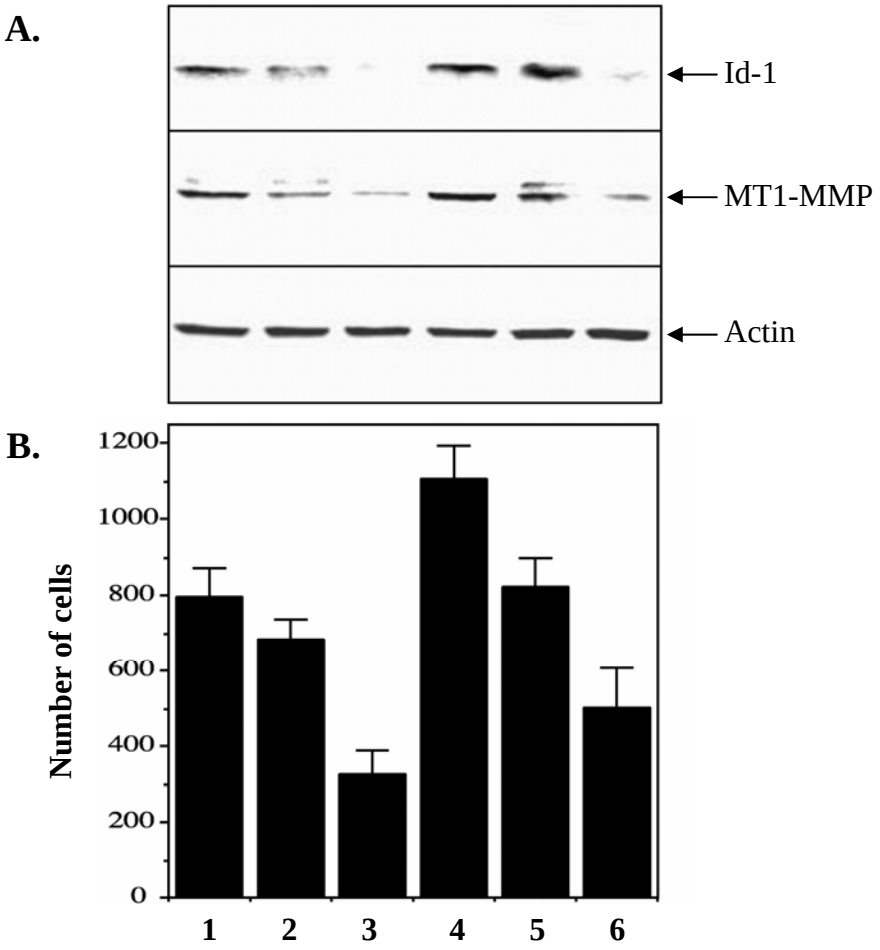


Fig. 14: Targeting Id-1 expression in MDA-MB231 cells down-regulated MT1-MMP expression that was correlated with the invasive ability of the cells.
A, Id-1 as well as MT1-MMP expression in six different subpopulations isolated from the pool populations of MDA-MB231 breast cancer cells infected with pLXSN-Id-1-antisense construct (as described for Fig. 13A). Actin was used as an internal control.
B, Boyden chamber invasion assay comparing the invasive ability of the six different subpopulations of pLXSN-Id-1-antisense-infected MDA-MB231 cells.

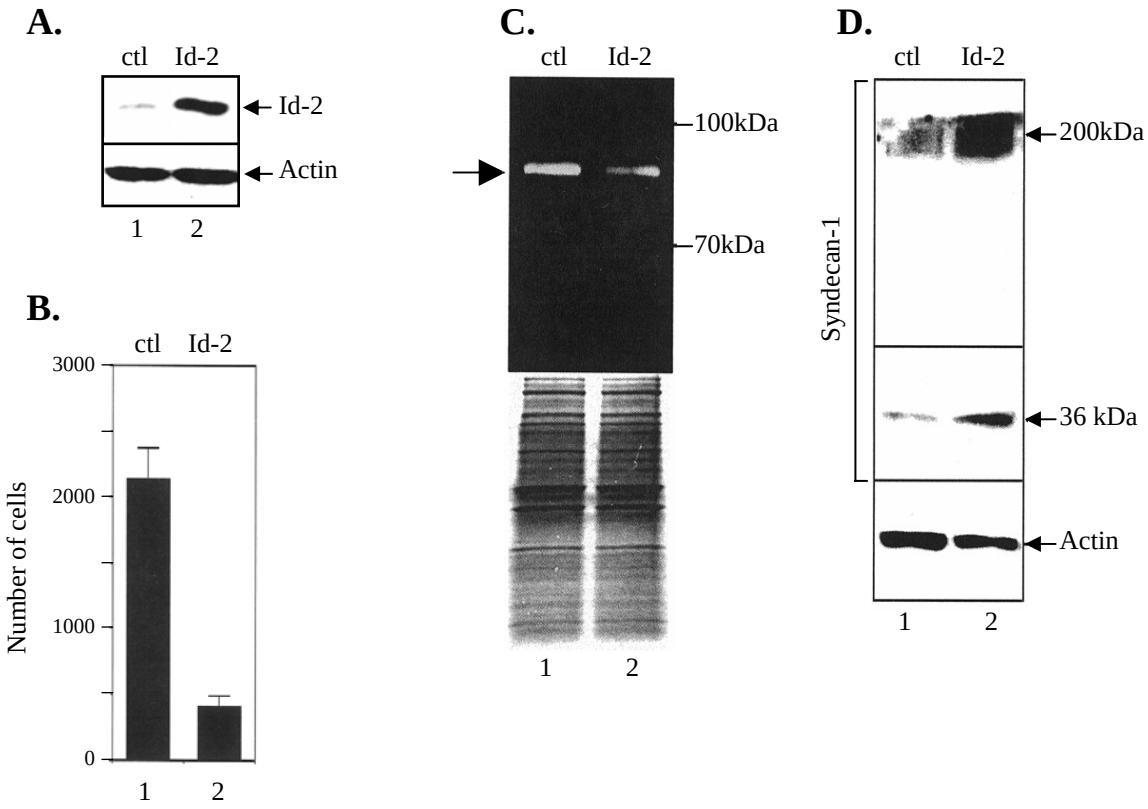


Fig. 15: Id-2 protein reduced the invasive phenotype of human metastatice breast cancer cells.

A, Id-2 protein expression in MDA-MB231 cells infected with pLXSN-control vector (*lane* 1) and infected with Id-2 sense construct (*lane* 2). Actin was used as an internal control.

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 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 (using antibody H-174). The *middle panel* shows the expression of the minor species at 36kDa (using antibody C-20).

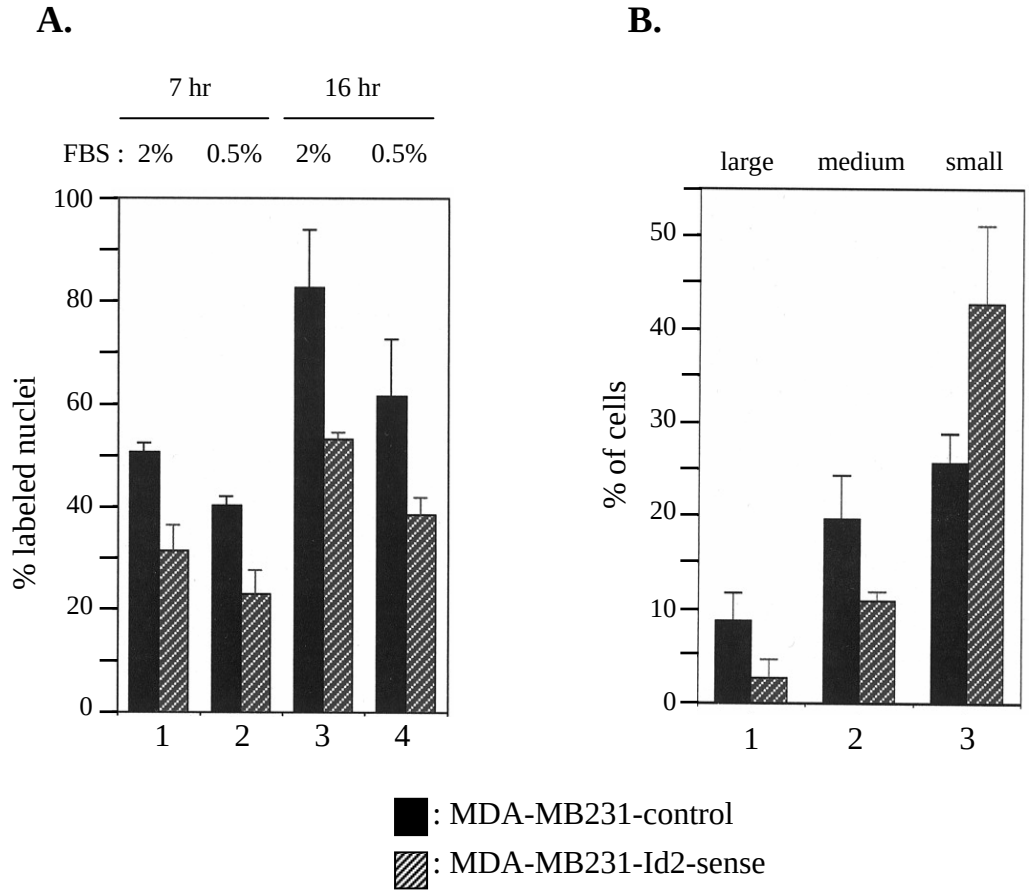


Fig. 16: Id-2 protein reduced the proliferative capacity of human metastatic breast cancer cells.

A, percentage of cells in S phase in MDA-MB231-control (*black bars*) and MDA-MB231-Id2-sense cells (*hatched bars*). Cell 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 hours (*columns* 1 and 2) or 16 hours (*columns* 3 and 4) of incubation.

B, anchorage-independent growth assays comparing MDA-MB231-control (*black bars*) and MDA-MB231-Id2-sense cells (*hatched bars*). Colonies formed were classified as follows: large colonies (>50μm in diameter), *columns* 1; medium-size colonies (0.25-0.5μm in diameter), *columns* 2; and small colonies (<25μm in diameter), *columns* 3.

DCIS

grade III invasive carcinomas

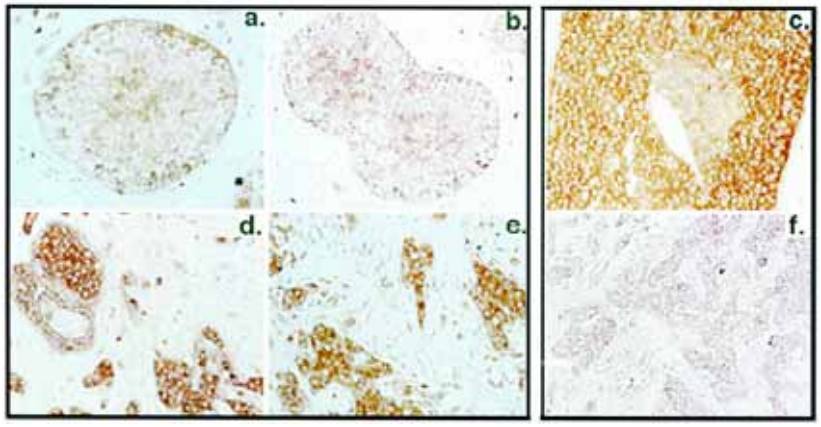
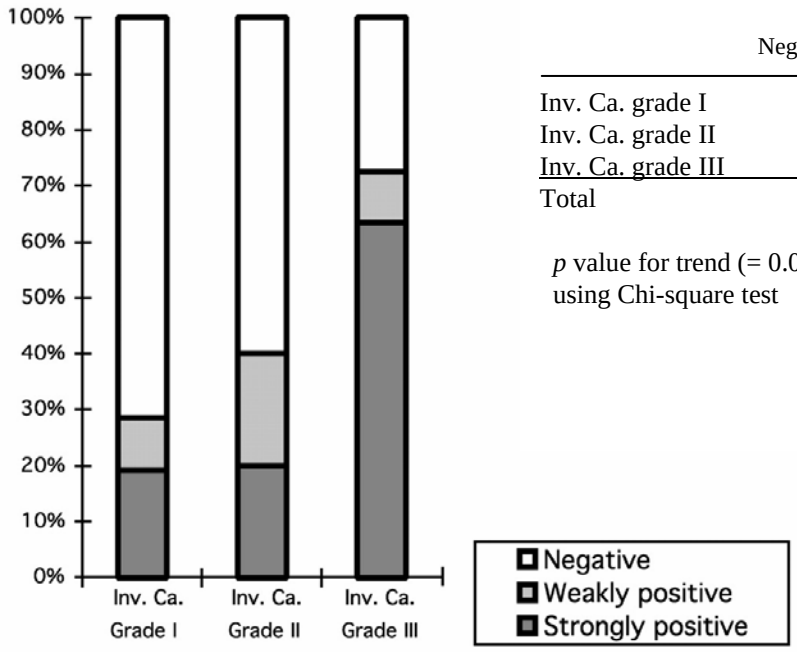


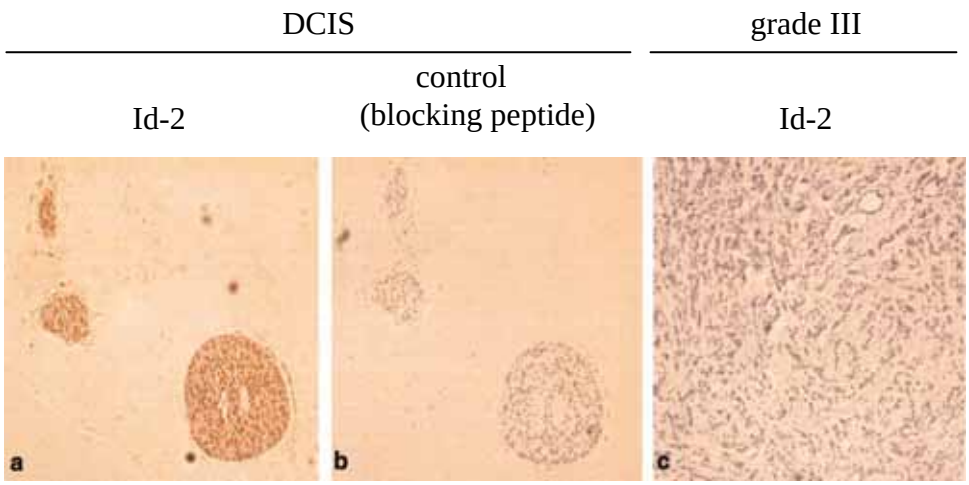
Fig. 17: Id-1 expression in human breast cancer biopsies
Representative sections from DCIS and grade III invasive carcinomas were analyzed by immunohistochemistry using an antiserum directed against Id-1. The majority of DCIS were negative (*panels a* and *b*), whereas only one out of a total 10 showed strong positivity in its large ductal structure (*panel* c). The majority of the infiltrating carcinomas showed strong Id-1 immunoreactivity (*panels* d and e), whereas the minority of the invasive tumors were negative (*panel* f). In *panel* d, a differentiated glandular structure is negative, whereas infiltrating cells show strong immunoreactivity.



	Negative	Weakly positive	Strongly positive	Total
Inv. Ca. grade I	15	2	4	21
Inv. Ca. grade II	9	3	3	15
Inv. Ca. grade III	6	2	14	22
Total	30	7	21	58

p value for trend (= 0.0126) was calculated using Chi-square test

Fig. 18: Id-1 expression in human infiltrating breast cancer biopsies
A total of 58 infiltrating carcinomas were analyzed by immunohistochemistry using an antiserum directed against Id-1. The majority of the grade I invasive carcinomas (Inv. Ca.) showed negative or weakly positive signals for Id-1 (white and light gray, respectively), whereas the majority of the grade III invasive carcinomas showed strongly positive Id-1 immunoreactivity (dark gray).



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

Fig. 19: Id-2 expression in human breast cancer biopsies
Id-2 was 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**). The slides were counterstained with hematoxyline.

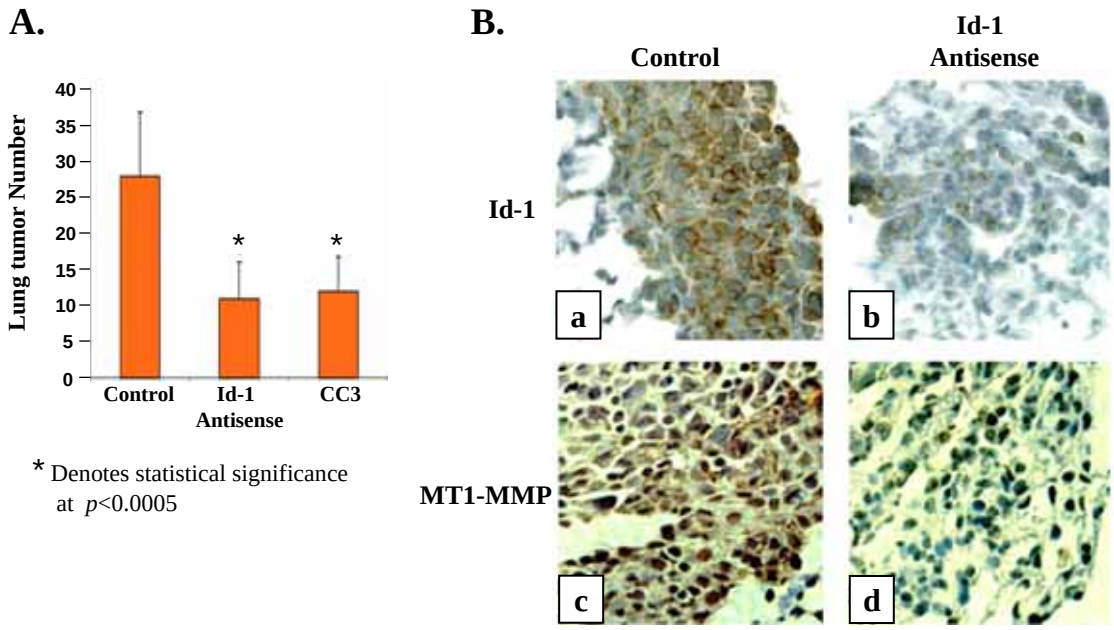


Fig. 20: Systemically targeting Id-1 expression significantly reduced the metastatic spread of 4T1 breast cancer cells in a syngeneic BALB/c mouse models.

A, The 4T1 murine metastatic breast cancer cells, which expressed high levels of Id-1 protein, were first inoculated systemically into BALB/c mice. The tumor-bearing mice were then injected i.v. 3 days after tumor inoculation with various constructs by using CLDC. The graph shows the mean number of lung metastases per mouse treated with the control gene luciferase (18 mice); with Id-1 antisense (17 mice); or with the metastasis suppressor gene CC3 (16 mice) constructs. The graph represents two independent experiments. *, $p<0.0005$ compared with control.

B, Immunohistochemistry analysis of Id-1 levels (a and b) as well as MT1-MMP (c and d) in 4T1 breast tumor cells harvested from the lungs of CLDC-injected mice. The protocol was as in **A**, except the tumor-bearing mice were injected with the corresponding liposome-DNA complexes 2 days before killing. Immunohistochemistry on tumors from CLDC control-treated mice (a and c) and from CLDC-Id-1-antisense-treated mice (b and d) was carried out as described in *Methods*.

Table 2: Expression and function of Id-1 and Id-2 in breast epithelial cells.

Cell type		Function, expression and phenotype	Reference
Non transformed mammary epithelial cells	Id-1	Induction of cell proliferation or apoptosis depending on the cell density Induction of cell migration and invasion Suppression of mammary epithelial cell differentiation Mammary gland development Highly expressed during mammary development in virgin mice, early pregnancy and involution	44, 64 43 42 44
	Id-2	Highly expressed in differentiated mammary epithelial cells in culture Mammary gland development Highly expressed during the second part of pregnancy and lactation Necessary for normal architecture Lactation defects in Id2-/- mice	44 53 44 77 52
Transformed mammary epithelial cells	Id-1	Induction of cell proliferation and invasion Correlation with tumor grade (overexpressed in aggressive breast tumors) Strong unfavorable prognostic marker for node negative breast cancer patients Repression of aggressive and metastatic phenotype by Id-1 targeting	46 46 47 88 47
	Id-2	Inverse correlation with tumor grade (suppressed in aggressive breast tumors) Decrease cell migration and invasion	53 53

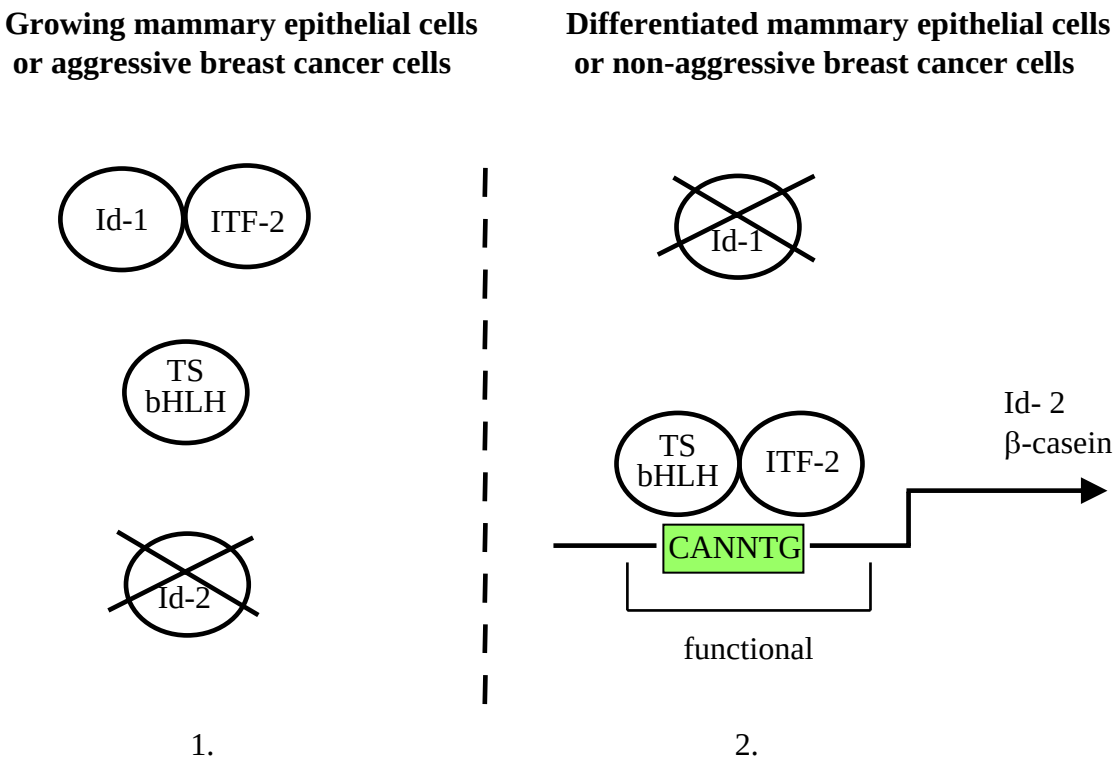


Fig. 21: Model representing the link between Id-1 down-regulation and expression of several target genes in normal mammary epithelial cells as well as breast cancer cells

Id-1 binds to ITF-2, ubiquitous bHLH protein, to form the non functional hetero-dimer (1). The down-regulation of Id-1 would lead to the release of ITF-2. After its release from Id-1, the tissue-specific bHLH (TS bHLH) protein could then dimerize with ITF-2 and consequently activates the transcription of genes involved in the functional and morphological differentiation of mammary epithelial cell, such as Id-2 and β-casein (2).