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Suppression of Mammary Epithelial Cell Differentiation by the Helix-Loop-Helix Protein Id-1

PIERRE-YVES DESPREZ, EIJI HARA,† MINA J. BISSELL, AND JUDITH CAMPISI*

Department of Cancer Biology, Life Sciences Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

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Cell proliferation and differentiation are precisely coordinated during the development and maturation of the mammary gland, and this balance invariably is disrupted during carcinogenesis. Little is known about the cell-specific transcription factors that regulate these processes in the mammary gland. The mouse mammary epithelial cell line SCp2 grows well under standard culture conditions but arrests growth, forms alveolus-like structures, and expresses β-casein, a differentiation marker, 4 to 5 days after exposure to basement membrane and lactogenic hormones (differentiation signals). We show that this differentiation entails a marked decline in the expression of Id-1, a helix-loop-helix (HLH) protein that inactivates basic HLH transcription factors in other cell types. SCp2 cells stably transfected with an Id-1 expression vector grew more rapidly than control cells under standard conditions, but in response to differentiation signals, they arrested growth and formed three-dimensional structures similar to those of control cells. Id-1-expressing cells did not, however, express β-casein. Moreover, 8 to 10 days after receiving differentiation signals, they lost three-dimensional organization, invaded the basement membrane, and then resumed growth. SCp2 cells expressing an Id-1 antisense vector grew more slowly than controls; in response to differentiation signals, they remained stably growth arrested and fully differentiated, as did control cells. We suggest that Id-1 renders cells refractory to differentiation signals and receptive to growth signals by inactivating one or more basic HLH proteins that coordinate growth and differentiation in the mammary epithelium.

Differentiated cells have a wide range of growth potentials, depending on their lineage and state of maturation. Thus, growth and differentiation are not necessarily antithetic. Nonetheless, the loss of growth control that is a hallmark of tumorigenesis invariably entails deranged differentiation (14). How and why this occurs is not well understood, particularly for the epithelial cells that give rise to the majority of mammalian cancers. This is certainly the case for mammary epithelial cells. Mammary epithelial cells undergo coordinate changes in growth and differentiation during embryogenesis, at puberty, and throughout much of adulthood, with striking changes occurring during pregnancy and lactation. They are also a common target for carcinogenesis in adult females. Very little is known about the genes that determine the lineage, differentiated characteristics, and growth potential of mammary epithelial cells.

Cell lineage and differentiation are commonly controlled by the sequential and combinatorial action of transcription factors. In some cells, specific transcription factors control both cell proliferation and differentiation. For example, C/EBP transcription factors mediate adipocyte and hepatocyte differentiation, and some C/EBP family members are potent growth inhibitors (11, 36, 49). Similarly, some basic helix-loop-helix (bHLH) transcription factors (26) regulate both growth and differentiation. bHLH factors include several widely expressed proteins, such as the c-*myc* proto-oncogene family and E proteins, as well as lineage- and cell-type-specific factors, such as the *Drosophila achaetescute* complex proteins and their mammalian homologs. Among the most extensively studied bHLH proteins are the myogenic determining factors, of which MyoD is the prototype. MyoD directly activates the transcription of skeletal-muscle-specific genes (9). It also suppresses cell proliferation (42). Growth inhibition by MyoD may be due to its ability to repress transcription of the c-*fos* proto-oncogene (47) or induce the $p21^{sdi1/cip1/waf1}$ inhibitor of cyclin-dependent protein kinases (16, 34).

bHLH transcription factors act as heterodimers. The HLH motif is the interface for dimerization; dimerization allows the basic regions to form a composite DNA-binding domain which determines the sequence to which the dimer binds (8, 50). bHLH factors also dimerize with another class of proteins termed Id. Id proteins contain HLH motifs but lack basic regions. Because bHLH-Id heterodimers do not form functional DNA-binding domains, Id proteins inhibit the function of bHLH transcription factors. In contrast to bHLH proteins, fewer than half a dozen Id proteins have been identified, and they are all widely expressed (2, 6, 13, 45, 52). Id proteins may be ubiquitous regulators of bHLH function whose effects depend on the complement of bHLH factors expressed by a given cell. Control of bHLH function by Id proteins is best understood for myoblasts. Proliferating myoblasts express Id-1, which binds an E protein that is the primary dimerization partner of MyoD (32). Id expression declines in response to differentiation signals (2, 45), thereby permitting the formation of MyoD-E protein heterodimers. Constitutive expression of Id prevents MyoD-dependent transcription and myogenic differentiation (22).

Using Id-1 as a tool, we obtained the first evidence that one or more bHLH factors may coordinate the growth and differentiation of mammary epithelial cells. We used for our studies a homogeneous murine mammary epithelial cell line, SCp2 (10), which was cloned from a heterogeneous culture derived

* Corresponding author. Mailing address: Life Sciences Division, Department of Cancer Biology, Building 70A-MS1118, Lawrence Berkeley Laboratory, Berkeley, CA 94720. Phone: (510) 486-4416. Fax: (510) 486-4475. Electronic mail address (Internet): JCAMPISI@LBL.GOV.

† Present address: Imperial Cancer Research Fund, Molecular Oncology Laboratory, London WC2A 3PX, United Kingdom.

from mid-pregnancy mammary glands (7, 39). SCp2 cells grow well on tissue culture plastic in serum-containing medium. However, when given an exogenous basement membrane and lactogenic hormones in serum-free medium, they cease proliferation, form alveolus-like structures, and functionally differentiate, as judged by the expression and secretion of milk proteins. SCp2 cells differentiate similarly to epithelial cells in mammary glands during pregnancy or lactation, as well as to cells in primary culture and in heterogeneous parental culture (1, 10, 37, 39). Here, we show that SCp2 differentiation entails a decline in the level of Id-1 expression that is essential for functional differentiation and the stability of the growth arrest associated with differentiation.

MATERIALS AND METHODS

Cell culture. SCp2 cells were grown in Dulbecco's modified Eagle's and Ham's F12 media (DME-F12; 1:1) containing 5% heat-inactivated fetal bovine serum, insulin (5 μg/ml; Sigma, St. Louis, Mo.), and gentamicin (50 μg/ml; Sigma), as previously described (10). The Englebreth Holm Swarm (EHS) tumor was passaged in C57BL mice as described by Kleinman et al. (27), and extracellular matrix (ECM) was isolated from the tumor (EHS-ECM) as described previously (27, 46).

Cells were plated at $5 \times 10^4/cm^2$ in a mixture containing DME-F12, 2% fetal bovine serum, and 5 μg of insulin per ml and allowed to attach for 16 to 20 h. In some experiments, cells were made quiescent by shifting them to serum-free DME-F12 plus insulin for 3 days and then were induced to differentiate by shifting them to serum-free DME-F12 plus the lactogenic hormones insulin, hydrocortisone (1 μg/ml; Sigma), and prolactin (bovine, 3 μg/ml; National Institutes of Health, Bethesda, Md.) and either 1% EHS-ECM or 30 μg of laminin (Sigma) per ml (4). Prior serum deprivation increased the rate and synchrony of differentiation. Alternatively, proliferating cells were washed, suspended in serum-free DME-F12 plus lactogenic hormones, and plated directly atop a layer of EHS-ECM. Unless noted otherwise, cells were assessed for differentiation 5 days after exposure to EHS-ECM and lactogenic hormones.

Transfection. Murine Id-1 cDNA (2, 17) was cloned in either the sense or antisense orientation downstream of the mouse mammary tumor virus (MMTV) promoter in pMSRB (18) from which the insert was removed (MMTV-Id vector). SCp2 cells were transfected with the blasticidin S resistance gene under the control of the simian virus 40 early promoter (pSV2bsr) (21), alone (control) or with a sense or antisense MMTV-Id vector, by calcium phosphate precipitation (15, 39). The cells (50% confluent in 100-mm-diameter dishes) were transfected with 3 μg of pSV2bsr DNA without or with 30 μg of MMTV-Id DNA. Blasticidin S hydrochloride (2 μg/ml; Funakoshi, Tokyo, Japan) was added 48 h after transfection. Two to 3 weeks later, about 500 colonies each of control cells and cells transfected with MMTV-Id sense vector and MMTV-Id antisense vector were pooled and expanded.

Immunoprecipitation. Cells were labeled with [^{35}S]methionine, radiolabeled proteins were immunoprecipitated with a rabbit polyclonal antiserum reactive against murine Id-1 (22), and proteins were analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Hara et al. (19). Briefly, proliferating cells were shifted to 0.5% serum for 36 h and labeled for 5 h with 100 μCi of Tran ^{35}S-label (>1,000 Ci/mmol; ICN) per ml in methionine-free medium 199 containing insulin, prolactin, and hydrocortisone. Cells were lysed, and lysates were sonicated and clarified by centrifugation. Equal amounts of acid-precipitable radioactivity were incubated with Id-1 antiserum, and the immune complexes were collected on protein A-Sepharose, washed, and released by detergent for analysis by SDS-PAGE.

RNA isolation and analysis. Total cellular RNA was purified as described by Chomczynski and Sacchi (5). The RNA (10 μg) was separated by electrophoresis through formaldehyde-agarose gels and transferred to nylon membranes, and the membranes were hybridized to ^{32}P-labeled probes prepared by random oligonucleotide priming, were washed, and were exposed for autoradiography as described by Sambrook et al. (38).

The β-casein probe was 540-bp mouse cDNA (from J. Rosen, Baylor College of Medicine, Houston, Tex.), the Id-1 probe was murine Id-1 cDNA (2, 17), the keratin 18 probe was mouse cDNA (40), and the histone 3 probe was the *Sal*I-*Pvu*II fragment of the murine histone 3.2 cDNA (31).

Immunofluorescence. Cells were cultured on glass coverslips, washed with phosphate-buffered saline (PBS), fixed with acetone-methanol (1:1, vol/vol) for 5 min at −20°C, and washed with PBS. A murine monoclonal anti-rat β-casein immunoglobulin G (1:1,000 dilution) (23) was used to detect β-casein by indirect immunofluorescence, as described previously (10, 44). The coverslips were dipped in 0.5 μg of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) per ml, washed in water, and viewed by epifluorescence to detect DAPI-stained nuclei and β-casein-expressing cells.

DNA synthesis and autoradiography. Cells were labeled with [^{3}H]methylthymidine (10 μCi/ml; 60 to 70 Ci/mmol) for 24 h, washed twice with PBS, and processed for immunofluorescence. After immunostaining, the coverslips were air dried, coated with NTB2 emulsion (1:2 dilution; Kodak), and exposed for 16 to 24 h. The coverslips were developed with D-19, fixed in Rapid-Fix (Kodak), and viewed by phase-contrast microscopy.

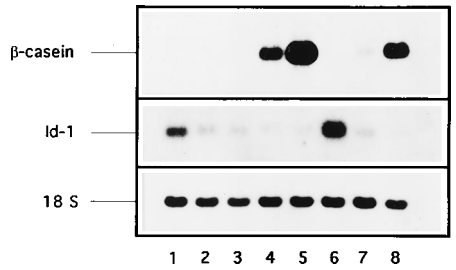

FIG. 1. Reciprocal expression of Id-1 and β-casein mRNA in growing and differentiating mammary epithelial cells. SCp2 cells growing under standard tissue culture conditions were shifted to serum-free medium for 3 days and then to serum-free medium containing lactogenic hormones and either laminin or EHS-ECM, as described in Materials and Methods. RNA was isolated 6, 24, or 48 h later and analyzed on Northern blots for the abundance of β-casein (1.4-kb) and Id-1 (1.2-kb) mRNA. The blots were probed for the abundance of 18 S rRNA to control for RNA integrity, loading, and transfer. Lane 1, growing cells: lane 2, cells deprived of serum for 3 days; lanes 3 through 5, serum-deprived cells given hormones and laminin for 6, 24, and 48 h, respectively; lanes 6 through 8, serum-deprived cells given hormones and EHS-ECM for 6, 24, and 48 h, respectively.

RESULTS

Id-1 expression declines in quiescent and differentiated SCp2 cells. Id-1 mRNA was expressed by exponentially growing SCp2 cells and was evident as a readily detectable 1.2-kb transcript on Northern (RNA) blots of total cellular RNA (Fig. 1, lane 1). When the cells were deprived of serum, the level of Id-1 mRNA decreased markedly within 3 days (Fig. 1, lane 2). Serum deprivation reduced cell proliferation: after 3 days, [^{3}H]thymidine incorporation declined and there was no increase in cell number (data not shown). Thus, Id-1 expression and growth were positively correlated in SCp2 cells, as they are in human fibroblasts (19).

Serum deprivation, and its attendant decline in proliferation, did not induce differentiation. Thus, serum-deprived SCp2 cells did not form three-dimensional structures (data not shown) or express β-casein, a marker of functional differentiation (Fig. 1, lane 2). Serum deprivation did, however, permit a more rapid and synchronous response to differentiation signals (9a).

The signals for SCp2 cell differentiation are lactogenic hormones (insulin, hydrocortisone, and prolactin) and a basement membrane (commonly laminin or the EHS tumor-derived matrix EHS-ECM) in serum-free medium (10). Growth factors antagonize the differentiation response. To assess Id-1 expression during differentiation, SCp2 cells were serum deprived and then given lactogenic hormones and laminin. The cells were analyzed for Id-1 and β-casein mRNA 6, 24, and 48 h thereafter (Fig. 1, lanes 3 through 5).

Id-1 mRNA remained virtually undetectable throughout the experiment. β-Casein mRNA was undetectable 6 h after cells received laminin and lactogenic hormones but was readily detectable within 24 h and was highly expressed by 48 h. The level of 18S rRNA did not change during the experiment and thus served to control for RNA integrity, loading, and transfer.

EHS-ECM contains about 90% laminin (3, 28) and is less costly than laminin. Although EHS-ECM is contaminated with a variety of proteins, including tumor-derived growth factors (51), it has been used extensively by us and others because it

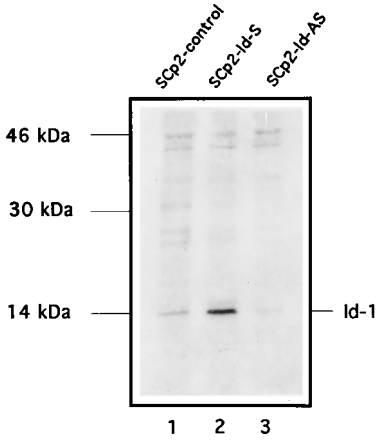

FIG. 2. Id-1 protein expression in SCp2 cells transfected with control, Id-1 sense, and Id-1 antisense vectors. Control, SCp2-Id-S, and SCp2-Id-AS cells were metabolically labeled and lysed, and the lysates were immunoprecipitated with Id-1 antiserum and analyzed by SDS-PAGE, as described in Materials and Methods.

induces both morphological and functional differentiation (3, 27, 30). In parallel with the above-described experiment, serum-deprived SCp2 cells were given lactogenic hormones and EHS-ECM. In contrast to its response to laminin, Id-1 mRNA was induced 6 h after exposure to EHS-ECM (Fig. 1, lane 6). This induction was transient, and the amount of Id-1 mRNA declined to nearly undetectable levels within 24 h (Fig. 1, lane 7). Unlike laminin, EHS-ECM did not induce β-casein mRNA until 48 h after it was added (Fig. 1, lane 8).

The transient induction of Id-1 mRNA by EHS-ECM very likely is due to contaminating growth factors (51). This idea is supported by our preliminary finding that either epidermal growth factor or transforming growth factor α and EHS-ECM induced Id-1 mRNA to similar extents in serum-deprived SCp2 cells, independently of lactogenic hormones (data not shown). We speculate that the transient induction of Id-1 by EHS-ECM may delay or transiently suppress functional differentiation.

Ectopic Id-1 expression suppresses differentiation without stimulating growth. To directly examine the role of Id-1 in mammary epithelial cell growth and differentiation, we transfected SCp2 cells with murine Id-1 cDNA under the control of the MMTV promoter. In our study, this promoter was active whether cells were on plastic or EHS-ECM and showed only a modest glucocorticoid response. Unless noted otherwise, all experiments were done in the presence of the glucocorticoid hydrocortisone (43). One of two vectors was transfected into cells. In one vector, the cDNA was transcribed in the sense orientation. In the other, transcription was in the antisense orientation. These vectors were cotransfected with pSV2bsr, a vector conferring resistance to the antibiotic blasticidin S. pSV2bsr transfected alone served as an additional control. Stable blasticidin-resistant colonies were selected and pooled.

Cells transfected with the Id-1 cDNA in the sense orientation (SCp2-Id-S cells) expressed the 14-kDa Id-1 protein at a severalfold-higher level than did cells transfected with pSV2bsr alone (control), as judged by immunoprecipitation of radiolabeled cell lysates (Fig. 2, lanes 1 and 2). By contrast, cells transfected with the Id-1 cDNA in the antisense orientation (SCp2-Id-AS cells) expressed Id-1 protein at a severalfold-lower level than did controls (Fig. 2, lane 3). Thus, the Id-1 sense and antisense cDNAs increased and decreased, respectively, the level of Id-1 protein in transfected SCp2 cells.

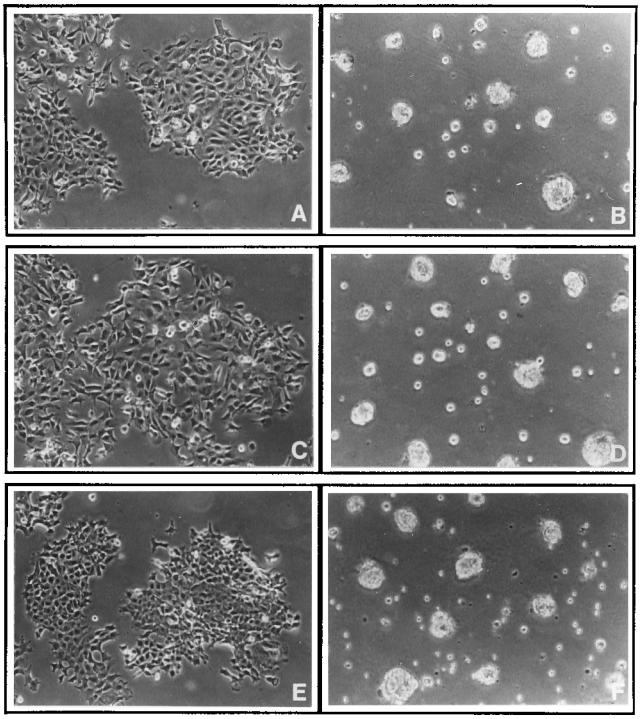

FIG. 3. Morphology of SCp2 cells transfected with control, Id-1 sense, and Id-1 antisense vectors under growing and differentiation-inducing conditions. Control, SCp2-Id-S, and SCp2-Id-AS cells were plated on tissue culture plastic in serum-containing medium and were photographed while proliferating exponentially (A, C, and E). Parallel cultures of proliferating control, SCp2-Id-S, and SCp2-Id-AS cells were plated atop a layer of EHS-ECM in serum-free medium plus lactogenic hormones and were photographed 5 days later (B, D, and F). (A and B) Control cells; (C and D) SCp2-Id-S cells; (E and F) SCp2-Id-AS cells. Magnification, ×52.

Control, SCp2-Id-S, and SCp2-Id-AS cells differed somewhat in their doubling times during exponential growth. Control cells, like untransfected SCp2 cells (10), doubled every 15 to 16 h. SCp2-Id-S cells grew more rapidly, doubling every 12 to 13 h, whereas SCp2-Id-AS cells grew more slowly, doubling every 20 h. There were also morphological differences. When grown on tissue culture plastic, SCp2-Id-AS cells formed colonies that were more compact than those of control cells, although both types of cells had a typically epithelial cuboidal morphology (Fig. 3A and E). SCp2-Id-S cells, by contrast, grew as more disperse colonies and were less cuboidal (Fig. 3C). Finally, the growth of control and SCp2-Id-AS cells declined markedly within 3 days after serum deprivation, whereas serum-deprived SCp2-Id-S cells grew exponentially for 4 to 5 days until they reached confluence (data not shown).

Despite these differences, the initial growth and morphological response to differentiation signals appeared to be similar for control, SCp2-Id-S, and SCp2-Id-AS cells. Within 5 days after being plated on EHS-ECM in lactogenic hormones, all three cell types ceased proliferating. Thus, although the ectopic expression of Id-1 prevented growth arrest due to serum deprivation, it had no effect on the growth arrest induced by differentiation signals. Moreover, all three cell types were capable of forming three-dimensional structures (Fig. 3B, D, and F). These structures appeared to be similar at low magnification, although closer examination showed differences among them (see below).

RNA was isolated 5 days after the transfected cells were given differentiation signals and analyzed for β-casein, keratin

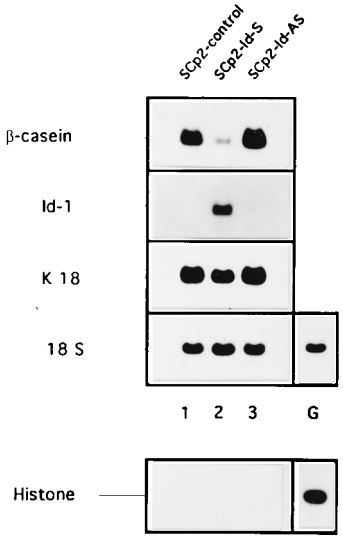

FIG. 4. Expression of β-casein, Id-1, keratin 18 (K 18), and histone 3 mRNA in differentiated SCp2 cells transfected with control, Id-1 sense, and Id-1 antisense vectors. Control, SCp2-Id-S, and SCp2-Id-AS cells were plated atop a layer of EHS-ECM in the presence of lactogenic hormones; 5 days later, RNA was isolated and analyzed for the abundance of β-casein, Id-1, keratin 18, and histone 3 mRNA, as described in Materials and Methods. Hybridization with an 18S rRNA probe served to control for RNA integrity, loading, and transfer. Lane G, RNA from proliferating SCp2 cells. RNA was analyzed for histone 3 mRNA as a positive control for hybridization of the histone probe.

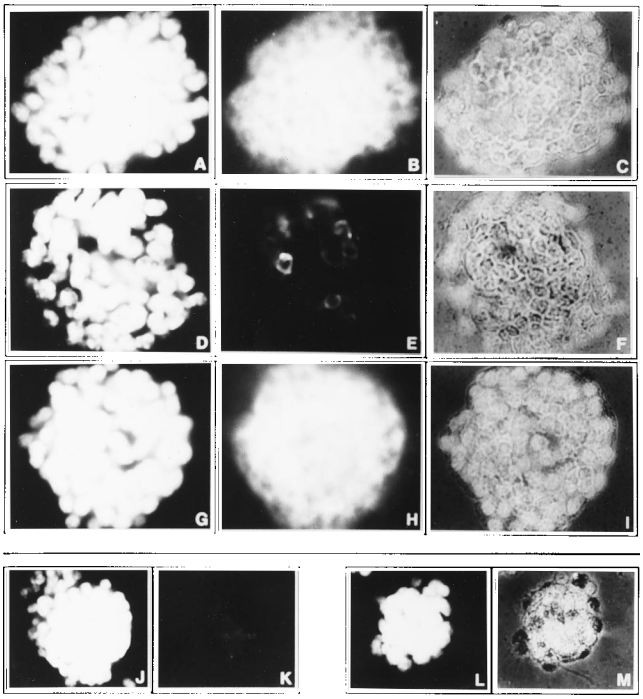

FIG. 5. Single-cell analysis of β-casein expression and DNA synthesis in SCp2 cells transfected with control, Id-1 sense, and Id-1 antisense vectors. Control, SCp2-Id-S, and SCp2-Id-AS cells were serum deprived and provided with differentiation signals as described in the legend to Fig. 3; 4 days later, they were labeled with [^{3}H]thymidine for 24 h and then fixed and stained with DAPI and processed for immunofluorescence by using a β-casein antibody and autoradiography, as described in Materials and Methods. Shown are DAPI fluorescence (A, D, G, J, and L), β-casein immunofluorescence (B, E, and H), control antibody immunofluorescence (K), and autoradiography (C, F, I, and M). (A to C) Control cells; (D to F) SCp2-Id-S cells; (G to I) SCp2-Id-AS cells; (J and K) control cells; (L and M) differentiated SCp2 cells treated with transforming growth factor α for 24 h and serving as a positive control for DNA synthesis in three-dimensional alveoli. Note that the SCp2-Id-S cells in panel F are negative for [^{3}H]thymidine incorporation (compare with the positive control in panel M), which is consistent with the lack of histone 3 expression (Fig. 4 and 6A and B). The dark areas in panel F are due to background grains and discontinuities in the cell aggregate, which are not seen in aggregates of control or SCp2-Id-AS cells. Magnifications, ×220 for panels A to I and ×138 for panels J to M.

18, and histone 3 mRNA (Fig. 4). These transcripts served as indicators of functional differentiation, the epithelial character of the cells, and cell proliferation, respectively.

As expected, control cells (Fig. 4, lane 1) expressed keratin 18 mRNA and high levels of β-casein mRNA, whereas Id-1 and histone 3 mRNA were undetectable. SCp2-Id-AS cells (lane 3) also expressed keratin 18 and even higher levels of β-casein, and Id-1 and histone 3 mRNA were undetectable in these cells as well. The inability of the double-stranded probe to detect an antisense transcript in SCp2-Id-AS cells suggests that the Id-1 antisense RNA may be very labile, as reported for other antisense RNAs (20). Despite this lability, the antisense Id-1 RNA reduced the level of Id-1 protein (Fig. 2), which was also expected from findings in other systems (20). In either case, control and SCp2-Id-AS cells behaved similarly. They arrested growth and thus did not express histone 3, and they functionally differentiated, as judged by abundant β-casein expression.

By contrast, SCp2-Id-S cells (Fig. 4, lane 2) expressed barely detectable levels of β-casein. This was not due to a loss of epithelial characteristics, because SCp2-Id-S cells expressed keratin 18 and formed three-dimensional structures in response to differentiation signals (Fig. 3D). Likewise, the failure to express β-casein was not due to growth stimulation by the Id-1 transgene, as judged by the lack of histone 3 mRNA (Fig. 4) and [^{3}H]thymidine incorporation (Fig. 5F). SCp2-Id-S cells expressed the Id-1 transgene mRNA (1.4 kb) throughout the experiment, but the endogenous Id-1 mRNA (1.2 kb) was not expressed under these conditions (Fig. 4). When proliferating on plastic, SCp2-Id-S cells expressed both the endogenous and transfected Id-1 genes, which were distinguishable on Northern blots (data not shown).

To confirm that Id-1 suppresses functional differentiation without stimulating growth, control, SCp2-Id-S, and SCp2-Id-AS cells were treated for 5 days with EHS-ECM and lactogenic hormones and analyzed at the single-cell level for β-casein and DNA synthesis (Fig. 5). In alveoli formed by control or SCp2-Id-AS cells, nearly all cells expressed β-casein, which was detectable by a monoclonal anti-β-casein antibody (Fig. 5B and H). Moreover, when [^{3}H]thymidine was added 24 h before fixation, none of the cells in these alveoli incorporated radiolabel, which was detectable by autoradiography (Fig. 5C and I). As a positive control, differentiated SCp2 cells were stimulated to initiate DNA synthesis by transforming growth factor α (Fig. 5M) (30a). SCp2-Id-S cells also failed to incorporate [^{3}H]thymidine (Fig. 5F), which is consistent with the lack of histone 3 expression. However, very few β-casein-positive SCp2-Id-S cells were evident (Fig. 5D and E).

Taken together, the results indicate that mammary epithelial cells that ectopically express Id-1 remain capable of arresting growth and forming three-dimensional structures in response to ECM, at least within an initial 5-day period, but fail to undergo complete functional differentiation.

Growth arrest and morphological differentiation are unstable in Id-1-expressing cells. When examined at high magnification, it was apparent that the three-dimensional structures formed by SCp2-Id-S cells differed from those formed by con-

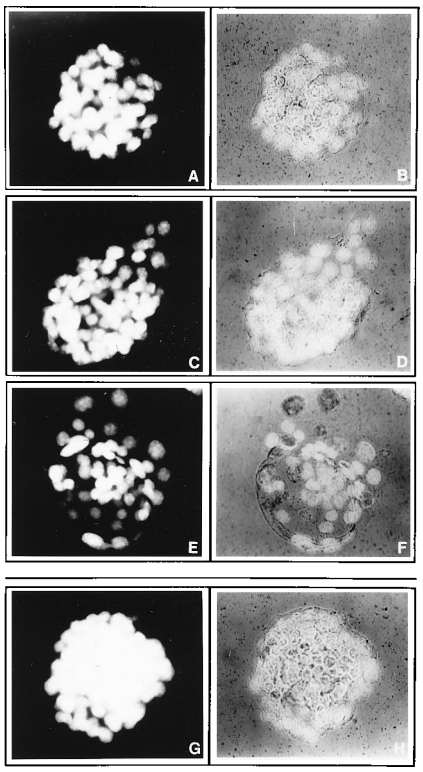

FIG. 6. Instability of three-dimensional structures and growth arrest. SCp2-Id-S cells and control cells were serum deprived and provided with differentiation signals as described in the legend to Fig. 3; the cells were labeled with [^{3}H]thymidine for 24 h preceding the 5-, 8-, and 10-day time points after exposure to lactogenic hormones and EHS-ECM. The cells were then fixed and stained with DAPI and processed for autoradiography, as described in Materials and Methods. DAPI fluorescence is shown in panels A, C, E, and G; autoradiography is shown in panels B, D, F, and H. (A through F) SCp2-Id-S cells after 5 (A and B), 8 (C and D), and 10 (E and F) days of exposure to lactogenic hormones and EHS-ECM. (G and H) Control cells after 10 days of exposure to lactogenic hormones and EHS-ECM. With some batches of EHS-ECM, disintegration of three-dimensional structures and resumption of DNA synthesis occurred 1 to 2 days earlier or later than the times shown here. Magnification, ×170.

trol or SCp2-Id-AS cells (Fig. 5D). In particular, SCp2-Id-S cells formed structures that were less compact, with weaker cell-cell interactions.

More striking was the effect of ectopic Id-1 expression on the stability of the response to differentiation signals. Alveoli formed by control or SCp2-Id-AS cells were stable. These structures persisted for more than 2 weeks in culture, as long as they were maintained in EHS-ECM and lactogenic hormones. During this time, the cells remained growth arrested (Fig. 6G and H) and continued to express β-casein (data not shown). By contrast, the three-dimensional structures formed by SCp2-Id-S cells were unstable. Between 8 and 10 days after exposure to EHS-ECM and lactogenic hormones, the aggregates became increasingly disorganized. At this time, cells at the periphery began to break from the aggregate and invade the surrounding matrix (Fig. 6C and D). This disaggregation and invasion occurred in the absence of cell proliferation. Ten to 12 days after receiving differentiation signals, many of the SCp2-Id-S-cell aggregates had disintegrated. At this time, many of the invading cells initiated DNA synthesis (Fig. 6E and F).

We conclude that Id-1 prevents the functional differentiation of mammary epithelial cells, destabilizing growth arrest and morphogenesis. Because the only known function of Id proteins is to inactivate bHLH factors, we suggest that growth and differentiation in mammary epithelial cells are controlled by one or more bHLH proteins.

DISCUSSION

In several models of terminal differentiation or tumorigenesis, strong growth stimuli, such as activated oncogenes or mitogens, often completely block differentiation by stimulating unregulated growth. In vivo, however, tumor cells display a wide range of growth and differentiation properties. In the early stages of tumorigenesis especially, the balance between growth and differentiation may be only subtly perturbed (14). Thus, in order to understand tumorigenesis, we must understand how growth and differentiation are balanced in normal cells.

We developed an epithelial cell line for studying growth and differentiation in the mammary gland. SCp2 cells were cloned from a mammary gland-derived mixed-cell population containing functional epithelial cells (10, 39). SCp2 cells respond to hormones and the basement membrane protein laminin very similarly to the parent cells and the epithelial cells of glands during pregnancy or lactation. This response entails an arrest of cell growth, morphogenesis, and tissue-specific gene expression (1, 35). Here, we identify Id-1 as a growth-regulated gene in SCp2 cells that appears to promote growth indirectly by partly suppressing differentiation.

Id-1 encodes a protein that, like other Id family members, negatively regulates bHLH transcription factors (2, 45). Id genes are expressed by many cell types, generally in association with growth (6, 13, 25, 48), and appear to regulate growth and differentiation. For example, Id-1 and Id-2 are induced by mitogens in quiescent fibroblasts, and this induction is essential for the onset of DNA replication (19). In addition, Id expression declines with replicative senescence (19) and with the differentiation of osteogenic cells (24, 33), B cells (53), myoblasts (2, 9), hepatocytes (29a), and neuroblasts (12). In myoblasts and myeloid precursors, constitutive Id-1 expression stimulates growth and blocks terminal differentiation (22, 29).

Id-1 is expressed in proliferating epithelial tissues in early mouse embryos (13, 52), but little is known about Id expression in differentiated epithelial cells. We found that growing SCp2 cells expressed Id-1 but that quiescent and differentiated cells did not. This expression pattern parallels that in the mammary gland: Id-1 was expressed in glands from virgin animals and in involuting glands but not in glands during pregnancy or lactation (45a). The transient induction of Id-1 by EHS-ECM (which is contaminated with growth factors [51]) is consistent with the findings that Id-1 and Id-2 are growth factor inducible in other cells (6, 19, 25) and that epidermal growth factor and transforming growth factor α induce Id-1 in serum-deprived SCp2 cells (30b).

To assess the function of Id-1 in mammary epithelial cells, we transfected SCp2 cells with vectors expressing either a sense or an antisense Id-1 cDNA. The MMTV promoter maintained high, near-constitutive levels of expression whether cells were growing or differentiated. The transfected cells—control, SCp2-Id-S, and SCp2-Id-AS—were pools of several hundred clones, and thus their response was not a peculiarity of an individual clone. (However, we recently isolated clones that behave much like the starting cells.) SCp2-Id-S and SCp2-Id-AS cells expressed higher and lower levels, respectively, of Id-1 protein.

The effect of Id-1 in mammary epithelial cells was novel. Higher- or lower-than-normal levels of Id-1 had a small stimulatory or inhibitory effect, respectively, on the growth rate of SCp2 cells. In addition, constitutive Id-1 expression prevented

the cells from arresting growth when deprived of serum. However, Id-1 had no effect on the ability of cells to arrest growth or undergo initial morphogenesis in response to differentiation signals. This is in contrast to results with myoblasts and lymphoid cells, in which Id-1 abolishes the growth arrest associated with differentiation (22, 29). Thus, mammary epithelial cells and the signals that induce their differentiation are, so far, unique in their ability to override growth stimulation by Id-1. Despite the initial growth arrest and morphologic response, Id-1 did prevent complete functional differentiation, as judged by the tissue-specific marker β-casein. This is the first example of an Id gene perturbing the balance between growth and differentiation in epithelial cells.

The blocking of functional differentiation caused by Id-1 was not due to unregulated growth and is unlikely a result of the direct repression of β-casein. SCp2-Id-S cells displayed other phenotypic correlates of aberrant differentiation, including weakened cell-cell interactions and ability to migrate through the EHS-ECM. It was noteworthy that migration was evident before the cells resumed DNA synthesis. It appeared, then, that SCp2-Id-S cells remained growth arrested until they left the aggregate and began to migrate through the basement membrane.

Id-1 may act to maintain growth potential, rather than directly stimulate growth, in mammary epithelial cells, which is analogous to the function proposed for basonuclin in basal keratinocytes (48). Towards this end, Id-1 may maintain the expression of growth-stimulatory genes or repression of growth-inhibitory genes. These genes, in turn, may prime, but be insufficient to stimulate, cell proliferation. The recent findings that cyclin D antagonizes MyoD activity (41) and that MyoD inhibits growth in part by inducing $p21^{sdi1/cip1/waf1}$ (16, 34) offer cyclin D and p21 as potential indirect targets of Id-1. We do not yet know how the other phenotypic consequences of Id-1 expression—weakened cell-cell interactions and ability to migrate through the basement membrane—relate to the instability of the growth arrest.

The only known function of Id proteins is to inactivate bHLH transcription factors. We suggest, therefore, that one or more bHLH proteins may regulate functional differentiation in mammary epithelial cells at a step that maintains growth arrest and permits milk protein expression. We are currently attempting to identify proteins that interact with Id-1 in SCp2 cells to better understand the mechanism(s) by which Id-1 overexpression leads to disrupted differentiation independent of a direct effect on growth.

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