ID Proteins Regulate Diverse Aspects of Cancer Progression and Provide Novel Therapeutic Opportunities

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The inhibitor of differentiation (ID) proteins are helix-loop-helix transcriptional repressors with established roles in stem cell self-renewal, lineage commitment, and niche interactions. While deregulated expression of ID proteins in cancer was identified more than a decade ago, emerging evidence has revealed a central role for ID proteins in neoplastic progression of multiple tumor types that often mirrors their function in physiological stem and progenitor cells. ID proteins are required for the maintenance of cancer stem cells, self-renewal, and proliferation in a range of malignancies. Furthermore, ID proteins promote metastatic dissemination through their role in remodeling the tumor microenvironment and by promoting tumor-associated endothelial progenitor cell proliferation and mobilization. Here, we discuss the latest findings in this area and the clinical opportunities that they provide.

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The inhibitor of differentiation (ID) family of proteins are critical regulators in a wide range of developmental and cellular processes. They regulate stem cell homeostasis and fate commitment in various cell types, including neuronal,[1] hematopoietic,[2,3] and embryonic[4] cells, where they function by both inhibiting cell-autonomous differentiation programs[4] and by coordinating the cell's interaction with the extracellular milieu and its niche.[5,6] In this review, we will focus on recent discoveries demonstrating that these functions of IDs are retained by many cancers to promote proliferation and self-renewal and to facilitate signaling from the tumor microenvironment.

The four members of the vertebrate ID family (ID1, ID2, ID3, and ID4) belong to the basic helix-loop-helix (bHLH) family of transcription factors. All four members share the highly conserved bHLH region and have similar molecular weights of between 13–20 kDa.[7,8] Outside the HLH domain, there are extensive sequence differences among the four members of the ID proteins. Different members of the ID proteins are expressed in distinct expression patterns in a tissue-specific and stage-dependent manner, hence controlling different cellular and physiological processes.[9–11] The bHLH transcription factors are key regulators of lineage- and tissue-specific gene expression and act as obligate dimers binding DNA through composite basic domains to regulate the transcription of target genes containing E-boxes (CANNTG) in their promoters. ID proteins dimerize with bHLH proteins, but because ID proteins lack a basic DNA-binding domain, ID-bHLH heterodimers fail to bind DNA, thereby inhibiting the transcriptional activity of the bHLH proteins. As such, ID proteins are dominant negative regulators of bHLH function.[12] ID proteins interact with the ubiquitously expressed E protein transcription factors (E12, E47, E2-2, and HEB) which can act as homodimers (in B cells) or as heterodimers with tissue-restricted bHLH proteins such as MyoD (muscle) and NeuroD (nerve). A number of reports demonstrate noncanonical functions for ID proteins, including binding to non-HLH transcription factors such as Rb-family pocket proteins,[13] Ets factors,[14] or RNA[15] although the broader significance of these findings to ID protein biology is yet to be explored. The biochemical mechanisms of ID protein activity remain largely unelucidated and comprise an area of intensive investigation.

DEREGULATION OF IDs IN HUMAN CANCER

ID family members exhibit unique spatio-temporal patterns of tissue expression during development[16] and malignancy,[17] although evidence suggests biochemical redundancy *in vitro*.[8] The mechanisms governing ID protein expression are complex—an extensive body of literature shows that *ID* gene transcription is exquisitely sensitive to signals from the extracellular environment, including transforming growth factor-β (TGF-β),[18,19] steroid hormones,[20] receptor tyrosine kinases,[21,22] and oncoproteins[23] (**Figure 1**). The stability of IDs is also tightly controlled by the APC/Cdh1 E3 ubiquitin ligase complex,[24] resulting in short half-lives for ID proteins

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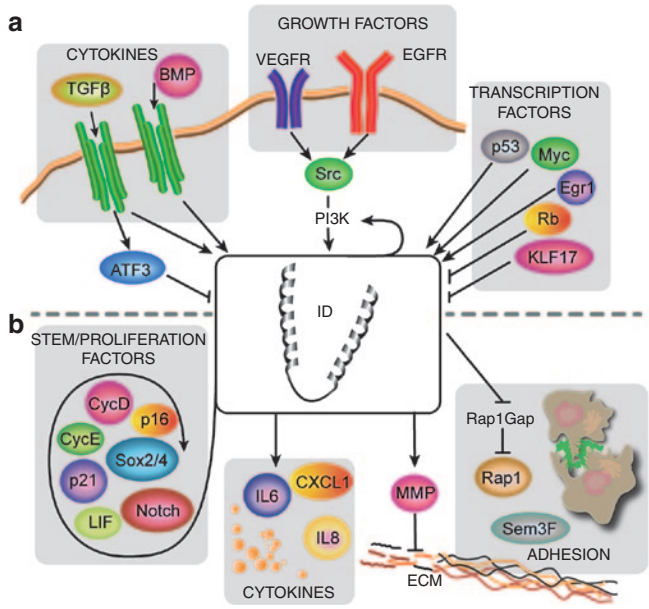

Figure 1 Regulation of inhibitor of differentiation (ID) expression and their function in cancer biology. (**a**) ID proteins are sensitive to a diverse array of extracellular signals, including steroid hormones, growth factors, and members of the TGF-β superfamily. ID proteins are also downstream of well-established oncogenic pathways such as RAS-Egr1, MYC, and Src-PI3k as well as tumor suppressors RB p53 and KLF17. (**b**) ID proteins regulate cellular pathways that are essential to the development and progression of cancer. IDs regulate self-renewal and cell-cycle through a number of known stem and proliferation factors such as Notch, Sox2/4, LIF, cyclin genes and the CDK inhibitors $p21^{waf1}$ and $p16^{INK4A}$. In addition, IDs remodel the tumor microenvironment by inducing the expression of pro-angiogenic cytokines such as IL6 and CXCL1 which increase endothelial cell proliferation and migration and that might influence the biological properties of other cell types in the tumor microenvironment. ID proteins have also been shown to promote invasion by degrading the extracellular matrix through induction of several members of the maxtrix metalloproteinase (MMP) protein family such as MMP-2, MMP-9, and MMP-14. ID genes control a stem cell-intrinsic transcriptional program that preserves stem cell adhesion to the niche in neural stem cells and in glioma. ID proteins activate the Ras-related protein RAP1 by suppressing the GTPase activating protein RAP1GAP, thereby promoting adhesion of cells to a supportive endothelial niche.

in most tissues. In certain physiological and malignant stem cell populations, ID proteins are stabilized by the ubiquitin-specific peptidase 1 deubiquitinase which counters ubiquitin-mediated ID destruction.[25] Ubiquitin-specific peptidase 1 is overexpressed in a subset of primary osteosarcomas, where it stabilizes ID1, ID2, and ID3, leading to repression of p21 and the osteogenic differentiation program.[25]

Analysis of clinical specimens has shown that high expression of ID proteins, particularly ID1, correlates with aggressive clinical behavior and poor patient outcome in many cancers (**Table 1**) Furthermore, data from our group shows ID1 expression is upregulated between primary breast cancers and their matched brain metastases (unpublished data) suggesting a functional role for ID1 in the metastatic process. However, analysis of ID1 expression in human cancers is complicated by several factors. ID1 can be expressed by rare neoplastic cells in subsets of breast,[26] glioma,[27] and bladder[17] cancers, which can be difficult to identify using tissue microarrays. Furthermore, tumor-associated endothelial cells often show high ID1 expression, confounding expression analysis in whole tumor extracts.

Table 1 ID protein expression in primary human malignancies

ID protein	Tumor type	Alteration	Prognosis	Reference
ID1	Bladder	Increased	Poor	117
	Brain	Increased	Good	59
	Breast	Increased	Poor	26,63, 67–69, 118,119
	Colon and rectal	Increased	Poor	65,120
	Gastric	Increased	Poor	121,122
	Head and neck	Increased	Poor	123
	Kidney	Increased	Poor	124
	Lung	Increased	Poor	125–128
	Leukemia	Increased	Poor	119
	Liver	Increased	Poor	129,130
	Nasopharyngeal	Increased	Poor	123
	Esophageal	Increased	Poor	131
	Ovarian	Increased	Poor	132
	Pancreatic	Increased	Poor	133
	Prostatic	Increased	Poor	75,81,134
	Thyroid	Increased	Poor	135,136
ID2	Brain	Increased	Poor	57
	Breast	Reduced	Good	137
	Colon and rectal	Increased	Poor	120
	Hodgkin's lymphoma	Increased	Not applicable	138
	Pancreatic	Increased	Poor	139
	Prostatic	Increased	Poor	81
ID3	Brain	Increased	Poor	57
	Colon and rectal	Increased	Poor	65
	Gastric	Increased	Poor	121
	Burkitt lymphoma	Mutated/loss of function	Not applicable	28–30
	Pancreatic	Increased	Not applicable	140
	Prostatic	Increased	Poor	134
ID4	Brain	Increased	Poor	141
	Breast	Reduced	Poor	142
	Colon and rectal	Reduced	Poor	142
	Leukemia	Increased	Leukemic transformation	143
	Ovarian	Increased/ amplified	Not applicable	33
	Prostatic	Increased	Poor	144

ID, inhibitor of differentiation.

In contrast to ID1, other ID proteins possess diverse patterns of expression and function in cancer. Recent data from several groups demonstrates that ID3 is a tumor suppressor in Burkitt's lymphoma, and is inactivated through somatic mutation in up to 68% of cases.[28–30] ID3 inactivation promotes tumor cell survival

through ligand-independent signaling by the B-cell receptor to the PI3K pathway. Interestingly, ID3 is not mutated in other B-cell lymphomas, perhaps reflecting ID3's role in specific phases of B-cell maturation.[29] Similarly, ID4 is epigenetically silenced through promoter hypermethylation in subsets of cancers, including human leukemia,[31,32] suggesting a tumor suppressive function. Conversely, ID4 acts as a proto-oncogene in serous ovarian cancer where it is genomically amplified, overexpressed, and required for SOC cell line proliferation.[33] ID4 has been identified as a negative regulator of BRCA1 which is the most commonly mutated gene in familial breast and ovarian cancer.[34] In an unbiased ribozyme-based screen ID4 was discovered to inversely regulate expression of BRCA1 in an ovarian and breast cancer cell line.[35] In a separate study by Welcsh *et al.*, overexpression of BRCA1 increases the expression level of ID4, suggesting a negative feed-back loop in BRCA1 signalling.[36] Therefore, depending on the cell type and development stage, ID proteins can have different functional outcomes as oncogenes or tumor suppressors. These data demonstrate the importance of context in understanding ID protein function in cancer. IDs also serve as downstream targets of several known oncogenic pathways. Regulation of ID proteins has been shown to be mediated by several well-established oncoproteins such as MYC, RAS, RB, and SRC (**Figure 1**).[13,23,37] Tumor-suppressor genes that cause repression of ID transcription include p53, FOXO3, and growth inhibitory signals by TGF-β–SMAD.[38–40] ID proteins have been shown to regulate central hallmarks of cancer such as proliferation, cellular senescence, and survival, as reviewed previously by Perk *et al.*[41] Aberrant levels of ID proteins have been associated with the upregulation of a number of proliferation and pro-survival factors such as cyclins D1 and E,[42] PI3K–AKT,[43–46] and nuclear factor-κB (NF-κB),[47] and the inhibition of cyclin-dependent kinase inhibitors (CKI) p16INK4A, p21waf1, p27Kip1,[48–50] and pRb[51] (**Figure 1**).

Recent studies have provided critical insights into novel cellular and molecular events controlled by ID proteins, revealing complex roles within neoplastic cells and their local microenvironment. In particular, ID expression is critical in controlling cancer stem cell (CSC) phenotypes and is associated with the activation of angiogenesis, induction of cancer cell invasion and metastasis, and remodeling of the microenvironment.

IDs REGULATE THE CSC PHENOTYPE

There is now compelling evidence that specific subpopulations of tumor cells, known as CSC, drive the tumorigenic and metastatic potential of many tumors.[52] In many cases, signaling pathways active in tissue stem and progenitor cells are important in the maintenance of CSC pools. Recent data demonstrates an important role for ID proteins in tissue stem cells, which appears to be conserved in their malignant counterparts. Tissue stem cells reside in anatomical niches and lineage commitment of these cells is often coordinated with departure of stem cells from their niche. Hematopoietic[3] and adult neural stem cells[1] are identified by high expression of Id1 and recent work has identified a critical role for ID proteins in niche interactions and self renewal of hematopoietic stem cell and neural stem cell. Increasing evidence supports a role for bone remodeling cells in providing a niche for HSC. Mice deficient in *Id1* exhibit reduced long-term hemaotopoietic repopulating activity and enhanced myeloid differentiation,[3] associated with altered bone formation[53] and altered bone stromal cytokine expression.[5] Niola *et al.* have recently identified that Id1, Id2, and Id3 maintain adhesion of adult neural stem cells to an endothelial niche.[6] ID proteins promote adhesion by activating RAP1, a GTPase involved in integrin-dependent adhesion, by suppressing the expression of its negative regulator RAPGAP1. Compound deletion of *Id* genes leads to exit of neural stem cells from the niche.[6]

A significant body of literature supports a key role for ID proteins in high-grade glioma (HGG), which is the most frequent adult brain tumor and which remains practicably incurable, with median survival of ~15 months for patients with the highest grade tumors, known as glioblastoma multiforme. HGGs fall into four molecular subtypes: proneural, neural, classical, and mesenchymal, each with unique clinical features and genomic defects. Numerous studies demonstrate that HGG are maintained by a minor subset of glioma-initiating cells (GICs) that share many traits with neural stem cells, including self-renewal, multilineage differentiation, and a dependence on a perivascular niche.[54] GICs are commonly identified by the capacity to form spheres in suspension culture and/or through expression of cell surface stem cell markers CD133 or CD44.

Gliomas express high levels of ID proteins[55] and various studies have provided evidence that ID proteins regulate the genesis and maintenance of GICs. GICs are enriched for co-expression of ID1 and CD44, which preferentially localize to the perivascular environment.[27] Furthermore, coexpression of ID1 and CD44 predicts poor prognosis in glioblastoma multiforme, albeit weakly.[27] The cytokine TGF-β is a major player in the regulation of Id proteins in HGG, where it upregulates expression of Id1 and promotes the self renewal capacity of GICs[27] (**Figure 2a**). This result is in contrast to "normal" epithelial cells where TGF-β is known to repress ID1 expression through engagement of the Smad protein corepressor ATF3.[19] In glioblastoma multiforme, ATF3 is commonly epigenetically silenced,[56] switching the TGF-β regulation of Id1 from repression to induction. Further *in vitro* studies support a role for ID1 and ID3 in GIC biology as pharmacological inhibition of TGF-β or genetic ablation of ID1 and ID3 reduces human HGG sphere formation and invasiveness *in vitro*,[27,57] which is in turn associated with reduced expression of the neural stem cell marker Sox2.[58] However, Barrett *et al.* provide evidence that *in vitro* sphere-forming assays must be interpreted with caution. By crossing a knock-in mouse model in which the Id1 promoter drives GFP expression to two distinct transgenic mouse models of HGG, the authors demonstrate that while $Id1^{hi}$ cells are enriched for *in vitro* sphere-forming capacity compared to $Id1^{low}$ cells, they are not enriched for the capacity to transplant disease into naive recipients.[59] Surprisingly, the authors found that the Id^{low} glioma cells have greater tumorigenic potential than the Id^{high} glioma cells (which have greater self-renewal capacity). Though both populations are capable of transmitting disease, the more proliferative Id^{low} glioma cells are more tumorigenic. One of the caveats of this study is the lack long-term transplantation assay and could imply that Id1 does not mark the CSC in this model. Accordingly, deletion of Id1 and Id3 *in vivo* led to a very modest increase in survival in these models. In keeping with the animal data, low ID1 expression associates with poorer outcome in the proneural subtype of HGG,[57,59] although these data are based on analysis of mRNA expression in total tumor extracts, which has caveats as previously discussed.

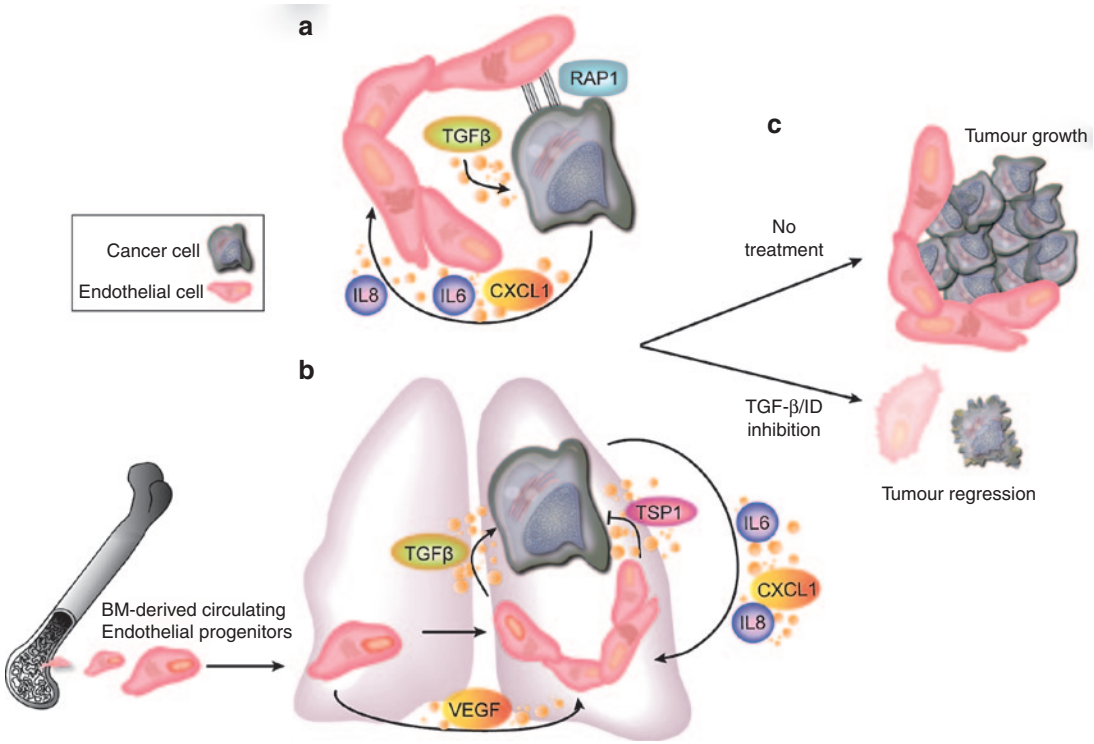

Figure 2 A proposed conserved cellular mechanism for inhibitor of differentiation (ID) proteins in tumorigenesis and metastasis. (**a**) IDs control the glioma cancer stem cell niche in a cell autonomous as well as paracrine manner. TGF-β, expressed by endothelial cells, upregulates the expression of ID proteins in glioma stem cells, thereby maintaining glioma stem cell self renewal via RAP1-dependent adhesion to the niche. ID expression may also re-inforce the vascular niche by the induction of pro-angiogenic factors like IL6 and CXCL1. (**b**) ID proteins may play a similar role in coordinating an endothelial niche for disseminated breast cancer cells in the lung. Endothelial cells can enforce quiescence of disseminated breast cancer cells in the lung through the expression of thrombospondin-1 (TSP1). In contrast, activated or sprouting endothelial cells downregulate TSP1- and instead express TGF-β which promotes the escape of disseminated tumor cells from dormancy, in an ID-protein dependent manner. ID proteins in turn can activate the expression of proangiogenic factors CXCL1 and IL8 to drive endothelial activation and sprouting. Endothelial activation can be further promoted by the recruitment from the bone marrow to nascent metastatic sites of Id^{+} EPCs, which require ID1 for mobilization and proliferation. EPCs support angiogenesis by the expression of VEGF and through direct luminal incorporation. ID signaling in activated endothelial cells further represses TSP-1. (**c**) Targeting ID expression and their associated pathways in tumor cells and endothelial cells from bone marrow and tumor blood vessels will disrupt both cell-autonomous and cell-nonautonomous programs, which may eventually produce additive or even synergistic antitumor effects. VEGF, vascular endothelial growth factor.

Several studies suggest that ID2, ID3, and perhaps ID4 may also be critical in controlling GIC malignancy. Id3[60] and Id4[61] are able to reprogram Ink4a/Arf-deficient mouse astrocytes into GIC-like cells through a variety of proposed mechanisms. These include cell autonomous derepression of Sox2, cyclin E, and Notch signaling by Id4,[62] and by induction of the pro-angiogenic factors IL6, IL8, and CXCL1 by Id3[60] (**Figures 1** and **2a**), which may promote the development of a vascular niche for GICs, which will be discussed later. To further explore the functional redundancy between Id proteins, Niola *et al.* first created a new transplantable mouse model in which p53 loss cooperates with expression of oncogenic h-Ras to generate murine tumors with transcriptional signatures resembling human mesenchymal HGG.[57] By further engineering inducible Id1, Id2, and Id3 silencing, they showed that coordinated loss of three Id proteins leads to marked reductions in proliferation and stem cell marker expression, inhibition of *in vitro* sphere-forming capacity and a dramatic improvement in animal survival. This apparent discrepancy with the limited phenotype observed following deletion of Id1 and Id3 by Barrett *et al.* may be explained by differences in the requirement for Id proteins between different models of HGG or by compensation between family members, requiring deletion of 3 or more ID members to reveal a phenotype.

Mirroring the effects of Id deletion in neural stem cells,[6] deletion of Ids in HGG is associated with increased Rap1Gap expression, decreased Rap1 activity and loss of adhesion to endothelial cells (**Figure 2a**). In support of the generality of this result, RapGap1 expression is dramatically downregulated in human HGG and a five-gene expression signature including ID2, ID3, and Rap1Gap strongly predicts outcome in HGG.[57] This suggests that the mechanism of ID function in neural stem cells may be maintained by CSCs.

There is increasing evidence for ID proteins, in particular ID1 and ID3, in the promotion of the CSC phenotype of epithelial cancers. We have shown that Id1 cooperates with oncogenic h-Ras in transformation of mammary epithelia to generate highly metastatic breast cancers.[63] Id1 was required for tumor maintenance, as inactivation of the Id1 transgene in established tumors led to growth arrest followed by regression associated with widespread cellular senescence, which is essentially the irreversible loss of self-renewal and proliferative capacity.[63] Similarly, Stankic *et al.* recently demonstrated that Id1 expression generates breast cancer cells with tumor-initiating properties independent of an epithelial-to-mesenchymal transition (EMT) program.[64] ID1 and ID3 are required for the re-initiation of proliferative capacity in experimental breast cancer lung metastases.[26] John Dick *et al.* recently reported that ID1

and ID3 are also required for the self renewal and tumor-initiating capacity of colon CSCs through cell-cycle restriction driven by the cell-cycle inhibitor p21.[65] In these studies, silencing of Id1 and Id3 sensitized colon cancer-initiating cells to the chemotherapeutic agent oxaliplatin. Taken together, there is a recurring role for ID proteins across diverse tumor types in the maintenance of the CSC phenotype. Although by and large the mechanistic details remain to be determined, these studies identify self-renewal pathways controlled by ID1 and ID3 as potential targets for the development of therapies to eradicate cancer-initiating cells.

IDs REGULATE TUMOR METASTASIS

The metastatic cascade is a complex multistep process involving intravasation, dissemination of the cancer cells into circulation, extravasation followed by initiation and outgrowth in distant metastatic organs. In many solid cancers, cancer cells disseminate early in the life of a tumor, before clinical presentation, and surgical resection,[66] followed by a long period of dormancy prior to metastatic relapse. ID genes have demonstrated roles in many facets of metastatic dissemination, including tissue remodeling and invasion, initiation of proliferative capacity at the distant site, as well as functions in host endothelial cells that contribute to angiogenesis.

CANCER CELL-AUTONOMOUS ROLES FOR ID PROTEINS IN METASTASIS

To investigate the transcriptional changes associated with metastatic dissemination, Minn *et al.*[67] used murine xenografts of MDA-MB-231 human breast cancer cells to select cell subpopulations highly metastatic to lung. Transcriptional profiling analysis revealed 95 genes differentially expressed by lung-tropic sublines, among which ID1 was the sole transcription regulator. Functional studies by Minn and others now clearly show that ID1 and ID3 are required for breast tumor initiating functions, both in primary tumor formation and during metastatic colonization of distant organs in breast, gastric, and pancreatic cancer models.[26,67–71]

Emerging evidence suggests that activation of the EMT program can promote invasion and metastasis by promoting the acquisition of a CSC-like state.[72,73] As such, EMT may confer on epithelial cells a set of characteristics that enable them to disseminate from primary tumors and seed metastases.[74] ID1 is highly expressed in metaplastic breast cancers,[17] a rare subtype with a poorly differentiated mesenchymal-like phenotype. There is evidence that ID1 may regulate EMT, both directly through interaction with Cav-1 in prostate cancer cells,[75] induction of cadherin-switching in immortalized esophageal epithelial cells,[76] suppression of E-cadherin and ZO-1 in human kidney cells,[77] and indirectly through loss of KLF17[69] in breast cancer cells. ID1 and ID3 also regulate MMPs, a major protein family associated with EMT that regulates remodeling of the ECM, degradation of the basement membrane and stromal cell layers, allowing the infiltration of cancer cells during invasion.[78,79] Desprez *et al.*[80] originally reported that constitutive expression of ID1 results in upregulation of a novel MMP protein, MT1-MMP, and invasion through basement membrane (**Figure 1b**). High expression of ID1 also induces increased secretion of MMP-2 in prostate cancer[81] and MMP-9 in leukemia.[82]

However, several recent studies have demonstrated that epithelial–mesenchymal plasticity is crucial at different stages of metastasis, in particular, the reversal of EMT is necessary for efficient metastatic colonization.[83] Stankic *et al.* have recently shown that ID1, under the control of TGF-β signaling, mediates epithelial–mesenchymal plasticity and that ID1 expression is associated with an epithelial phenotype in breast cancer lung metastases.[64] ID1 induces a mesenchymal-to-epithelial transition at the metastatic site by antagonizing the activity of Twist, a bHLH transcription factor, but not at the primary site, where this state is controlled by the zinc finger protein Snail1. This observation has also shed light on a possible role of Id proteins in regulating the dynamic interactions among epithelial and mesenchymal gene programs.

ID CONTROL OF THE METASTATIC MICROENVIRONMENT

In addition to their cell-autonomous roles in metastatic dissemination, ID proteins may play a role in stromal control of metastatic progression. Recent studies suggest the existence of a perivascular niche in which lung-metastatic breast cancer cell dormancy is enforced by the expression of thrombospondin-1 (TSP-1), an antiangiogenic factor, by endothelial cells.[84] Ghajar *et al.* demonstrated that metastatic cells can be activated to proliferate by vessel remodeling and sprouting via a TGF-β-dependent mechanism[84] (**Figure 2b**), which bears striking parallels to the endothelial niche in which glioma stem cells reside (**Figure 2a**). Although the involvement of ID proteins in the secretion of TSP-1 or the response of disseminated breast cancer cells to TGF-β is yet to be explored in this setting, ID1 is known to suppress TSP-1 expression[85–87] so it is tempting to speculate that ID signaling in activated endothelial cells may be responsible for the suppression of TSP-1 in the escape from metastatic dormancy.

ID proteins also play a role in the expression of proangiogenic factors by neoplastic cells. ID4 and ID3 promote tumor angiogenesis via secretion of IL8 and CXCL1 by glioma[60,88] and breast cancer cells,[15] which may support tumor growth via the promotion of vessel remodeling and reinforcement of the cancer niche, whether it be glioma growing in the brain or breast cancer cells growing in the lung (**Figure 2a**,**b**). Given the proven role for ID proteins in niche interactions in primary cancers, exemplified by glioma, the role of ID proteins in establishing niche interactions in metastatic sites is an important area of future study.

ID EXPRESSION AND FUNCTION IN ANGIOGENESIS

Angiogenesis is a rate limiting step in tumor progression required to provide nutrients necessary for tumor growth and access to the circulation for metastatic cells. ID proteins are commonly expressed by endothelial cells and are upregulated in tumor-associated vessels. The mechanism controlling Id expression in tumor vasculature are not fully elucidated, however bone morphogenic proteins (BMPs), members of the TGF-β superfamily, induce ID1 expression in endothelial cells through receptor-mediated activation of Smad1, Smad4, and Smad5 binding to bone morphogenic protein response elements in the ID1 promoter.[89,90] Bone morphogenic protein-dependent upregulation of ID1 expression stimulates endothelial cell proliferation and sprouting *in vitro* and angiogenesis *in vivo*.[91,92] This activity of ID1 may be dependent on binding to the HLH protein E2-2; ID1 binds to E2-2 *in vitro* and can reverse the blockade of experimental angiogenesis

in vivo resulting from E2-2 overexpression.[93] In addition, vascular endothelial growth factor secreted by tumors activates the mitogen-activated protein kinase pathway and may impinge on the ID1 and ID3 promoters of endothelial cell at the EGR-1 site, as has been demonstrated in other cell types.[94,95] Id2 loss leads to downregulation of vascular endothelial growth factor in pituitary tumors suggesting a possible feedback loop between Ids and vascular endothelial growth factor-mediated by Hif-1α.[96,97]

Mice lacking Id1, Id2, and Id3 show extensive embryonic hemorrhaging,[98] suggesting a strict requirement for ID proteins in endothelial biology. In xenograft models of tumor growth, animals with reduced Id1 and Id3 dosages showed a significant loss of tumor vascular integrity and fail to develop metastatic lesions.[99] However, spontaneous models of solid tumors, including breast (MMTV-Neu), prostate (TRAMP), and multiple cancers (PTEN deficiency) demonstrate a more varied and complex dependency on ID proteins for angiogenesis.[100–102] In these models, the expression of ID proteins in the tumor vessels varies with tumor type and grade: *e.g.*, ID is expressed in the neovessels of breast, intrauterine, and poorly differentiated prostate cancers, but not in pheochromocytomas or well-differentiated prostate tumors.[100,101] Interestingly, while ID deficiency led to the development of hypoxia and/or hemorrhage in tumors with high endothelial ID expression, it did not significantly impact on tumor progression. However, in the MMTV-Her2/neu model, partial loss of Id function in combination with chemical inhibition of stress-activated pathways lead to dramatic regression of aggressive tumors.[102]

These models revealed a functional contribution for bone marrow (BM)-derived endothelial progenitor cells (EPCs) in tumor angiogenesis, which increased with grade.[101] vascular endothelial growth factor secreted by tumors leads to a dramatic upregulation of Id1 and Id3 proteins in the BM, presumably in BM-derived EPCs which results in their mobilization into the circulation and subsequently into tumors.[103] EPCs provide stability to nascent vessels by direct luminal incorporation into sprouting vessels but also regulate the angiogenic switch at a critical early stage of tumor growth via paracrine secretion of pro-angiogenic growth factors[104] (**Figure 2b**). While the contributions of EPCs to neovessel formation in primary and metastatic tumors have been reported to be variable,[105] remarkably, specific ablation of EPCs *in vivo* results in severe angiogenesis inhibition and impaired tumor growth.[106]

EPCs from tumor-bearing mice[104,107] and isolated from cancer patients[108] express high levels of ID1. The Id1 KO mice were critical in demonstrating that BM-derived progenitors are the source of tumor endothelium, as Id1 KO mice failed to mobilize these progenitors, while BM transplantation of Id1 KO mice with wild type BM rescued the observed vascular defects.[103] Following these observations, acute and conditional shRNA-mediated silencing of Id1 in the adult BM resulted in EPC mobilization defects associated with severe angiogenesis inhibition, impaired primary tumor growth, and progression of micrometastases to macrometastases, suggesting a critical role for these cells in angiogenesis-mediated tumor growth.[104] Importantly, ID1 deficiency reduced levels of mobilized EPCs and not other hematopoietic cells, as observed in Id KO animals which maintain normal hematopoiesis.[109] The selectivity of Id1 gene expression for EPCs has been exploited to specifically express transgenes in EPCs *in vivo*.[107] Use of the Id1 promoter to drive expression of suicide genes, or shRNAs targeting EPC-intrinsic factors including VEGFR2, reduced circulating EPCs and showed significant defects in angiogenesis-mediated tumor growth.

How IDs control the generation of EPCs is also beginning to be explored. ID1 is required for the maintenance of long term repopulating hematopoietic stem cells ($lin^- Sca^+ kit^+ CD34^-$)[2,3] in the BM through suppression of p21. Loss of ID1 upregulates p21, which in turn drives the stem cells towards a more committed myeloid state, as assessed by gene expression profiling, an event that is associated with the depletion of cells capable of endothelial cell fate commitment.[110] These results suggest that ID1 is required in early hematopoietic stem cells to restrain the commitment to the myeloid lineage and preserve a pool of cells that give rise to endothelial progenitors in response to vasculogenic growth signals. To understand signaling mechanisms responsible for Id1-mediated EPC functions, a recent study has demonstrated the role of Id1/PI3K/Akt/NF-κB/survivin signaling pathway in EPC proliferation.[111]

ID SIGNALING PATHWAYS AS THERAPEUTIC TARGETS IN CANCER

The transcriptional regulators ID1 and ID3 are attractive targets for cancer therapy as they are required for angiogenesis, tumor invasiveness, and metastasis. Furthermore, ID proteins are undetectable in most normal tissues, but are highly expressed in many cancer cells and cancer-associated blood vessels suggesting that targeting ID proteins may provide a large therapeutic window in which to treat cancers while minimizing toxicity (**Figure 2c**). ID proteins have cell-autonomous roles in proliferation and CSC homeostasis, and genetic studies in glioblastoma,[27,57] breast,[26,63] and colon cancer[65] demonstrate that Id activity is required for the maintenance of certain cancers. A novel systemic siRNA delivery system was used to demonstrate the feasibility of systemic Id protein targeting. Knockdown of ID4 in established ovarian cancer xenografts led to long-term tumor remission in 80% of mice.[33] While these data suggest that inactivation of Id proteins may serve as a novel therapeutic strategy, transcription factors are notoriously difficult to target with small molecule inhibitors. Several groups have used a variety of alternative methods to disrupt Id factor protein complexes in cancer cells. For example, treatment with cell permeable peptides aimed at disrupting the interaction of ID1/ID3 with the bHLH protein E47 led to marked activation of E47 transcriptional activity and the induction of cell cycle arrest and apoptosis in breast[112] and ovarian[113] cancer cells. The broader effectiveness of targeting ID protein binding depends on the nature of the biochemical or transcriptional complexes in which ID proteins act, which in many instances has not been elucidated and remains a major knowledge gap in this field.

Others have targeted ID1 expression rather than function. Anido *et al.* used clinically approved small molecule inhibitiors of TGF-β receptor (TGFβRI) to downregulate Id1 and Id3 in a mouse model of glioblastoma multiforme, leading to reduced tumor initiation and tumor growth in an ID-dependent manner.[27] Treatment of mice bearing orthotopic HGG tumors with a small molecule known to downregulate Id1, cannabidiol, led to marked inhibition of tumor growth *in vivo*.[58] Similarly, Mistry *et al.* show that small molecule inhibitors of the ubiquitin specific protease ubiquitin-specific peptidase 1 promote degradation of ID1 and inhibition of acute myeloid leukemia cell line growth and survival

in vitro and *in vivo*.[114] The relative importance of ID protein downregulation to the therapeutic efficacy of cannabidiol and ubiquitin-specific peptidase 1 inhibitors remains to be determined.

Since ID proteins play a role in supporting vasculature and BM-derived EPCs, several groups have targeted the critical roles for ID proteins in cancer-associated endothelial cells which form the stem cell niche and provide nutrients to growing metastatic tumors.[115] To model the efficacy of therapeutic targeting of Id1+ EPCs, Mellick *et al.* targeted the toxic thymidine kinase gene to EPCs using the Id1 promoter, leading to potent inhibition of angiogenic-mediated tumor growth.[107] Systemic delivery of Id1 antisense oligonucleotide fused to an endothelial delivery peptide downregulated Id1 in tumor endothelial cells *in vivo* and led to remarkable inhibition of primary tumor growth and metastasis.[116] Targeting of ID proteins in appropriate tumor types, such as high grade serous ovarian cancer, basal breast cancer and glioma may deplete the CSC pool, its niche and supporting vasculature, opening new therapeutic opportunities in these aggressive cancers (**Figure 2c**).

CONCLUSIONS AND FUTURE DIRECTIONS

The studies highlighted in this review demonstrate the complex context-dependency of ID protein expression and function in cancer, where ID proteins can play opposing roles at times in neoplastic progression. ID proteins have fundamental roles in sensing and integrating extracellular cues to control the phenotype of cancers, via cell autonomous and extrinsic pathways, such as formation of the metastatic niche, increased proliferation and self renewal, metastasis, and angiogenesis. The corruption of the normal functions of ID proteins to maintain a more undifferentiated state in a cancer cell (CSCs) make ID proteins an attractive therapeutic target. A detailed understanding of the mechanism of action of ID proteins in different cancer subtypes will be essential to exploit their therapeutic potential.

While *ID* genes are deregulated through genetic or epigenetic events in a subset of cancers, emerging evidence suggests that ID expression may be required by a broader range of cancers where they are not necessarily genomically altered or overexpressed, a process known as lineage-dependency. In such cancers, ID proteins and associated molecular networks may be promising new therapeutic targets, permitting simultaneous targeting of neoplastic cells, and their supportive microenvironment.

ID proteins are also valuable tools in deconvoluting tumor heterogeneity, and their association with CSCs may lead us to therapies directed at the CSC subpopulation of cancers. Many questions remain to translate better biological insights of ID function into therapeutics. In particular, further studies into the role of IDs in the escape from metastatic dormancy and their relationship to the cell of origin of cancer promise to yield exciting biological breakthroughs and valuable insights in the clinical management of cancer progression.

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