American Journal of Pathology, Vol. 163, No. 6, December 2003

Id4 Regulates Mammary Epithelial Cell Growth and Differentiation and Is Overexpressed in Rat Mammary Gland Carcinomas

Liang Shan, Minshu Yu, Cunping Qiu, and Elizabeth G. Snyderwine

From the Chemical Carcinogenesis Section, Laboratory of Experimental Carcinogenesis, National Cancer Institute Center for Cancer Research, Bethesda, Maryland

Id4 belongs to a family of helix-loop-helix (HLH) proteins that impact cellular growth and differentiation via regulation of basic HLH transcription factors. Herein the rat *Id4* gene was cloned (GenBank Accession No. AF468681). The expression of rat *Id4* was examined in rat mammary gland tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a carcinogen found in the human diet. By real-time polymerase chain reaction analysis, relative expression of *Id4* mRNA in carcinomas, adenomas, and normal tissue was 27, 6, and 1, respectively. Immunohistochemical analysis indicated statistically elevated nuclear expression for Id4 protein in carcinomas in comparison to adenomas and normal mammary gland. In carcinomas, Id4 nuclear expression was positively correlated with proliferation, invasiveness, and tumor weight (Fisher Exact Test or Spearman Correlation, $P < 0.05$). The consequence of enforced expression of *Id4* on mammary epithelial cell proliferation, differentiation, and growth in soft agar was examined in HC11 cells, a well-characterized model for studying various aspects of mammary epithelial cell biology. After transient and stable transfection of HC11 cells, *Id4* overexpression increased cell proliferation and inhibited lactogenic hormone-mediated differentiation as revealed by inhibition of β-casein promoter activity and β-casein expression. In addition, enforced expression of *Id4* in HC11 cells induced a statistically significant increase in colony growth in soft agar. The results implicate *Id4* in rat mammary gland carcinogenesis and suggest that *Id4* may contribute to carcinogenesis by inhibiting mammary epithelial cell differentiation and stimulating mammary epithelial cell growth. *(Am J Pathol 2003, 163:2495–2502)*

Basic helix-loop-helix (bHLH) transcription factors play an important role in cellular development and differentiation in virtually all eukaryotic organisms.[1] The Id proteins ("inhibitors of differentiation or DNA binding") were identified as helix-loop-helix proteins lacking a basic domain that antagonize the action of bHLH transcription factors by forming DNA binding incompetent heterodimers.[1,2] To date, four Id family members (Id1–4) have been identified in mammals[1,2]. Id proteins coordinate many cellular functions including cell growth, differentiation, cell cycle progression, and invasiveness.[2–6] The mechanism of action of Id proteins is largely regarded to be via dominant-negative regulation of bHLH transcription factors.[2]

Biochemical and genetic data suggest that Id proteins are in general positive regulators of cell proliferation and negative regulators of differentiation.[1–6] Id expression is regulated in a cell cycle-dependent manner and is necessary for G1-S transition.[1,7] In addition to binding to bHLH transcription factors, certain *Id* genes bind to other classes of transcription factors and cell cycle regulators.[1] One member of the Id family, Id2 has been shown to directly interact with the retinoblastoma tumor suppressor protein reversing the growth arrest and cell cycle block elicited by this protein.[8]

Of the four identified Id proteins, the least is known about Id4. Mouse and human *Id4* were cloned in the mid 1990s.[9–11] In adult mice, the expression of *Id4* was shown to be distinct from *Id1–3*.[9,12,13] *Id4* has been implicated in the regulation of differentiation of several tissue types including adipocytes, oligodendrocytes, astrocytes, and spermatogonia.[13–16] In addition, one previous study has shown elevated nuclear expression of Id4 during carcinogenesis specifically in seminomas, a malignant counterpart of early spermatogonia.[13]

Id genes are regarded as prime candidates for oncogenesis.[1,2,4] Indeed, deregulated expression of *Id* mRNA and protein has been observed in various human malignancies, including breast cancer.[13,17–23] Both *Id1* and *Id2* have been shown to regulate mammary epithelial cell proliferation, differentiation, and apoptosis.[24–27] To date, *Id1* has been implicated in the aggressive phenotype of human breast cancer cells and is more frequently expressed in infiltrating carcinomas than ductal carcinomas *in situ*.[21] Whether *Id4* plays a role in human breast cancer

Accepted for publication September 10, 2003.

Address reprint requests to Elizabeth G. Snyderwine, Chemical Carcinogenesis Section, Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, Building 37, Room 4146, 37 Convent Drive, Bethesda, MD 20892-4262. E-mail elizabeth_snyderwine@nih.gov.

is not yet known. However, *Id4* has been recently shown to regulate the expression of *Brca1*, a gene linked to human breast cancer susceptibility.[28] *Brca1* also appears to regulate the expression of *Id4*, and it has been speculated that a *Brca1-Id4* regulatory loop may be disrupted in human breast cancer.[29] The co-regulation of *Id4* and *Brca1* raises the intriguing possibility that *Id4* influences mammary gland carcinogenesis.

In previous studies using cDNA microarray analysis, we examined the expression profiles in rat mammary gland cancers induced by the experimental carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) and the cooked meat-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP).[30] An expressed sequence tag (EST) clone (RGIDV72) was highly overexpressed in both PhIP and DMBA-induced rat mammary gland carcinomas with nine of nine tumors showing at least fivefold higher mRNA expression than normal mammary gland. As described herein, this EST was cloned and identified as the rat *Id4* gene (GenBank Accession No. AF468681). Rat *Id4* was highly overexpressed in rat mammary gland carcinomas and its expression was correlated with tumor malignancy. *Id4* was further shown to regulate the growth and differentiation of mammary gland epithelial cells in culture. The findings implicate *Id4* in rat mammary gland carcinogenesis and suggest that *Id4* overexpression may impact carcinogenesis by inhibiting mammary epithelial cell differentiation and augmenting cell growth.

Materials and Methods

Rat Mammary Gland Carcinomas and Normal Tissue

Archival Sprague-Dawley rat mammary gland tumors (tubulopapillary carcinomas and adenomas) induced previously with PhIP[30–32] were used in this study. Normal mammary gland tissue was collected from mature virgin (150 days old), mid-pregnant (10 to 15 days), and lactating (with 10- to 12-day old pups) Sprague-Dawley rats; in addition, the glands from Sprague-Dawley rats were collected on day 6 of involution. Tumor histopathology and microscopic invasion were assessed as described previously.[32]

Cloning of the Rat Id4 Gene

Cloning of rat *Id4* was carried out using a PCR-cloning strategy. Total RNA and high molecular weight DNA were isolated from normal mammary glands using TRIzol extraction reagent (Invitrogen, Carlsbad, CA) and phenol/chloroform method, respectively. First-strand cDNA was synthesized using SuperScript First-Strand Synthesis Kit (Invitrogen). To obtain the exon sequence, six pairs of overlapping primers were designed from rat ESTs which showed high homology (≥ 90%) to mouse *Id4* by BLAST search as follows: F1 5′-acagcgatccaccttagtc-3′ and R1 5′-acaagcggtagagcgagct-3′; F2 5′-tcgatttctggagcttggaac-3′ and R2 5′-gagtgtagcagtcgttca-3′; F3 5′-tgtgcctgcagtgcgatatg-3′ and R3 5′-agctcagcggcagagaatg-3′; F4 5′-ttacggcgctcaacactgac-3′ and R4 5′-acggtgaatgcttgtgaactg-3′; F5 5′-acgttgtctcggcttgtcattc-3′ and R5 5′-tctgagcctcagatactagatac-3′; F6 5′-agtgagtgacatttcataccctg-3′ and R6 5′-aagacagcagttctgtagactc-3′. To amplify the intron sequences, the above F4 and R3 were used for intron 1 and the following one pair of primers was designed for intron II based on the exon sequence: iF 5′-acagcattctctgccgctga-3′ and iR 5′-gaatgacaagccgagacaac-3′. PCR fragments were cloned into the pCR 2.1 vector using the TA Cloning Kit (Invitrogen). Plasmid DNA was isolated using QIAprep Spin Miniprep Kit or QIAGEN Plasmid Maxi Kit (QIAGEN, Valencia, CA). Sequencing was performed using ABI PRISM Big Dye Terminator Sequencing Kit in an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Immunohistochemistry

Paraffin-embedded sections from the tumors and normal mammary glands were immunohistochemically stained using LSAB2 system (DAKO, Carpinteria, CA). The optimal dilutions for antibodies against Id4 (Santa Cruz Biotechnology, Santa Cruz, CA) and proliferating cell nuclear antigen (PCNA) (DAKO) were 1:50 and 1:100, respectively. Omission of the primary antibody was used as a negative control. All slides were examined and scored in a blinded fashion without knowledge of the sample at the time of assessment. Nuclear immunoreactivity was expressed as the percentage of nuclear-stained positive cells by evaluating 5 to 10 randomly selected high-power fields (×400) (2000 to 5000 total cells counted).

Real-Time Quantitative PCR Analysis

Real-time PCR analysis was carried out using the TaqMan7 Gold RT-PCR Kit on the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) as described in detail previously.[33] Briefly, the primers and probes were designed using Perkin Elmer Primer Express7 software as forward primer, 5′-aggctcgtgcccaccat-3′; reverse primer, 5′-cgataacgtgctgcaggatct-3′; and probe, CCGCCCAACAAGAAAGTCAGCAAAGTG. The threshold cycles (C_T) were recorded for Id4 and GAPDH. GAPDH was used for normalization. Relative gene expression (calculated as $2^{-\Delta\,\Delta CT}$) was derived by the method outlined in ABI Prism Sequence Detection System User Bulletin #2. For statistical analysis, comparison was done between $\Delta\ C_T$ of the tumors and control tissue. The range for the relative expression was derived from the SE of the ΔC_T values and calculated as the exponential function of $-\Delta\ \Delta\ C_T \pm$ SE.

Cell Culture, Luciferase Assay of β-Casein Gene Promoter Activity, and Semi-Quantitative RT-PCR

HC11 mammary epithelial cell line (a subclone of COMMA-1D cells derived from mid-pregnant mice) and HC11-

Lux cells (HC11 cells stably transfected with a β-casein promoter luciferase construct (p-344/-1βc-Lux)) were kindly provided by David S. Salomon (National Cancer Institute, Bethesda, MD) and Nancy E. Hynes (Friedrich Miescher Institute, Basel, Switzerland).[34,35] Both cell lines were routinely maintained in growth medium which consisted of RPMI 1640 (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 5 μg/ml bovine insulin (Invitrogen), 10 ng/ml epidermal growth factor (EGF, Invitrogen) and 50 μg/ml gentamicin (Invitrogen). To induce cell differentiation, cells were grown to confluence and maintained in growth medium for 1 or 2 days and then cultured for 48 hours in differentiation medium containing RPMI 1640, 10% heat-inactivated FBS, 5 μg/ml bovine insulin, 1 μmol/L dexamethasone (Sigma, St. Louis, MO), 5 μg/ml bovine prolactin (Sigma) and 50 μg/ml gentamicin.[34–36] β-casein gene expression, a marker of mammary epithelial cell differentiation, was analyzed using semi-quantitative RT-PCR and β-casein gene promoter activity. Semi-quantitative RT-PCR was performed for 20 cycles and the PCR fragments were separated on 2% agarose gel. The RT-PCR primers used for the β-casein gene were 5′-actgtatcctctgagactg-3 and 5′-tctaggtactgcagaaggtc-3. The GAPDH primers was purchased from Clontech (Palo Alto, CA) and used for internal control. β-casein gene promoter activity was assayed in triplicate using the luciferase assay system (Promega, Madison, WI) with activity being expressed as light units/μg protein.[34–36]

Construction of Id4 *Expression Vectors, Transient Transfection, and Generation of Stably Transfected Cell Lines*

The coding region of the rat *Id4* was amplified using primers 5′-aggaagcgcgcgatgaag-3′ and 5′-acacctggccaacgcagct-3′, and cloned into the *Srf* enzyme restriction site of the pCMV-Script mammalian expression vector (Stratagene, Cedar Creek, TX) in the sense-orientation. The inserted sequence and orientation were verified using *Sac*II enzyme restriction and sequencing. Transfection was carried out for 24 hours in growth medium using Lipofectamine and Plus reagents with 1.6 μg of pCMV-Script-*Id4* construct plasmid DNA according to Invitrogen's protocol. Cells were cultured for an additional 24 hours in growth medium only and cell growth and colony-forming efficiency were subsequently assayed. After culturing in differentiation medium for 48 hours, the effect of transient *Id4* overexpression on cell differentiation was evaluated by measuring β-casein promoter activity in HC11-Lux cells with the luciferase assay. Transfection was confirmed by Western blot analysis of Id4 protein and semi-quantitative RT-PCR analysis of the rat *Id4*.

To establish stably transfected cell lines, cells were selected using geneticin (G418, 600 μg/ml, Invitrogen). Individual cell clones were trypsinized using a cloning cylinder and re-cultured under continuous selection with G418. The expression levels of Id4 protein in these clones were determined by Western blot analyses. The effect of *Id4* overexpression on cell growth and colony-forming efficiency was assayed as described below and the effect on cell differentiation was evaluated by Northern blot analysis of β-casein[36] expression.

Northern and Western Blot Analysis

Both Northern and Western blot analyses were carried out as described previously.[36] The β-casein gene probe was generated by RT-PCR using the primers described above, and the probe was ^{32}P-labeled using ready-to-go DNA Labeling Beads (Amersham Biosciences, Piscataway, NJ) as described previously.[36] The optimal dilutions of Id4 primary and rabbit secondary antibodies in the Western blot analysis were 1:200 and 1:2000, respectively. As loading control, the membrane was stripped and re-probed using antibody against β-actin (Santa Cruz).

Cell Proliferation and Soft Agar Assay

Cell proliferation was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Cells were seeded into the 96-well plates in growth medium at a starting density per well of 1×10^4 cells and cell proliferation measured at 24-hour intervals. Colony-forming efficiency of both transiently and stably transfected cells was analyzed using soft agar assay with a 0.66% agarose base and a 0.33% top soft agar layer (containing 4×10^5 cells) in 60-mm-diameter dishes. The dishes were incubated for 2 weeks and stained overnight with nitrobluetetrazolium before counting (Artec Colony Counter, Farmingdale, NY).

Statistical Analysis

Statistical analysis was carried out with SigmaStat Statistical Software Version 2 (Jandel, San Rafael, CA).

Results

Molecular Cloning of Rat Id4

In previous studies using cDNA microarray analysis, a rat EST (clone RGIDV72, GenBank Accession No. AW916745) was observed to be overexpressed in chemically induced rat mammary gland carcinomas.[30] BLAST searching with the forward and reverse sequences of the EST (634 bp) showed no known rat genes with high homology. However, the reverse sequence of this clone was highly homologous with a region on *Id4* exon 3 from mouse (90%) and human (89%) suggesting that the unknown rat EST was a fragment of rat *Id4*. To clone the rat *Id4* gene, a BLAST homology search was then performed for all published rat EST sequences in GenBank, EMBL, and DDBJ databases using the mouse *Id4* cDNA sequence as a template (www.ncbi.nlm.nih.gov). A set of rat EST sequences was subsequently obtained which showed a high homology (>90%) to different areas of mouse *Id4* cDNA, although several gaps in the sequence

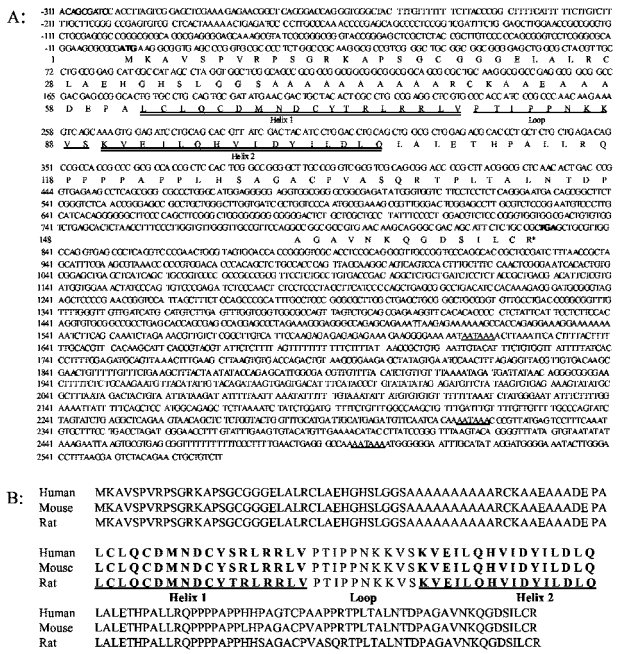

Figure 1. Rat *Id4* gene sequence and structure (GenBank Accession No. AF468681). **A:** Nucleotide sequence of rat *Id4*. Rat *Id4* contained three exons and two introns with stop codon in exon 2. A portion of the upstream promoter region (−1 to −311) was also cloned and sequenced. In **bold** are the start (ATG) and stop (TGA) codons, and the three potential polyadenylational signals (AATAAA). **B:** Comparison of the Id4 protein structure among human, mouse, and rat. The helix-loop-helix domain is depicted. As is characteristic of the Id family, there was no basic region adjacent to the HLH motif. The rat Id4 protein shows a high homology to mouse (97.52%) and human (95.65%) and the HLH domain is highly conserved. Only one amino acid in helix 1 of rat Id4 protein was different from mouse and human. The HLH domain is highly conserved within the rat Id family. Distinct from the other Id proteins, Id4 displayed an alanine-rich region adjacent to the HLH motif.

were evident among the aligned ESTs. Six pairs of overlapping primers were designed based on the aligned EST sequences and after sequencing these fragments, the cDNA sequence of rat *Id4* was obtained. To determine the intron locations, the sequences for human, mouse and rat *Id4* were compared, and PCR primers flanking the rat introns were designed. After PCR and cloning, 2881 nucleotides of the genomic sequence of rat *Id4* were sequenced (Figure 1A). The DNA sequence of rat *Id4* was reported to GenBank (Accession No. AF468681).

Genomic Sequence and Protein Structure of Id4

The *Id4* gene encodes a 161 amino acid protein with the coding region encompassing exon 1 and a portion of exon 2 (Figure 1A). The 3′ untranslated region includes part of exons 2 and 3. The first intron resides within the protein-coding region, and the second intron lay within the 3′ untranslated region. The 5′-flanking region is rich in GC bases and no TATAA or TATTA box was seen in the putative promoter region upstream of the gene. The helix-loop-helix (HLH) domain is situated in the first exon. As is characteristic of the *Id* family, there was no basic region adjacent to the HLH motif. In the 3′ untranslated region, three potential polyadenylational signals (AATAAA) were observed. The rat Id4 protein is highly homologous to mouse and human Id4 with a percent similarity of 97.5% and 95.7%, respectively (Figure 1B). Among all rat *Id* family members (*Id1–4*), the HLH domain was highly conserved, particularly at helix 2. The conserved loop structure of different Id proteins indicated that the loop might play an important role in specifying the correct dimerization partner. Distinct from the other Id proteins, Id4 displayed an alanine-rich region adjacent to the HLH motif.

Id4 *Expression in Normal Rat Mammary Gland and Carcinogen-Induced Tumors*

Expression of Id4 protein in normal rat mammary gland and chemically induced rat mammary gland carcinomas and adenomas were examined by immunohistochemistry. Id4 protein, which could be observed in cytoplasm and nuclei, varied with the stage of mammary gland differentiation among virgin, pregnant, lactating, and involuting rats (Figure 2). In mature virgin rats, Id4 protein was largely located in the cytoplasm of epithelial cells (Figure 2A). Less than 5% of nuclei showed immunoreactivity (3.6 ± 0.9, mean ± SE, $N = 10$). However, nuclear immunoreactivity in epithelial cells of mammary glands from mid-pregnant rats was nearly 50% (47 ± 1.1, $N = 3$) (Figure 2B). In lactating rats, less than 1% of epithelial cell nuclei were stained (0.7 ± 0.1, $N = 3$) and cytoplasmic staining was weak (Figure 2C). In the involuting rat mammary glands, only a weak cytoplasmic staining ($N = 5$) was observed (Figure 2D). In PhIP-induced rat mammary gland tumors, Id4 nuclear-positive cells were diffusely distributed throughout the carcinomas (Figure 2E). In the majority of carcinomas examined (72%, 43 of 60 carcinomas), Id4 protein expression was detected in at least 10% of tumor epithelial cell nuclei. In contrast, only three adenomas (two tubular adenomas and one fibroadenoma) showed nuclear staining. The majority of adenomas (77%, 10 of 13) showed less than 10% of nuclei stained positive, and adenomas showed either no clear staining or cytoplasmic staining in comparison to the negative control (Figure 2, F and G, respectively). Consistent with immunohistochemistry, carcinomas had higher expression of *Id4* message than did control mammary gland tissue. Microarray analysis of five PhIP-induced carcinomas showed increased relative expression of *Id4* in comparison to normal mammary gland (Figure 2H).

The percentage of Id4-positive nuclei and the mRNA expression detected by real-time PCR were further quantified in normal mammary gland, adenomas, and carcinomas (Figure 3). The mean percentage of Id4-positive nuclei in carcinomas was over 3- and 7.5-fold higher than in adenomas and control mammary gland, respectively (Figure 3A). By real-time PCR analysis, *Id4* mRNA expression was highest in carcinomas, followed by adenomas, and lowest in control mammary gland with an average relative expression of 27, 6, and 1, respectively (Figure 3B). In carcinomas, Id4 nuclear expression was positively correlated with the tumor cell proliferation as measured

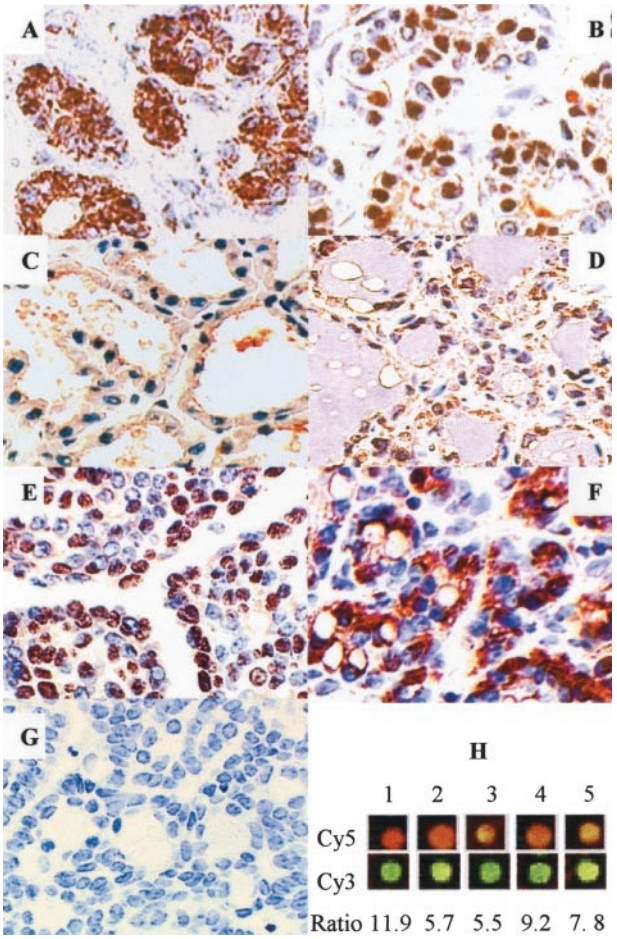

Figure 2. Immunohistochemical analysis of Id4 expression in normal rat mammary gland and tumors. **A:** Mammary gland from mature virgin rat shows largely cytoplasmic staining of Id4. **B:** Mammary gland from mid-pregnant rat shows ~50% nuclear expression. **C:** Mammary gland from lactating rat (with 12-day-old pups) shows little nuclear staining and weak cytoplasmic staining. **D:** Mammary gland from involuted rat shows weak cytoplasmic staining. **E:** Representative carcinoma from PhIP-treated rats with high nuclear expression of Id4. **F:** Representative tubular adenoma from a PhIP-treated rat showing cytoplasmic Id4 staining. **G:** Negative control showing immunostaining without primary antibody of a representative carcinoma. The nuclei are counterstained using Mayer's hematoxylin. Magnification, ×400. **H:** Microarray analysis of five PhIP-induced carcinomas showed increased relative expression of Id4 in comparison to normal mammary gland. Analysis included both Cy5 (**top panel**) or Cy3 (**bottom panel**) labeling of tumor RNA as described previously[30] and average increase in *Id4* expression ranged from 5.5 to 11.9.

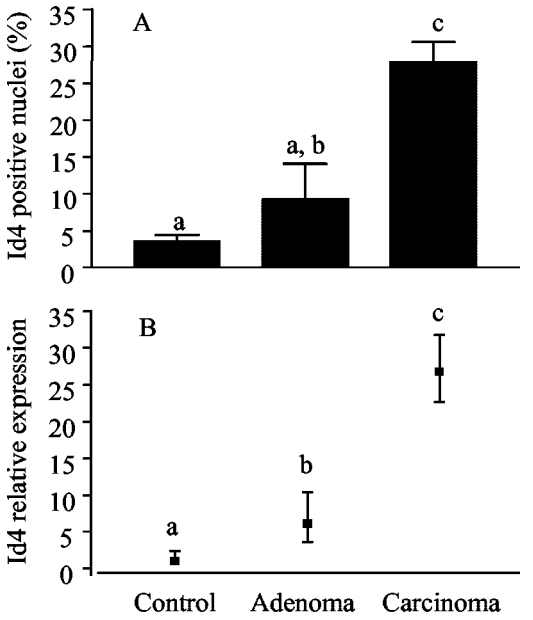

Figure 3. Expression of *Id4* in normal rat mammary gland and PhIP-induced rat mammary gland tumors. **A:** Percentage of Id4-positive nuclei in control (normal rat mammary gland), adenomas, and carcinomas as detected immunohistochemically. Values are the means ± SE of 10, 13, and 60 samples, respectively. **B:** Real-time PCR analysis of *Id4* expression in normal mammary gland, adenomas, and carcinomas. Values are the exponential function of $-\Delta\ \Delta C_T \pm$ SE of $N = 3$, 5, and 23 samples for control, adenomas, and carcinomas, respectively. Distinct letters above the data indicate significantly different (one-way analysis of variance on ranks or one-way analysis of variance, $P < 0.05$).

by PCNA labeling index (Table 1). In addition, Id4 nuclear expression was statistically associated with a higher frequency of microscopic stromal invasion and a greater tumor weight.

Effect of Id4 *Overexpression on Mammary Epithelial Cell Growth and Differentiation*

To examine in more detail the consequence of *Id4* overexpression on mammary epithelial cells, studies were carried out in HC11 cells transiently and stably transfected with *Id4*. This mammary epithelial cell line was chosen because it is a non-tumorigenic, well-characterized cell line capable of *in vitro* differentiation. In the presence of lactogenic hormones (ie, differentiation medium), HC11 cells undergo differentiation with the production of milk proteins such as β-casein. The increased expression of β-casein is a marker of HC11 cell differentiation, and the HC11-Lux cells (HC11 cells harboring the β-casein promoter-luciferase construct) provide a facile means to evaluate β-casein expression.[34,35] By Western blot analysis, endogenous Id4 expression was detected in HC11 cells cultured in growth medium, however, after treatment with differentiation medium, Id4 protein declined to barely detectable levels (data not shown). HC11-Lux cells transiently transfected with the pCMV-*Id4* construct showed elevated expression of the Id4 protein, whereas cells transfected with *Id4* in the reverse orientation or empty vector control (pCMV) did not show elevated Id4 expression (Figure 4A). After culturing in differentiation medium, the β-casein promoter activity (Figure 4B) and mRNA level (Figure 4C) were significantly lower in cells transfected with pCMV-*Id4* than in cells transfected with the empty vector indicating that enforced expression of *Id4* inhibited mammary epithelial cell differentiation. Cell proliferation was significantly higher in cells transiently transfected with pCMV-*Id4* than with the empty vector control (Figure 4D). Furthermore, colony-forming efficiency of pCMV-*Id4* transfected cells in the soft agar assay was 1.6- and 1.3-fold higher than empty vector control for ≥0.1- and ≥0.2-mm diameter colonies, respectively (Figure 4E).

To further confirm the effect of enforced expression of *Id4* on cell growth and differentiation, clonal lines of HC11 cells stably transfected with pCMV-*Id4* were established.

Table 1. Correlation Between Id4 Nuclear Expression and Tumor Cell Proliferation, Invasion, and Weight in PhIP-Induced Mammary Gland Carcinoma

Tumor parameter	Id4 nuclear expression >10%	Id4 nuclear expression ≤10%	Fisher test (p)	Spearman correlation
PCNA labeling index (%)				
≥30% (N = 26)	92%	8%		
<30% (N = 34)	56%	44%	0.003	0.424, $p < 0.05$
Tumor invasion				
Yes (N = 14)	93%	7%		
No (N = 26)	58%	42%	0.03	0.317, $p < 0.05$
Tumor weight (gram)				
≥ 2.0 (N = 34)	79%	21%		
< 2.0 (N = 20)	50%	50%	0.035	0.293, $p < 0.05$

Fisher Exact Test was applied to tumors showing a cut-off of > or ≤ 10% Id4 nuclear expression and categorized for labeling index, invasion and weight as indicated. Spearman Rank Order Correlation was applied to all Id4 nuclear expression values without categorization. N, number of carcinomas examined.

By Western blot analysis, these clones showed higher Id4 protein expression than the parental HC11 cells (Figure 5A). Three lines (2–1, 2–3, and 2–6) that showed the highest expression of Id4 were selected for further analysis. After lactogenic hormone treatment, the induction of β-casein gene expression was markedly lower in clones expressing Id4 than in parental HC11 cells or HC11 cells stably transfected with the empty vector indicating that overexpression of *Id4* inhibited differentiation (Figure 5B). As was observed after transient transfection, all *Id4*-overexpressing clones showed a statistically significantly higher rate of proliferation in comparison to parental HC11 cells or HC11-pCMV cells carrying the empty vector (Figure 5C). No difference in proliferation was observed between HC11 and HC11-pCMV cells. In the soft agar assay, colony formation was 2- to 3-fold higher in *Id4*-overexpressing clones than in parental or HC11-pCMV cells for both ≥0.2 and ≥0.5 mm diameter colonies; differences that were statistically significant (Figure 5D). The total number of colonies formed by each clone appeared to be proportional to the level of Id4 protein expressed.

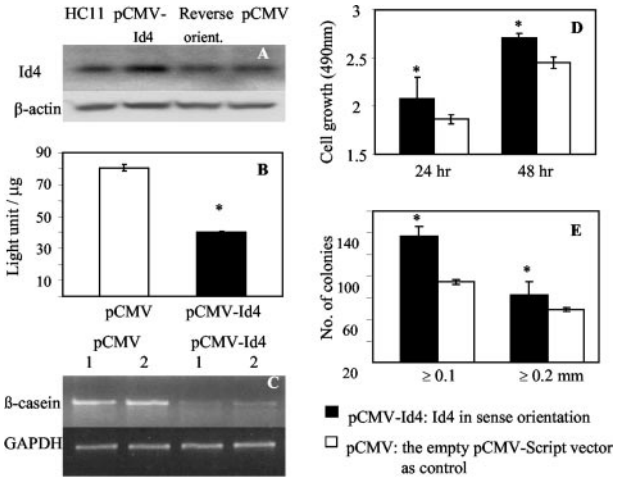

Figure 4. Effect of *Id4* overexpression on HC11 cell growth and differentiation after transient transfection. **A:** Western blot analysis of Id4 showing higher expression of Id4 after transient transfection of HC11-Lux cells with *Id4* (pCMV-*Id4*) than with pCMV empty vector or pCMV with *Id4* in the reverse orientation (orient.). **B:** Luciferase assay of β-casein promoter activity in HC11-Lux cells cultured in differentiation medium showing inhibition by enforced *Id4* expression (values are means ± SD, N = 5). **C:** Semi-quantitative RT-PCR showing decreased expression of β-casein mRNA. Duplicate determinations were carried out on separate pools of transfected cells. **D:** Cell growth assay showing higher proliferation of HC11 cells transfected with *Id4*. Cells were cultured in growth medium and proliferation was assayed at 24 and 48 hours, respectively (values are the means ± SD, N = 5). **E:** Soft agar assay indicating increased colony-forming efficiency in HC11 overexpressing *Id4* in comparison to HC11 cells carrying empty vector (values are the means ± SD, N = 3). Colonies sizes were counted at ≥0.1 mm and at ≥0.2 mm. **Asterisk** indicates statistically significant differences (Student's t-test, $P < 0.05$).

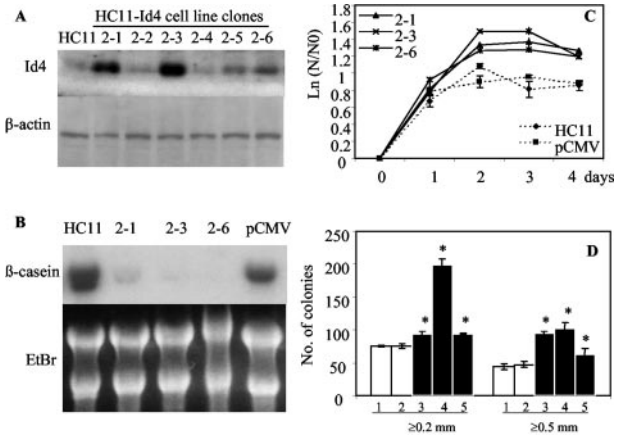

Figure 5. Effect of *Id4* overexpression on HC11 cell growth and differentiation after stable transfection. **A:** Western blot analysis of Id4 expression in established stably transfected clonal cell lines and in parental HC11 cells. The highest Id4 expression was seen in clones 2–1, 2–3, and 2–6. **B:** Northern blot analysis of β-casein gene expression in HC11 cells, Id4-overexpressing clones, and in a representative clone harboring the pCMV empty vector. Cells were cultured in differentiation medium for 48 hours before analysis. Equal loading was confirmed by ethidium bromide (EtBr) staining. **C:** Growth of cell lines was determined in growth medium over a 4-day period. Values are the means ± SD, N = 5. At 2, 3, and 4 days, proliferation was statistically higher in all Id4-overexpressing clones than in parental HC11 cells or HC11-pCMV cells with empty vector (analysis of variance, $P < 0.05$). N, absorbance at each time point; N_0, absorbance at 2 hours after cell plating. **D:** Soft agar assay indicating higher colony-forming efficiency in clones overexpressing Id4 than in parental HC11 cells or HC11 cells with empty vector (values are the means ± SD, N = 3). **Bars** labeled **1** through **5** correspond to parental HC11 cells, HC11 cells with empty vector, clone 2–1, clone 2–3, and clone 2–6, respectively. Colonies sizes were counted at ≥0.2 mm and at ≥0.5 mm. **Asterisk** indicates statistically significant differences from parental or empty vector cells (analysis of variance, $P < 0.05$).

Discussion

Id4 has recently been proposed to be a gene involved in breast cancer because of a reciprocal regulation of *Id4* and *Brca1*.[28,29] In the current study we demonstrate that *Id4* is overexpressed both at the mRNA and protein level in PhIP-induced rat mammary gland carcinomas relative to age-matched normal mammary gland and adenomas. Furthermore, we demonstrate that the overexpression of *Id4* correlates with malignancy and with proliferation, growth, and invasiveness of rat mammary gland carcinomas. These findings support a link between *Id4* and breast cancer and raise the possibility that overexpression of *Id4* may play a role in the development of this malignancy. Preliminary immunohistochemical studies carried out by us using human tissue microarrays (National Cancer Institute, Bethesda, MD) suggested elevated nuclear expression of Id4 protein in human breast cancers (unpublished observations). Further studies are needed to ascertain whether *Id4* is similarly involved in human breast cancer.

To better understand the impact of *Id4* overexpression on the mammary gland, the effect of enforced expression of *Id4* was examined in HC11 mammary gland epithelial cells. Although several studies have described the ability of various *Id* genes to inhibit differentiation and induce cell growth in several different cell lines,[15–17,24,25,37] to our knowledge no previous study has examined the effect of enforced *Id4* expression on growth and differentiation of mammary epithelial cells. The HC11 cell line proliferates in culture in the growth medium and at confluency can be induced to differentiate in the presence of medium containing lactogenic hormones.[34,35] Enforced expression of *Id4* in HC11 cells was shown to enhance cell proliferation and growth in soft agar. This enhanced growth *in vitro* is consistent with the observed *in vivo* correlation of *Id4* expression with proliferation of rat mammary gland carcinomas. Similar to our findings, overexpression of *Id4* has been shown to enhance the growth of an ovarian cancer cell line in soft agar.[28] In addition to the effects of *Id4* on cell growth, *Id4* inhibited hormone-mediated differentiation of mammary epithelial cells as shown by the inhibition of β-casein promoter activity and gene expression in HC11 cells transiently and stably transfected with *Id4*. It is further notable that reduction in the expression of *Id4* coincided with differentiation of HC11 cells and with lactation in the rat mammary gland, a differentiated state of the gland. Therefore our data support the notion that *Id4* may be a regulatory factor for mammary gland differentiation.

It is well recognized that the stage of mammary gland differentiation is an important determinant in rat mammary gland carcinogenesis. In the rat model, susceptibility to chemical carcinogenesis is highest during pubescence when the gland is less differentiated and undergoing rapid growth.[38] Therefore, the ability of *Id4* to inhibit differentiation concomitant with increasing proliferation may facilitate mammary gland carcinogenesis. Rat mammary gland adenomas showed significantly lower expression of *Id4* than did carcinomas. Adenomas arise from more differentiated cell types such as those in alveolar buds and lobules whereas carcinomas are derived largely from less differentiated ductal epithelium.[38,39] It is tempting to speculate that tumor malignancy in the rat model may in part be influenced by *Id4*-mediated regulation of mammary epithelial cell differentiation.

The molecular mechanism of action of Id proteins are, in a general sense, known to involve regulation of bHLH transcription factors and effects on cell cycle regulatory proteins. Id proteins including Id4 are likely to inhibit the commitment or differentiation that the bHLH proteins promote.[1,2] Nuclear localization appears to influence the action of Id4 in the mammary gland. Nuclear expression of Id4 was higher in carcinomas than in normal tissue, and the nuclear expression was altered during normal differentiation of the rat mammary gland. Movement of Id4 between the nucleus and cytosol has been observed with differentiation of oligodendrocytes and associated with cell cycle withdrawal.[16] Id4 has no nuclear localization sequences, and therefore its presence in the nucleus is indicative of possible heterodimerization with bHLH proteins.[40] Preliminary microarray analysis of *Id4*-overexpressing HC11 cells (*versus* parental HC11) show differences in expression of several genes regulating cell growth (unpublished observations).

An inverse association between *Brca1* and *Id4* expression has been previously suggested.[28] PhIP-induced carcinomas have been reported to express reduced levels of Brca1 protein.[41] However, *Brca1* is not mutated in chemically induced rat mammary gland carcinomas.[41,42] Although it has been suggested that a *Brca1-Id4* regulatory loop may be disrupted in some human breast cancers resulting in the down-regulation of both genes[29], the elevated Id4 and low Brca1 expression in PhIP-induced carcinomas suggests that this regulatory loop is not disrupted in the rat model. Further studies are required to ascertain the possible impact of *Id4-Brca1* regulation on the development of rat mammary gland cancer.

Studies now suggest that *Id* genes are strongly expressed in undifferentiated, growing, and tumor cells.[1–6,21,22] *Id4* fits the paradigm of the *Id* gene family. We have shown that *Id4* is highly overexpressed in rat mammary gland cancer, its expression varies with stage of mammary gland differentiation *in vivo*, and it is involved in the regulation of mammary epithelial cell differentiation, proliferation, and growth. The mechanisms contributing to *Id4* overexpression in rat mammary gland cancers are not yet known. We analyzed *Id4* for mutations, and although a single nucleotide polymorphism (T/C) was detected in the third nucleotide from the stop codon in rat DNA, no mutations were specific to carcinomas (data not shown). Further studies are warranted to better define the molecular mechanisms of *Id4* in rat mammary gland carcinogenesis and to ascertain its potential role in human breast cancer.

Acknowledgments

We thank Drs. David S. Salomon and Nancy E. Hynes for providing the HC11 cells, and Dr. Snorri S. Thorgeirsson for helpful discussions.

References

1. Norton JD: ID helix-loop-helix proteins in cell growth, differentiation, and tumorigenesis. J Cell Sci 2000, 113:3897–3905
2. Norton JD, Deed RW, Craggs G, Sablitzky F: Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol 1988, 8:58–65
3. Yokota Y: Id and development. Oncogene 2001, 20:8290–8298
4. Rivera R, Murre C: The regulation and function of the Id proteins in lymphocyte development. Oncogene 2001, 20:8308–8316
5. Lasorella A, Uo T, Iavarone A: Id proteins at the cross-road of development and cancer. Oncogene 2001, 20:8326–8333
6. Benezra R, Rafii S, Lyden D: The Id proteins and angiogenesis. Oncogene 2001, 20:8334–8341
7. Norton JD, Atherton GT: Coupling of cell growth and apoptosis functions of Id proteins. Mol Cell Biol 1998, 18:2371–2381
8. Toma JG, El-Bizri H, Barnabé-Heider F, Aloyz R, Miller FD: Evidence that helix-loop-helix proteins collaborate with retinoblastoma tumor suppressor protein to regulate cortical neurogenesis. J Neurosci 2000, 20:7648–7656
9. Riechmann V, van Crüchten I, Stablitzky F: The expression pattern of Id4, a novel dominant helix-loop-helix protein is distinct from Id1, Id2 and Id3. Nucleic Acids Res 1994, 22:749–755
10. Mantani A, Hernandez M-C, Kuo W-L, Israel MA: The mouse Id2 and Id4 genes: structural organization and chromosomal localization. Gene 1998, 222:229–235
11. Pagliuca A, Bartoli PC, Saccone S, Valle GD, Lania L: Molecular cloning of Id4, a novel dominant-negative helix-loop-helix human gene on chromosome 6p21.3-p22. Genomics 1995, 27:200–203
12. Chaudhary J, Johnson J, Kim G, Skinner MK: Hormonal regulation and differential actions of the helix-loop-helix transcriptional inhibitors of differentiation (Id1, Id2, Id3, and Id4) in Sertoli cells. Endocrinology 2001, 142:1727–1736
13. Sablitzky F, Moore A, Bromley M, Deed RW, Newton JS, Norton JD: Stage- and subcellular-specific expression of Id proteins in male germ and Sertoli cells implicates distinctive regulatory roles for Id proteins during meiosis, spermatogenesis, and Sertoli cell function. Cell Growth Differ 1998, 9:1015–1024
14. Chen H, Weng Y-C, Schatteman GC, Sanders L, Christy RJ, Christy BA: Expression of the dominant-negative regulator Id4 is induced during adipocyte differentiation. Biochem Biophys Res Commun 1999, 256:614–619
15. Andres-Barquin PJ, Hernandez M-C, Israel MA: Id4 expression induces apoptosis in astrocytic cultures and is down-regulated by activation of the cAMP-dependent signal transduction pathway. Exp Cell Res 1999, 247:347–355
16. Kondo T, Raff M: The Id4 HLH protein and the timing of oligodendrocyte differentiation. EMBO J 2000, 19:1998–2007
17. Desprez P-Y, Lin CQ, Thomasset N, Sympson CJ, Bissell MJ, Campisi J: A novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1. Mol Cell Biol 1998, 18:4577–4588
18. Polsky D, Young AZ, Busam KJ, Alani RM: The transcriptional repressor of p16/Ink4a, Id1, is up-regulated in early melanomas. Cancer Res 2001, 61:6008–6011
19. Langlands K, Down GA, Kealey T: Id proteins are dynamically expressed in normal epidermis and dysregulated in squamous cell carcinomas. Cancer Res 2000, 60:5929–5933
20. Maruyama H, Kleeff J, Wildi S, Friess H, Büchler MW, Israel MA, Korc M: Id-1 and Id-2 are overexpressed in pancreatic cancer and in dysplastic lesions in chronic pancreatitis. Am J Pathol 1999, 155:815–822
21. Lin CQ, Singh J, Murata K, Itahana Y, Parrinello S, Liang SH, Gillett CE, Campisi J, Desprez PY: A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. Cancer Res 2000, 60:1332–1340
22. Wilson JW, Deed RW, Inoue T, Balzi M, Becciolini A, Faraoni P, Potten CS, Norton JD: Expression of Id helix-loop-helix proteins in colorectal adenocarcinomas correlates with p53 expression and mitotic index. Cancer Res 2001, 61:8803–8801
23. Ouyang XS, Wang X, Lee DTW, Tsao SW, Wong YC: Overexpression of Id1 in prostate cancer. J Urol 2002, 167:2598–2602
24. Parrinello S, Lin CQ, Murata K, Itahana Y, Singh J, Krtolica A, Campisi J, Desprez P-Y: Id-1, ITF-2, and Id-2 comprise a network of helix-loop-helix proteins that regulate mammary epithelial cell proliferation, differentiation, and apoptosis. J Biol Chem 2001, 276:39213–39219
25. Desprez P-Y, Hara E, Bissell MJ, Campisi J: Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. Mol Cell Biol 1995, 15:3398–3404
26. Singh J, Itahana Y, Parrinello S, Murata K, Desprez P-Y: Molecular cloning and characterization of a zinc finger protein involved in Id-1-stimulated mammary epithelial cell growth. J Biol Chem 2001, 276: 11852–11858
27. Mori S, Nishikawa S-I, Yokota Y: Lactation defect in mice lacking the helix-loop-helix inhibitor Id2. EMBO J 2000, 19:5772–5781
28. Beger C, Pierce LN, Krüger M, Marcusson EG, Robbins JM, Welcsh P, Welch PJ, Welte K, King M-C, Barber JR, Wong-Staal F: Identification of Id4 as a regulator of BRCA1 expression by using a ribozyme-library-based inverse genomics approach. Proc Natl Acad Sci USA 2001, 98:130–135
29. Welcsh PL, Lee MK, Gonzalez-Hernandez RM, Black DJ, Mahadevappa M, Swisher EM, Warrington JA, King M-C: BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. Proc Natl Acad Sci USA 2002, 99:7560–7565
30. Shan L, He M, Yu M, Qiu C, Lee NH, Liu ET, Snyderwine EG: cDNA microarray profiling of rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and 7,12-dimethylbenz[a]anthracene. Carcinogenesis 2002, 23:1561–1568
31. Ghoshal A, Preisegger KH, Takayama S, Thorgeirsson SS, Snyderwine EG: Induction of mammary tumors in female Sprague-Dawley rats by the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine and effect of dietary fat. Carcinogenesis 1994, 15:2429–2433
32. Snyderwine EG, Thorgeirsson UP, Venugopal M, Roberts-Thomson SJ: Mammary gland carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine in Sprague-Dawley rats on high- and low-fat diets. Nutr Cancer 1998, 31:160–167
33. Qiu C, Yu M, Shan L, Snyderwine EG: Allelic imbalance and altered expression of genes in chromosome 2q11–2q16 from rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine. Oncogene 2003, 22:1253–1260
34. Ball RK, Friis RR, Schoenenberger CA, Doppler W, Groner B: Prolactin regulation of β-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J 1988, 7:2098–2095
35. Merlo GR, Graus-Porta D, Cella N, Marte BM, Taverna D, Hynes NE: Growth, differentiation, and survival of HC11 mammary epithelial cells: diverse effects of receptor tyrosine kinase-activating peptide growth factors. Eur J Cell Biol 1996, 70:97–105
36. Shan L, Rouhani SA, Schut HAJ, Snyderwine EG: 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP) modulates lactogenic hormone-mediated differentiation and gene expression in HC 11 mouse mammary epithelial cells. Cell Growth Differ 2001, 12:649–656
37. Lister J, Forrester WC, Baron MH: Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. J Biol Chem 1995, 270:17939–17946
38. Russo IH, Russo J: Developmental stage of the rat mammary gland as determinant of its susceptibility to 7, 12-dimethylbenz[a]anthracene. J Natl Cancer Inst 1978, 61:1439–1449
39. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ: Biology of disease: comparative study of human and rat mammary tumorigenesis. Lab Invest 1990, 62:244–278
40. Pagliuca A, Cannada-Bartoli P, Lania L: A role for Sp and helix-loop-helix transcription factors in the regulation of the human Id4 gene promoter activity. J Biol Chem 1998, 273:7668–7674
41. Okochi E, Miyamoto K, Wakazono K, Shima H, Sugimura T, Ushijima T: Reduced Brca1 protein expression in 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine-induced rat mammary carcinomas. Mol Carcinog 2002, 34:211–218
42. Chen K-S, Shepel LA, Haag JD, Heil GM, Gould MN: Cloning, genetic mapping, and expression studies of the rat Brca1 gene. Carcinogenesis 1996, 17:1561–1566