Id proteins in cell growth and tumorigenesis

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Since the gene encoding Id1 was cloned in 1990, Id proteins have been implicated in regulating a variety of cellular processes, including cellular growth, senescence, differentiation, apoptosis, angiogenesis, and neoplastic transformation. The development of knockout and transgenic animal models for many members of the Id gene family has been particularly useful in sorting out the biologic relevance of these genes and their expression during normal development, malignant transformation, and tumor progression. Here we review the current understanding of Id gene function, the biologic consequences of Id gene expression, and the implications for Id gene regulation of cell growth and tumorigenesis.

Introduction

Cellular differentiation programs are tightly controlled through the coordinated regulation of gene expression. Basic helix-loop-helix (bHLH) transcription factors regulate the differentiation programs of multiple cell lineages (reviewed in Norton, 2000). These proteins share a common sequence motif of a stretch of basic amino acids responsible for site-specific DNA binding adjacent to a helix-loop-helix dimerization domain. The Id family of helix-loop-helix proteins does not possess a basic DNA binding domain and functions as a dominant-negative regulator of basic HLH proteins through the formation of inactive heterodimers with intact bHLH transcription factors (Figure 1). The Id family of proteins (comprised of 4 members designated Id1–Id4) has been demonstrated to bind the ubiquitously expressed bHLH E-proteins or cell lineage-restricted bHLH transcription factors, leading to inhibition of lineage-specific gene expression and differentiation (Norton et al., 1998). Hence, the name Id refers to both *i*nhibition of *d*ifferentiation and *i*nhibition of *D*NA binding. Transcriptional inhibition by Id proteins is mediated via inhibition of DNA binding of bHLH or other activator proteins at E boxes (CANNTG), N boxes (CACNAG), or Ets sites (GGAA/T) present in the promoter regions of regulated genes (reviewed in Zebedee and Hara, 2001). Since cellular differentiation programs are frequently altered during the development of neoplastic disease, it is not surprising that Id proteins would play a role in this process. Indeed, a clue to the potential role of Id genes in tumorigenesis came with the observation that, in general, high Id expression levels are found in proliferative, undifferentiated cells—a feature which is characteristic of tumor cells (Israel et al., 1999). Over the past several years, the particular mechanisms underlying the effects of Id genes on cell growth and differentiation have been investigated. Here we review data supporting the critical role of Id gene regulation in the development of normal cellular differentiation programs. We also review mechanisms of Id gene regulation of cellular growth controls and the cell cycle machinery and evaluate the contribution of dysregulated Id gene expression to the process of tumorigenesis.

Id regulation of cellular differentiation

The first direct genetic evidence for a role of Id proteins in regulating cellular differentiation came from mutational studies of the *Drosophila emc* locus. *Drosophila emc*, a helix-loop-helix protein, functions in a manner similar to Id proteins in that it forms heterodimers with bHLH target proteins and prevents them from binding DNA and functioning transcriptionally. Loss- and gain-of-function mutants of *emc* in *Drosophila* showed that *emc* inhibits the functions of *Daughterless* and *achaete-scute* bHLH proteins, which are involved in sex determination and neurogenesis (Campuzano, 2001). Later, in mammalian cell culture systems, differentiation of various cell lineages was shown to be accompanied by downregulation of Id expression, while overexpression of Id proteins within these systems, including keratinocytes, myoblasts, myeloid precursor cells, mammary epithelium, and preadipose cells, was shown to inhibit their ability to differentiate under appropriate conditions (reviewed in Lasorella et al., 2001). More recently, in vivo studies using targeted expression of Id genes to thymocytes (Kim et al., 1999; Morrow et al., 1999), intestinal epithelia (Wice and Gordon, 1998), and B-lymphocytes (Sun, 1994) of mice have demonstrated inhibition of cellular differentiation in these systems.

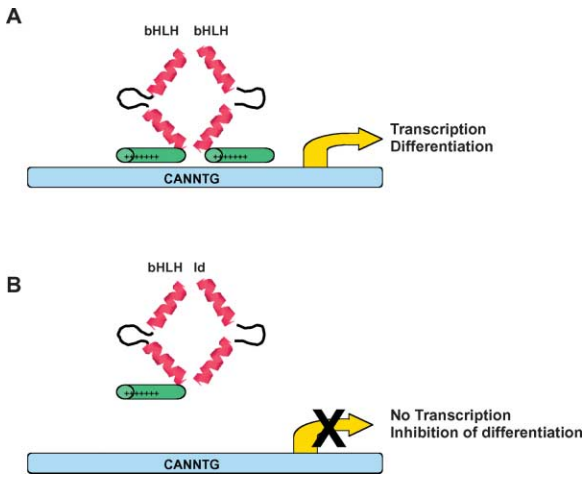

Figure 1. Model for Id effects on bHLH transcription

A: In the absence of Id proteins, dimers of bHLH proteins can bind DNA and activate transcription of differentiation-associated genes.
B: With expression of Id proteins, bHLH-Id dimers form which are unable to bind DNA and expression of differentiation-associated genes is inhibited.

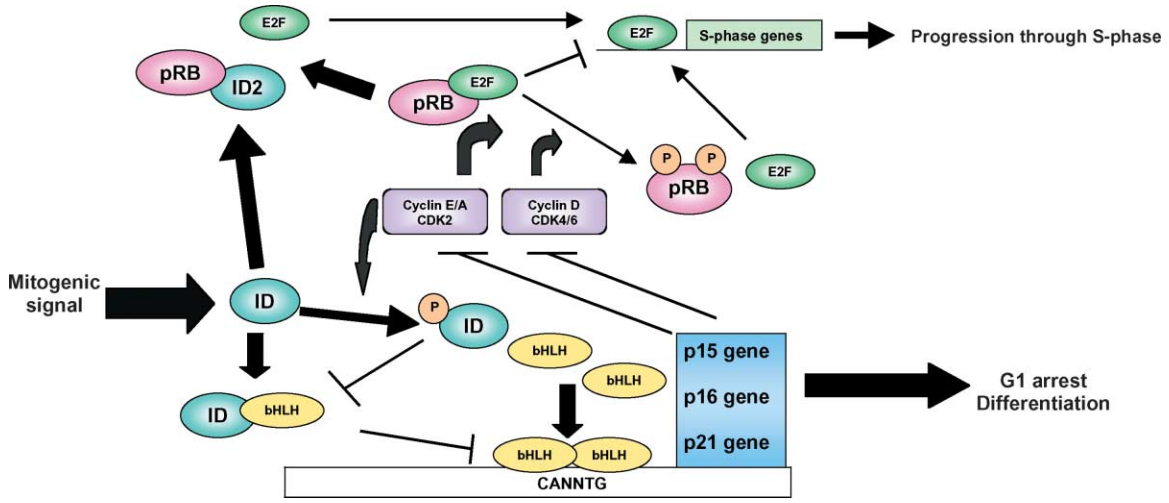

Figure 2. Model for Id gene function in cell cycle progression

Common cell cycle regulatory pathways in which Id gene regulation has been implicated are shown above. Id phosphorylation by cyclin-dependent kinases in late G1 is shown. This modified Id protein inhibits Id-bHLH dimer formation as shown. bHLH-mediated activation of the cdk-inhibitory proteins p15, p16, and p21 is also shown. This activity is also inhibited by Id proteins. The particular function of Id2 and its association with the retinoblastoma family of proteins (pRB) is also noted. Id2 interactions with pRB allow for release of E2F, expression of S phase genes, and cell cycle progression. All Id interactions noted support the role of Id proteins in cell cycle progression.

While several lineage-specific bHLH proteins involved in endothelial, muscle, neuronal, and hematopoietic cell differentiation have been identified (reviewed in Norton et al., 1998), no putative bHLH proteins involved in epithelial cell differentiation have been defined to date. Since previous work on Id gene function in human keratinocytes (Alani et al., 1999; Nickoloff et al., 2000), breast (Desprez et al., 1995), and intestinal epithelia (Wice and Gordon, 1998) suggests that lineage-specific bHLH proteins are likely to exist in these systems, it is anticipated that they will be identified and characterized in the near future.

Id proteins in development

Over the past few years, much work has focused on determining the role of Id proteins during development. In situ analyses of Id gene expression during mouse development have demonstrated widespread expression of Id1, Id2, and Id3 throughout the developing organism from early gestation through birth with considerable overlap in expression patterns of Id1 and Id3 and distinct expression of Id4 limited to the nervous system (Yokota, 2001). In an attempt to define the role of Id genes in development, several knockout animal models have been generated (Table 1). Deletion of the Id1 gene alone failed to produce an obvious phenotype, while mice that were null for Id2 possessed defects in immunity due to a lack of lymph nodes and Peyer's patches and a severely reduced population of natural killer (NK) cells and Langerhans cells, and mice null for Id3 possessed defects in B cell proliferation and humoral immunity (Hacker et al., 2003; Yokota, 2001). Given the overlapping expression patterns of Id1 and Id3 during embryogenesis, it was hypothesized that redundant functions existed between the Id1 and Id3 proteins. Indeed, mice that were null for both Id1 and Id3 were subsequently generated and found to possess embryonic lethality (E13.5) with aberrant neuronal differentiation and angiogenesis (Lyden et al., 1999). Specifically, neuroblasts were shown to

Table 1. Putative role for Id proteins in development and tumorigenesis

Gene deleted	Phenotype	Reference
Id1–/–	No major abnormalities, elevated TSP-1 expression	(Volpert et al., 2002; Yan et al., 1997)
Id2–/–	Lack lymph nodes, NK cells and Peyer's patches; defects in spermatogenesis; loss of Langerhans cells	(Hacker et al., 2003; Yokota et al., 1999)
Id3–/–	Abnormalities in humoral immunity and T cell development	(Kee et al., 2001; Pan et al., 1999; Rivera et al., 2000)
Id1 –/–, Id3 –/–	Lethal E13.5, vascular defects in forebrain, premature maturation of neurons	(Lyden et al., 1999)
Id1/Id3 loss of single or multiple alleles	Defects in vascularization and growth of tumor xenografts	(Lyden et al., 1999)
Id2–/–, Rb–/–	Compensatory resolution of Rb–/– defects in neurogenesis and hematopoiesis. Rescue of Rb–/– embryonic lethality but severe muscle loss leads to postnatal demise	(Lasorella et al., 2000)

Murine models have clarified the role of Id proteins in a variety of developmental processes and tumor growth and metastasis. Mouse models for Id genes are listed with their associated phenotypes.

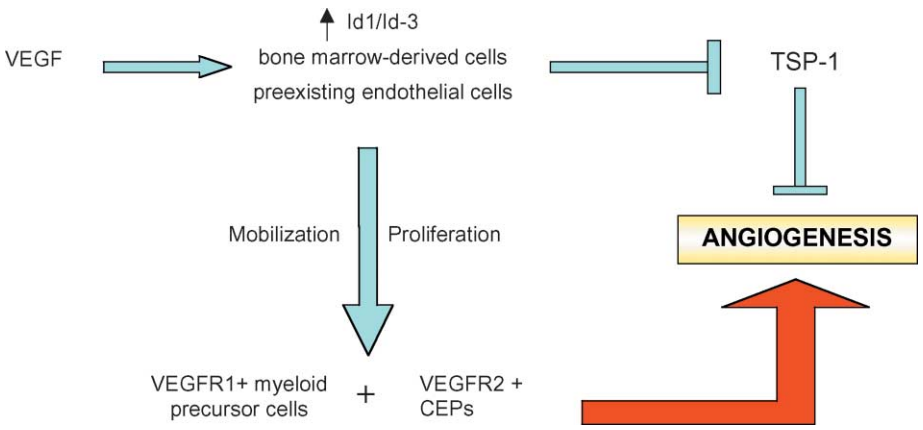

Figure 3. Model for Id gene regulation of tumor angiogenesis

Id gene expression has been shown to influence endothelial cells through the angiogenesis inhibitor thrombospondin-1 (TSP-1) and through VEGF receptor-mediated functions (VEGFR1,2) on myeloid precursor cells and circulating endothelial precursor cells (CEPs). Tumor cells release increased levels of VEGF, which signals bone marrow-derived cells and preexisting endothelial cells to upregulate Id1 and/or Id3 gene expression. Id genes subsequently promote angiogenesis in tumors through the mobilization and increased proliferation of bone marrow-derived cells or through effects on preexisting endothelial cells, including downregulated expression of the angiogenesis inhibitor thrombospondin-1 (TSP-1).

withdraw from the cell cycle prematurely with enhanced expression of neural-specific differentiation markers. In addition, these mice displayed vascular malformations in the forebrain and an absence of branching and sprouting of blood vessels into the neuroectoderm. While the expression patterns of neuronal and endothelial differentiation markers were evaluated in Id1/Id3 double null embryos and found to be altered, the specific targets of Id genes in these systems remain to be elucidated.

Id proteins and the cell cycle

A growing body of evidence has implicated Id proteins as playing a critical role in promoting G1-S cell cycle transitions. Early insights into the role of Id proteins in cell cycle control came with the cloning of Id3 as a mitogen-induced early response gene and later identification of Id1, Id2, and Id3 as being mitogen-responsive (Lasorella et al., 2001). Id genes were further implicated in promoting cell cycle progression, since antisense Id constructs introduced into serum-stimulated NIH 3T3 cells were shown to delay cell cycle reentry in these cells, while microinjection of anti-Id antibodies into serum stimulated NIH 3T3 cells led to late G1 withdrawal of these cells from the cell cycle (Lasorella et al., 2001). Id proteins have been more directly implicated in regulating cell cycle control, as the particular factors involved in these pathways have been elucidated. Id2 and Id3 have been shown to be phosphorylated in late G1 by cyclin-dependent kinase 2 (cdk2) (Deed et al., 1997; Hara et al., 1997), and Id2 has been found to reverse cellular growth inhibition by the retinoblastoma protein (pRb) through direct interaction with pRb, p107, and p130 via its HLH domain (reviewed in Lasorella et al., 2001). Moreover, Id2 had been shown to interact genetically with pRb since Id2/pRb double knockout animals display partial suppression of Rb null-associated embryonic lethality (Lasorella et al., 2000). Id1 has also been shown to inhibit E-protein and Ets-protein-mediated activation of the cdk inhibitor p16/INK4a (Alani et al., 2001; Ohtani et al., 2001), and Id1 null mouse embryo fibroblasts were shown to senesce prematurely due to increased expression of p16/INK4a (Alani et al., 2001). Since this tumor suppressor protein functions in the same pathway as pRb, these data suggest that Id proteins can inactivate this pathway either through direct interaction with pRb or through altered expression of genes that regulate Rb phosphorylation and, ultimately, function (Figure 2). Other cell cycle regulatory proteins involved in the pRb pathway have been shown to be affected by Id expression, and analysis of the promoter regions of the cell cycle inhibitory proteins p15, p16/Ink4a, and p21 (Pagliuca et al., 2000; Prabhu et al., 1997) has demonstrated activation by E-proteins, which is abrogated by Id proteins.

Id proteins and apoptosis

Id genes have been shown to promote apoptosis in a variety of settings. Transgenic mice with targeted Id1 expression in T cells showed a 96% reduction in the total number of thymocytes due to massive apoptosis (Kim et al., 1999). In addition, Id1 expression in dense mammary epithelial cell cultures induces apoptosis (Parrinello et al., 2001), Id3 expression induces apoptosis in B-lymphocytes (Kee et al., 2001), Id4 induces apoptosis in an astrocyte-derived cell line (Andres-Barquin et al., 1999), and Id1 induces apoptosis in neonatal and adult cardiac myocytes through a redox-dependent mechanism (Tanaka et al., 1998).

Table 2. Id gene expression in primary human malignancies

Tumor type	Deregulated Id	Reference
Breast cancer	Id1*	(Lin et al., 2000; Schoppmann et al., 2003)
Thyroid cancer	Id1*	(Kebebew et al., 2000)
Endometrial cancer	Id1*	(Takai et al., 2001)
Cervical cancer	Id1*	(Schindl et al., 2001)
Ovarian cancer	Id1*	(Schindl et al., 2003)
Squamous cell cancer (esophagus)	Id1*	(Hu et al., 2001)
Squamous cell cancer (nasopharynx)	Id1*	(Wang et al., 2002)
Melanoma	Id1	(Polsky et al., 2001)
Prostate cancer	Id1*	(Ouyang et al., 2002)
Ewing sarcoma	Id2	(Fukuma et al., 2003; Nishimori et al., 2002)
Squamous cell cancer (head and neck)	Id1, Id2, Id3	(Langlands et al., 2000)
Colorectal cancer	Id1*, Id2, Id3	(Wilson et al., 2001)
Astrocytic tumors	Id1*, Id2, Id3	(Vandeputte et al., 2002)
Pancreatic cancer	Id1*, Id2, Id3	(Maruyama et al., 1999)
Testicular seminomas	Id1*, Id2, Id3, Id4	(Sablitzky et al., 1998)

The above primary human tumors have been evaluated for Id gene expression by a variety of methods. Given concerns regarding specificity of reagents for Id1 detection in primary tumors, those tumors evaluated by antibody detection are designated with an asterisk (*).

The significance of apoptotic functions of Id proteins with respect to the development of human malignancies is still under investigation, and a potential tumor-suppressive function associated with high-level Id expression analogous to that seen with *myc* oncoproteins seems likely.

Id proteins and tumorigenesis

Id genes have been identified as potential protooncogenes, since overexpression of Id proteins in primary cells has been demonstrated to promote cellular immortalization; however, tumor-associated defects in Id genes have not been identified to date. Id1, Id2, and Id3 have each been shown to extended the lifespan of primary human keratinocytes (Alani et al., 1999; Nickoloff et al., 2000), and endothelial cells (Tang et al., 2002), while constitutive expression of Id1 alone has been shown to immortalize human keratinocytes and result in activation of telomerase activity and inactivation of the retinoblastoma protein (Alani et al., 1999). Id gene expression has been shown to be elevated in various tumor cell lines by Northern and Western analysis, including lung tumor cells, astrocytic tumor cells, colon cancer cells, Ewing sarcoma cells, and chondrosarcoma cells (Fukuma et al., 2003; Nishimori et al., 2002; reviewed in Lasorella et al., 2001). In addition, breast cancer cell lines that express Id1 have been shown to possess more invasive phenotypes than those that did not (Desprez et al., 1998), and Id1 promoter activity has been shown to be activated in metastatic breast cancers in association with loss of a retinoblastoma/histone deacetylase transcriptional repressor complex (Singh et al., 2002). In other recent work, Id1 has been shown to be a transcriptional target of Kaposi's sarcoma herpesvirus-associated genes in human endothelial cells (Tang et al., 2003). We await further clarification of the role of Id genes in the genesis of this malignancy.

Over the past several years, Id1 gene expression has been shown to be elevated in a variety of primary human tumors versus normal control tissue specimens, including breast cancers (Lin et al., 2000; Schoppmann et al., 2003), thyroid cancers (Kebebew et al., 2000), endometrial cancers (Takai et al., 2001), ovarian cancers (Schindl et al., 2003), cervical cancers (Schindl et al., 2001), squamous cell carcinomas of the esophagus (Hu et al., 2001) and nasopharynx (Wang et al., 2002), and melanomas (Polsky et al., 2001). Id2 has been found to be specifically upregulated in Ewing sarcomas, and the Id2 promoter has been shown to be a transcriptional target of EWS-ets fusion proteins (Fukuma et al., 2003; Nishimori et al., 2002). Id2 was also shown to be a downstream effector of myc proteins and was felt to be the major effector of N-myc in early studies of neuroblastomas (Lasorella et al., 2000, 2002); however, recent studies have reviewed the role of Id2 in the genesis of neuroblastomas and its utility as a prognostic marker in large-scale studies. These more recent large-scale data strongly suggest that Id2 is not a critical effector of N-myc in neuroblastomas and therefore not likely to be a useful prognostic marker in this disease (Vandesompele et al., 2003; Wang et al., 2003). Id1, Id2, and Id3 are all upregulated in pancreatic cancers (Maruyama et al., 1999), colorectal cancers (Wilson et al., 2001), astrocytic tumors (Vandeputte et al., 2002), and squamous cell carcinomas of the head and neck (Langlands et al., 2000), and all four Id genes are upregulated in testicular seminomas (Sablitzky et al., 1998), further supporting the notion of Id genes as cellular protooncogenes. In general, in situ evaluation of Id expression in human tumors has revealed a correlation between tumor invasiveness/aggressiveness/progression and Id expression (Table 2). While both in situ hybridization techniques and immunohistochemical techniques have been used to assess Id gene expression in primary human tissue specimens (reviewed in Lasorella et al., 2001), we have only had success using in situ hybridization techniques for confirming Id1 expression in tissue specimens. Of note, the commercially available antibody most widely used for Id1 immunohistochemical techniques has not been shown to be specific for Id1 in immunohistochemical studies published to date, and has been seen to have crossreactive bands on Western analysis and crossreactive staining in Id1 null mice (R.M.A. and H.A.S., unpublished results). Monoclonal antibodies against Id1 have recently been developed (Schaefer et al., 2001), and are currently being tested for specificity in Id1 null mouse models.

While the data in primary human tumors is suggestive of a link between Id gene expression and tumor development, few studies to date have confirmed this link. The most convincing link of Id genes to tumor development comes from transgenic mouse model systems in which Id gene expression was targeted to particular tissue types. In these studies, Id1 (Kim et al., 1999) and Id2 (Morrow et al., 1999) expression targeted to thymocytes demonstrated aberrant T cell development, massive apoptosis in targeted cells, and subsequent development of T cell lymphomas, while targeted expression of Id1 to B-lymphocytes again resulted in aberrant B cell development, massive apoptosis, and subsequent development of B cell lymphomas (Sun, 1994). Targeted expression of Id1 to intestinal epithelia resulted in the development of adenomas in a subset of these animals (Wice and Gordon, 1998). Since E2A null mice also develop T cell lymphomas in a fashion analogous to Id transgenic mice (Bain et al., 1997; Yan et al., 1997), it is likely that the Id effects in thymocytes are mediated through inhibition of the endogenous functions of E-proteins in T cell development. While the data in the transgenic mouse model systems described above is supportive of a role for Id genes in tumorigenesis, it must be considered that the complex functions of Id genes in inducing apoptosis and promoting cell growth are likely to influence the phenotypes of the particular targeted cells. Overall, these transgenic mouse models demonstrate that Id genes *can* induce malignant disease in vivo; however, whether they *do* induce these types of lesions in human cancers remains to be determined.

Id proteins and angiogenesis

Recent work has demonstrated the importance of Id gene expression in regulating exogenous tumor cell growth and metastasis (Lyden et al., 1999). Since mice lacking both Id1 and Id3 genes are embryonic lethal at day E13.5 and possess defects in angiogenesis, a series of experiments were carried out in mice lacking one to three Id1 and/or Id3 alleles to determine if such animals could support tumor angiogenesis. Surprisingly, mice lacking single or multiple Id1 and/or Id3 alleles were unable to support growth of tumor explants. Specifically, mice lacking even a single Id1 allele did not form tumors with B6RV2 lymphoma cell explants, and were unable to support metastases of Id1 null breast cancers. Additionally, Id1 +/– animals implanted with Lewis lung carcinoma cells survived twice as long as wild-type animals and had significantly fewer metastases. Since tumors that were either +/+ (B6RV2 and LLC) or –/– (B-CA) for Id1 were significantly perturbed in their ability to grow or metastasize in Id1 +/– animals and questions of immune toler-

ance of the tumor explants were ruled out, it was evident that the host itself was unable to support vascularization of the tumors. While these investigators did note the striking phenotypic similarity of Id1/Id3 double null embryos to α_v-integrin null embryos (Bader et al., 1998), their analyses revealed no alteration of Id expression in α_v-integrin null mice or vice versa. Furthermore, expression of matrix metalloprotease 2 (MMP2), a target of $\alpha_v\beta_3$ integrins on endothelial cells, was also unaffected in the aberrant ganglionic vasculature in Id1/Id3 null animals, suggesting other downstream targets of Id1 and Id3 as being important regulators of angiogenesis in that system. Recent data has identified thrombospondin-1 as being a major mediator of Id1 effects on angiogenesis, and the significance of this finding with respect to tumor growth in Id null mice is currently under investigation (Volpert et al., 2002). Other downstream effectors of Id1 were identified in the above studies, and we await clarification of their contribution to Id effects on tumor angiogenesis (Figure 3).

Conclusions

Id proteins have been demonstrated to play a role in a variety of biologic processes that have been implicated in regulating tumorigenesis; however, no putative alterations of Id genes have been identified in primary human tumors to date to certify Ids as true cellular protooncogenes. The most recent data on Id gene expression in primary human malignancies has received much attention, since these data suggest that Id proteins, or their downstream effectors, may be bona fide targets for treatment of a variety of human malignancies. While the associations between Id gene expression and cancer have been mounting, few experiments have proven, unequivocally, the link between Id expression and malignant progression. The data published to date in human tumors have been mostly observational and may be called into question given the variable reliability of reagents used in some studies. It is expected that, over the next few years, the field will move from mostly observational discoveries to functional studies of Id genes in tumors using the latest techniques for gene silencing in cell culture and in vivo model systems. In addition, studies using improved diagnostic tests and protein detection methods for Id gene products will allow for a clearer understanding of the true nature of Id gene expression in human cancers, their functions in malignant conversion and tumor progression, and their utility as molecular targets for cancer therapeutics.

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