Regulation of metastasis in colorectal adenocarcinoma: A collision between development and tumor biology

J. Joshua Smith, MD,a,d Natasha G. Deane, PhD,a,b,c Punita Dhawan, PhD,a,b,e and R. Daniel Beauchamp, MD,a,b,d,e Nashville, Tenn

From the Department of Surgery,a the Vanderbilt Ingram Cancer Center,b the Department of Radiology and the Vanderbilt Institute of Imaging Sciences,c the Department of Cell and Developmental Biology,d and the Department of Cancer Biology,e Vanderbilt University School of Medicine, Nashville, Tenn

COLORECTAL CARCINOMA (CRC) is the third leading cause of cancer-related deaths in the United States. Currently, an estimated 150,000 new cases of CRC and over 50,000 deaths are caused by this disease annually in the United States.1,2 Although localized tumor growth may cause significant organ dysfunction and even death, metastases cause most (~90%) human cancer deaths.3 The ability to metastasize is linked with the ability of cancer cells to invade adjacent tissues, gain access to vascular or lymphatic channels, and survive transit through the bloodstream so that they may extravasate, then reside, and finally colonize heterologous organs or tissues. Cancer cells acquire the capacity of invasion and metastasis through regulated processes related to tissue development and homeostasis. Identification of the key regulators of these processes may provide valuable prognostic information to patients with CRC as well as opportunities for enhanced intervention.

DEVELOPMENTAL BIOLOGY AND CARCINOGENESIS IN INTESTINAL EPITHELIAL CELLS

At least 3 major signaling pathways are critical for the development and maintenance of homeostasis in the gastrointestinal tract: the TGF-β superfamily, Wnt/β-catenin/TCF (T-cell–specific transcription factor), and Notch signaling pathways. These 3 signaling pathways contribute to the development, differentiation, and maintenance of homeostasis in intestinal epithelium, whereas mutations or aberrant regulation of these pathways contribute to tumor initiation and progression in the intestine.4 In this review, we will emphasize the interactions of 2 of these pathways, TGF-β superfamily and Wnt, and their potential roles in colorectal cancer cell invasiveness and metastatic potential. It is well recognized that other genetic alterations, signaling pathways and processes such as angiogenesis are also important contributors of carcinogenesis, tumor progression and metastasis, however, these processes will not be addressed in this brief review.

Adverse phenotypic behaviors have been linked to a process known as epithelial-to-mesenchymal transition (EMT). EMT is tightly controlled, reversible, and required for embryonic development, tissue reorganization, and wound healing. During EMT, cells lose epithelial polarity and acquire a mesenchymal phenotype with invasive characteristics.5 Cell–cell junctions are disrupted in EMT by mechanisms that involve loss of expression of the adherens junction protein, E-cadherin. Tight junctions lose polarity and function, and the cells that undergo EMT express mesenchymal markers, such as vimentin and α-smooth muscle actin (α-SMA). In many cases, cellular transformation recapitulates a molecular environment that favors EMT, which in turn disrupts normal epithelial cell polarity and results in the acquisition of invasive and metastatic potential. Thus, a growing body of evidence
implicates EMT in tumor cell migration, invasiveness, and metastatic behavior.6-8

OVERVIEW OF TGF-β SUPERFAMILY SIGNALING

The TGF-β superfamily is made up of 2 subfamilies of cytokines: the TGF-β/activin/nodal subfamily and the bone morphogenic protein (BMP)/growth and differentiation factor (GDF)/muellerian inhibiting substance (MIS) subfamily.9 For the purposes of this review, we will not focus on the accessory receptors (ie, betaglycan, cripto, or endoglin), interactions with inhibin, GDFs, or the muellerian subfamily (antimuellerian hormone/MIS) and their associations in the TGF-β superfamily. Specific TGF-β and BMP-related cytokines and their shared signaling intermediates function as critical developmental regulators, cell growth inhibitors, and tumor suppressors in normal tissue. Germline loss of certain components of the signaling intermediates for these cytokines often results in embryonic lethality because of their critical roles in development. However, somatic cell mutations or acquired loss of function may contribute to the development or progression of cancer.

The TGF-β family of ligands bind and signal through a heteromeric complex of ligand-specific

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Fig 1. Signaling specificity of Smad proteins as they relate to TGF-β ligands, ligand binding traps and the type I and type II receptors. Important ligand traps, ligands, and receptor complexes in TGF-β superfamily signaling are noted. Downstream R-Smads 1, 2, 3, 5, and 8 are grouped based on their signaling specificity. Adapted from Reference 9.
type I (ALK 1-7) and type II (ligand-specific) serine/threonine kinase receptors. Ligand access to the serine/threonine kinase receptors is regulated by a family of proteins known as ligand traps, which are proteins that selectively bind to specific TGF-β superfamily ligands that thereby block access to the receptors (Fig 1). For example, the ligand trap protein Noggin inhibits receptor activation of the BMP cytokines 2, 4, and 7. A type II receptor provides ligand specificity, forming an oligomeric complex with and activating the type I receptor by phosphorylation upon activation. Information from the tissue microenvironment to the cell nucleus by these growth inhibitors is then transmitted by specific Smad proteins (R-Smads) associated with the growth factor (type II) receptors. Nine members of the vertebrate Smads family intracellular signaling pathways have been identified, and by consensus, they are now referred to as Smad1 through Smad9. As a general rule, Smads1, 5, and 8 are R-Smads that function to transduce signals from the BMPs and their specific type II (BMPR-II, ActR-II/B) and type I (ALK3, ALK6, ALK2) receptors, which mediate cellular transition to an epithelial phenotype.

Smads2 and 3 are important R-Smad substrates of ALK5 that may be activated by either activin (through the type II ActR-IIIB receptor) or TGF-β selective type II receptors. Inhibitory Smads 6 and 7 (I-Smads) inhibit the signaling function of the receptor-activated Smads. Smad6 preferentially inhibits BMP signaling, whereas Smad7 can inhibit both TGF-β and BMP signaling by preventing receptor-mediated phosphorylation of the receptor-activated Smad proteins. When phosphorylated by the type I receptor, R-Smads associate with the common signaling intermediate Smad4, which translocates the entire R-Smad:Co-Smad complex to the nucleus, where it associates with 1 or more of several DNA-binding partners and activates the transcription of specific target genes important for both growth inhibition and EMT. Importantly, Smad4 translocates to the nucleus when complexed with the R-Smads in order to activate gene transcription. Steady-state levels of Smad proteins are regulated through the ubiquitin–proteasome degradation pathway.

TGF-β RECEPTORS AND SMAD4 LOSS IN COLORECTAL CANCER

The TGF-β family of peptides has a growth inhibitory role in intestinal epithelium. Kurokowa et al were the first to report that TGF-β was an inhibitor of cultured rat intestinal epithelial cells. We subsequently determined that inhibition of cultured intestinal cell proliferation after TGF-β treatment results from a mid-to-late G1 cell-cycle arrest associated with downregulation of cyclin D1 and inhibition of Cdk4-associated Rb kinase activity. In vivo, there is increased expression of both TGF-β1 and type II TGF-β receptor (TβRII) in intestinal epithelial cells as they migrate from the proliferative compartment toward the lumen in both the small intestine and the colon. This pattern of expression is inversely correlated with the mitotic activity in the gut epithelium. Taken together, these findings suggest that TGF-β plays a regulatory role in intestinal cell proliferation and perhaps differentiation.

TGF-β regulation of epithelial cell proliferation is altered by cellular transformation. TGF-β inhibits growth of nontumorigenic human colonic adenoma cells in culture; however, conversion of adenoma to a tumorigenic adenocarcinoma is associated with a decreased response to the inhibitory actions of TGF-β. Studies in human colon carcinoma cell lines have demonstrated a correlation between the differentiation state of tumors and the sensitivity to the antiproliferative and differentiation-promoting effects of TGF-β. Thus, loss of growth-inhibitory responses to TGF-β seems to be a common and important event that attends malignant transformation of epithelial cells.

One mechanism by which tumor cells become resistant to the growth inhibitory actions of TGF-β may be through downregulation or mutation of the TβRII. Several studies have suggested that a decrease in expression of TβRII is a key step for the neoplastic transformation of epithelial cells. Inactivation of the TβRII has been detected in a subgroup of colorectal carcinomas associated with the microsatellite instability or replication error phenotype found in approximately 13% of all colorectal cancers. Mutations of TβRII have also been identified in 15% of microsatellite stable colorectal cancers. Of potential importance are the observations that the subset of colorectal cancers that exhibit microsatellite instability (and TβRII mutations) tend to be proximal colon cancers and have a better prognosis (stage for stage) than most sporadic colorectal cancers that do not share these genetic defects. An important recent observation was made in a murine model of invasive mammary carcinoma wherein conditional loss of TβRII in breast cancer cells resulted in chemokine-mediated recruitment of myeloid cells into the tumor stroma and promotion of invasion and metastasis.
BMP SIGNALING IN THE INTESTINAL TRACT

The role of the BMP subfamily of ligands and receptors in the GI tract is less well known than that of the TGF-β subfamily, although recent experimental observations indicate their activity in growth, development, and cancer. BMP2 and its receptors are expressed in the mouse and human colon, predominantly in mature colonocytes at the epithelial surface. BMP2 promotes apoptosis and growth inhibition in cultured colon cancer cells. Howe et al44 and Sayed et al45 have identified germ-line mutations in the gene encoding ALK3 (BMP receptor 1A) in Juvenile Polyposis Syndrome (JPS). Of note, these studies also implicated Smad4 mutations in a significant subset of JPS patients. Villin promoter-directed expression of Noggin, which is a natural BMP antagonist, in the intestine of mice as well as targeted disruption of the BMP signaling pathway through conditional inactivation of the ALK3 gene in mice resulted in similar histopathology as is observed in JPS.46,47 Similarly, BMP2 expression was found to be decreased in microadenomas of familial adenomatous polyposis (FAP) patients. These studies indicate that BMP signaling is critical for homeostasis in the intestinal tract, and loss of this function leads to precancerous conditions, such as JPS and FAP.

EMT AND TGF-β SUPERFAMILY SIGNALING

In contrast with the tumor-suppressive effects of Smad4 intrinsic to TGF-β and BMP signaling, additional data demonstrate that Smad4 may also regulate EMT, perhaps through TGF-β and/or BMP-mediated signaling interactions with Ras/MAP kinase, PI3 kinase, or Wnt signaling pathways.48 Others have reported that TGF-β and BMP ligands and their respective ligand-trap peptides have homeostatic opposing effects on EMT in some systems with TGF-β promoting and BMPs that inhibit EMT49 (reviewed in Reference 10). The balance between a differentiated epithelial phenotype and the more aggressive and invasive mesenchymal phenotype is also influenced by the abundance and activity of locally produced cytokines. Rees et al50 recently reported evidence of EMT at the leading invasive front of esophageal adenocarcinomas that consisted of loss of membrane E-cadherin expression, increased α-SMA, and vimentin, along with diffuse stromal TGF-β1 immunostaining. In contrast, BMP7 immunostaining was absent at the invasive front with occasional cells that showed increased immunoreactivity in the central tumor areas.

Inhibitor of DNA binding/differentiation (Id) proteins (Id1-4) are downstream targets of TGF-β/BMP that regulate differentiation in osteoblasts, fibroblasts, epithelial cells, and endothelial cells in a cell-type–specific and contextual manner.51 Id proteins are helix-loop-helix proteins (HLH) that can antagonize basic helix-loop-helix (bHLH) transcription factors. Id proteins also mediate mitogenic signals and play critical roles in differentiation, development and cancer52,53 (reviewed in Reference 54). Id proteins also integrate Smad signals to create a permissive or refractory nuclear environment that orders cell fate and proliferation.55

The regulation of Id expression and the role of Id proteins in gene expression may help explain how different cell types or similar cells under diverse conditions have markedly dissimilar responses to TGF-β or BMP. For example, some cells respond to TGF-β by undergoing EMT, whereas others are simply growth inhibited and/or undergo an apoptotic response. The mechanisms that underlie these differential responses are not completely understood, but studies by Kowanetz et al55 indicate that Id proteins play an important role in these cell fate decisions. In the Kowanetz
et al studies, Smad4-dependent TGF-\(\beta\) induction of EMT was associated with sustained inhibition of Id2 and Id3 proteins, whereas Smad4-dependent BMP-7 treatment induced sustained upregulation of the same. Forced overexpression of Id2 or Id3 by adenoviral infection in this study was sufficient to block TGF-\(\beta\)1-mediated EMT, whereas knockdown of Id2 induced EMT in mouse mammary epithelial cells after treatment with either TGF-\(\beta\)1 or BMP-7.\(^{55}\) Thus, Id2 levels, along with combinations of transcriptional coregulatory factors, may determine the cellular response to TGF-\(\beta\)-family signaling, which dictates whether a cell will exhibit the EMT phenotype independent of ligand treatment. Accordingly, high levels of Id2 expression are correlated with favorable prognosis in patients with primary breast cancer, and overexpression of Id2 in MDA-MB-468 breast-cancer cells results in decreased invasive capacity of the transacted cells,\(^{56}\) presumably because of a blockade of TGF-\(\beta\)-mediated EMT. Interestingly, Id proteins 1, 2, and 3 are increased in colon cancers when compared with normal tissue\(^{57}\); however, the roles and the balance of the various Id proteins in gastrointestinal tumorigenesis are still unclear.\(^{51}\) Decreased expression of Id4 has been shown to correlate with differentiation and poor prognosis in CRC.\(^{58}\) Conversely, deletion of the Id2 gene in mice resulted in failure of terminal differentiation and expansion of the proliferative zone in the intestine of the developing fetus and neonatal mice. The Id2\(^{-/-}\) mice develop intestinal epithelial dysplasia and multiple adenomas between the age of 2 and 13 months, which defines a role for Id2 as a potential tumor suppressor in the gut.\(^{59}\)

**CANONICAL WNT SIGNALING IN INTESTINAL GROWTH AND DIFFERENTIATION AND IN COLORECTAL CANCER**

Wnt signaling is a critical regulator of early embryonic development. In adult organisms, Wnt signaling regulates maintenance of intestinal crypt-progenitor cell compartments and the control of cell fate along the intestinal crypt-villus axis.\(^{4}\) This pathway is highly conserved from invertebrates to vertebrate organisms, and intracellular levels of the key signaling mediator \(\beta\)-catenin are tightly controlled. The differentiated epithelial cell tightly regulates the levels of cytosolic \(\beta\)-catenin. The Wnt pathway is normally activated by Wnt ligand and receptor interactions in an embryologic or stem cell microenvironment. Cytoplasmic accumulation and nuclear translocation of \(\beta\)-catenin is a hallmark feature of Wnt signaling.\(^{60}\)

When Wnt signaling is activated, \(\beta\)-catenin levels transiently increase in the cell cytoplasm, and \(\beta\)-catenin translocates to the nucleus where it forms a transcriptional regulatory complex with the T-cell–specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) to activate transcription of cyclin D1, c-Myc, and other downstream targets.\(^{51,62}\) In the absence of Wnt signaling, \(\beta\)-catenin levels in the cytoplasm are exceedingly low, and the \(\beta\)-catenin pools are localized to the adherens junctions in a complex with E-cadherin. Normally, in the absence of a Wnt ligand, low cytoplasmic concentrations of \(\beta\)-catenin are maintained by the scaffold protein Axin. Axin coordinates the formation of a \(\beta\)-catenin destruction complex that consists of casein kinase 1a, glycogen synthase kinase 3\(\beta\), the tumor suppressor adenomatous polyposis coli (APC), and protein phosphatase 2A (Fig 2). Together, components of this complex act to phosphorylate \(\beta\)-catenin constitutively, which leads to its recognition by the \(\beta\)-TrCP subunit of the SCF ubiquitin ligase complex (SCF\(\beta\)-TrCP),\(^{63,65}\) catalytic transfer of polyubiquitin chains to \(\beta\)-catenin, and its rapid degradation by the 26S proteasome.\(^{66}\)

Inactivating mutations in the Apc (adenomatous polyposis coli) gene or activating mutations in the \(\beta\)-catenin gene result in failure to phosphorylate \(\beta\)-catenin with stabilization and accumulation of \(\beta\)-catenin in the cytoplasm and aberrant activation of Wnt signaling. Hypophosphorylation of \(\beta\)-catenin leads to accumulation in the cytoplasm and translocation to the nucleus with resultant regulation of target-gene expression with the TCF/LEF family of transcription factors.\(^{69}\) The significance of the role of APC in colorectal cancer is revealed by the fact that over 80% of cases of sporadic CRC have truncating mutations in the Apc gene, which has often been considered the gatekeeper in the genesis of CRC because it is the earliest detectable genetic lesion in most premalignant colorectal adenomas. Aberrant stabilization of \(\beta\)-catenin in CRC can result from inactivating APC mutation or stabilizing mutations in \(\beta\)-catenin (found in 10% of sporadic colon cancers).

**ADHERENS JUNCTIONS, Wnt AND EMT**

Adherens junctions are specialized forms of cadherin-based adhesive contacts important for tissue organization in developing and adult organisms. E-cadherin is an epithelium-specific cadherin that forms protein complexes with cytoplasmic proteins (catenins) that convert the specific, homophilic-binding capacity of the extracellular domain into stable cell adhesion. Critical proteins found at the adherens junction include E-cadherin, \(\alpha\)-catenin,
E-cadherin is a calcium-dependent adhesion protein. It is the major component of the adherens junction that facilitates cell-cell communication, and E-cadherin also functions as a tumor suppressor. Junctional proteins may also serve important roles other than as structural components of intercellular junctions. E-cadherin binds and sequesters cytoplasmic β-catenin, which renders it unavailable for transcriptional activity.

β-catenin, and p120 catenin. E-cadherin is a calcium-dependent adhesion protein. It is the major component of the adherens junction that facilitates cell-cell communication, and E-cadherin also functions as a tumor suppressor. Junctional proteins may also serve important roles other than as structural components of intercellular junctions. E-cadherin binds and sequesters cytoplasmic β-catenin, which renders it unavailable for transcriptional activity.
β-catenin unavailable for signaling in the canonical Wnt/β-catenin/TCF signaling cascade. Therefore, E-cadherin like the APC protein is an important tumor-suppressor protein. Both E-cadherin and APC have essential roles in preventing accumulation of cytoplasmic β-catenin, which thereby prevent inappropriate activation of the Wnt pathway.

We have recently found that forced expression of Smad4 in Smad4-deficient SW480 colon cancer cells induced E-cadherin expression and restored its membrane localization while reducing β-catenin levels. These effects were accompanied by a marked decrease in β-catenin/TCF activity. These data implicate Smad4 as a key modulator of both Wnt signaling and EMT in these colon cancer cells.

Decreased expression or loss of membrane localization of E-cadherin has been reported in breast, colon, esophageal, gastric, pancreatic, and other carcinomas. Loss of E-cadherin from the junctional complex in epithelial malignancies is associated with invasion, metastasis, and worse prognosis.

Loss of normal expression of E-cadherin may occur by any of several well-described mechanisms. The E-cadherin gene (CDH1) is located on chromosome 16q22.1. Germline mutations in CDH1 are found in families with hereditary diffuse gastric cancer. Somatic mutations in CDH1 have been identified in sporadic diffuse gastric carcinomas, lobular breast cancers, and carcinomas of the endometrium and ovaries. Notwithstanding these examples, mutational loss of E-cadherin expression is a relatively rare event in colorectal carcinoma, but more commonly, expression of E-cadherin is distinguished by epigenetic mechanisms. These mechanisms may include regulation at the transcriptional level and posttranslational regulation of E-cadherin membrane localization and stability.

Ireton et al and Davis et al have recently demonstrated that p120 catenin interaction with E-cadherin is essential for maintenance of E-cadherin stability and function as a tumor suppressor. Transcriptional repression is also a prominent regulatory mechanism by which E-cadherin expression may be suppressed in tumors. The zinc finger transcriptional repressors of the Snail/Slug family, Sip1 and ZEB1, bind to E-boxes in the E-cadherin promoter and interact with the transcriptional corepressor CtBP, which recruits histone deacetylases to facilitate silencing of E-cadherin expression.

In addition, 2 types of bHLH proteins, E12/E47 and Twist, have also been identified as interacting with the E-cadherin promoter E-box region to repress E-cadherin expression and to induce the EMT phenotype. In colorectal carcinoma, recent immunohistochemistry analysis of E-cadherin expression in a 577 tumor tissue microarray with 10 years of clinical follow-up revealed that normal membrane localization of E-cadherin was present in only 38% of samples, whereas ~34% exhibited cytoplasmic immunolocalization of E-cadherin and 23% had complete absence of E-cadherin immunostaining. The 5- and 10-year survival rates were significantly less for those patients whose tumors exhibited aberrant or absent E-cadherin expression.

Alteration of E-cadherin expression in experimental tumor models has profound effects on the invasive and metastatic potential of human cancer cells. Forced E-cadherin expression in E-cadherin-deficient breast cancer cells decreased invasiveness in collagen matrix, and forced expression of E-cadherin in human MDA-MB-231 breast cancer cells suppressed the development of bone metastases in athymic (nude) mice. We have found that suppression of SLUG (or SNAI2) expression by siRNA restores E-cadherin expression and partially rescues the transformed phenotype in Ras-transformed rat intestinal epithelial cells.

EPIGENETICS, E-CADHERIN, AND EMT

Histone acetylation contributes to epigenetic regulation of E-cadherin. The chromatin structure has a profound impact on gene transcription. Genomic DNA is coiled around the nucleosome, which is composed of histones. Specific modifications of histones, such as acetylation or deacetylation, influence how tightly the DNA is coiled around the nucleosomes. Acetylation of histones weakens the interactions between histones and DNA, thereby loosening the coils of DNA, which allows better access of transcription factors to the promoters of target genes. Histone acetylation is catalyzed by histone acetyltransferases. Conversely, histone deacetylation is catalyzed by histone deacetylases (HDACs). Histone deacetylation results in chromatin compaction and inactivation of gene expression. At least 11 HDACs have been identified in humans, which are classified into 2 general classes: class I (HDAC1, 2, 3, 8, and 11) and class II (HDAC4, 5, 6, 7, 9, and 10). HDAC1 and 2 participate in Sin3A, NuRD, and CoRest transcriptional corepressor complexes, whereas HDAC3 complexes participate with NCoR and SMRT corepressors. Corepressor complexes are recruited to specific gene-promoter regions by several target-specific transcription factors, such as the Snail/Slug family, Sip1, and ZEB1, which are all known to be involved in the regulation of EMT. Inhibition of HDAC activity may restore E-cadherin
expression in cancer cells in which it is epigenetically repressed. Ohira et al.\textsuperscript{111} demonstrated that trichostatin A treatment could restore E-cadherin expression in E-cadherin–deficient H661 lung carcinoma cells. Witta et al.\textsuperscript{112} also demonstrated that the HDAC inhibitor MS-275 could induce E-cadherin mRNA expression in 3 different lung-cancer cell lines, in which E-cadherin repression was associated with high-level expression of the ZEB1 transcription factor. They also observed that restoration of E-cadherin expression in the E-cadherin–deficient lung-cancer cells increased the sensitivity to the growth inhibitory and apoptotic effects of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, such as gefitinib.

**TUMOR BIOLOGY AT THE INVASIVE FRONT**

Increased nuclear β-catenin localization and Wnt signaling have been demonstrated at the tumor–stromal interface of the leading invasive edge of tumors and is associated with localized evidence of EMT (loss of E-cadherin at the cell membrane) and disruption of cell polarity.\textsuperscript{81} This group recently showed that well-to-moderately differentiated pT3 rectal adenocarcinomas express basement membrane proteins, but breakdown of the basement membrane in many rectal cancers occurs in discrete regions adjacent to cancer cells at the invasive front of tumors. At the sites of breakdown of the basement membrane (the invasive front), these investigators found localized expression of the transcription factor ZEB1 and decreased expression of 1 of its targets, Lama3 (a component of laminin 5 in the basement membrane).\textsuperscript{113} A provocative observation from the Spaderna report was that the basement membrane was rebuilt in both lymph node and distant metastases.113 Also, ZEB1 cooperates with the NAD+/NADH sensor CtBP, which is recruited to the E-cadherin promoter under hypoxic conditions to repress expression of E-cadherin, and the same hypoxic conditions increase the cell motility of lung cancer H1299 cells.\textsuperscript{114}

Evidence continues to accumulate that implicates localized hypoxic stress and anaerobic metabolism in colorectal and other cancers in association with poor prognosis.\textsuperscript{115-117} Furthermore, proliferating fibroblasts at the invading tumor front exhibit endogenous markers of hypoxia, acidity, and oxidative stress.\textsuperscript{118} Interplay between tumor epithelial cells and stromal cells creates a tumor promoting microenvironment. Balance of differentiated epithelial phenotype and the more aggressive and invasive mesenchymal phenotype is also influenced by abundance and activity of locally produced cytokines.

**THE TIGHT JUNCTIONS (TJS) AND EMT, INVASION AND METASTASIS**

TJs are the most apical cell–cell contacts in epithelial and many endothelial cell sheets.\textsuperscript{119} These junctions are important for their barrier function and serve as a gate for paracellular transport as well as a fence that modulates the lateral diffusion of proteins within the cell membrane (Fig 3). Loss of cellular polarity that results from EMT requires disruption of tight junctions. Recent studies have demonstrated frequently mislocalized expression of TJ proteins from their normal apical membrane localization to the cell cytoplasm during pathologic conditions and tumorigenesis, which indicates novel roles for TJ proteins. TJ components have also been implicated in the regulation of gene expression. For example, proteins such as ASH1 can translocate from TJs to the nucleus and bind to specific transcriptional factors or function as transcriptional factors themselves.\textsuperscript{120,121}

Claudins encode integral tight junction proteins with molecular masses ranging from 20 to 27 kDa, composed of 4 transmembrane domains and cytoplasmic N- and C-terminal ends.\textsuperscript{122} Each claudin exhibits a distinct tissue-specific pattern of expression. The C-terminal domains of claudin proteins serve as a binding site for a complex set of proteins that includes many PDZ-domain proteins (ZO-1, ZO-2, and ZO-3).\textsuperscript{123} Growth factor stimulation (EGF and TGF-β) induces differential changes in claudin expression and cellular localization.\textsuperscript{124,125} Claudins can also recruit and promote the activation of promatrix metalloproteinase-2, which is a key molecule involved in tumor invasion and metastasis.\textsuperscript{126}
CLAUDIN-1 REGULATES EMT AND METASTASIS

Based on our growing awareness of EMT and the need for cells to dissolve cell–cell junctions in order to invade, we investigated the relationships of the tight junction proteins of the claudin family and EMT in colorectal cancer. We were surprised to find that claudin-1 expression is increased in human colon carcinoma and that nuclear claudin-1 localization is a frequent occurrence in metastatic lesions. Genetic manipulation of claudin-1 expression in colon-cancer cell lines induces changes in cellular phenotype with structural and functional changes in markers of EMT. To determine whether claudin-1 protein expression has a causal role in colon tumor progression and invasion, we manipulated the levels of claudin-1 expression in primary (SW480) and metastatic (SW620) colorectal-cancer cell lines derived from a single patient. Increased expression of claudin-1 in the SW480 cells resulted in no changes in cell proliferation in culture but in increased invasiveness through extracellular matrix in transwell in vitro assays. Overexpression of claudin-1 in the SW480 cells to levels similar to those expressed in SW620 cells also resulted in decreased apoptosis in response to anoikis (detachment in cell culture) and in increased expression of matrix metalloproteinases. Claudin-1 overexpression in the SW480 cells also resulted in enhanced xenograft subcutaneous tumor growth and in increased hepatic metastases when cells were injected into the splenic circulation. In contrast, RNA interference-mediated inhibition of claudin-1 levels in metastatic SW620 cells resulted in opposite effects, which included decreased invasiveness in culture, increased apoptosis in response to anoikis, decreased xenograft tumor growth, and decreased hepatic metastases in the splenic injection assay. Increased claudin-1 expression was associated with increased Wnt signaling activity, whereas inhibition of claudin-1 expression resulted in decreased Wnt signaling activity in both

Fig 3. Tight junctions. Depicted are 2-dimensional and 3-dimensional renditions of a tight junction with associated proteins. Tight junctions are closely associated areas of 2 cells whose membranes join forming a virtually impermeable barrier to fluid. Tight junction denotes a type of junctional complex in vertebrates, whereas similar junctions in invertebrates are called separate junctions. Adapted from http://www.tier-guide.com and illustrations via personal communication with Punita Dhawan, PhD.
SW480 and SW620 cell lines. Of interest, claudin-1 has been identified as a probable target of β-catenin/TCF signaling, which supports a potential role for claudin-1 dysregulation in colorectal carcinogenesis.\textsuperscript{129} Taken together, the data suggest that Wnt/β-catenin/TCF not only regulates claudin-1 expression but also that, conversely, claudin-1 expression in colon cancer cells positively impacts Wnt/β-catenin/TCF signaling.

During the course of our examination of the role of claudin-1 in EMT and metastasis, we noted an inverse correlation of claudin-1 and Smad4 expression among several colon-cancer cell lines. For example, Smad4 null lines SW480, SW620, and HT29 all expressed abundant levels of claudin-1, whereas the Smad4-expressing lines HCT116, HCT115, the immortalized rat small intestinal cells, and the immortalized mouse colonocyte line did not express detectable levels of claudin-1. Furthermore, we found an inverse relationship between claudin-1 levels and Smad4 levels in tumor lysates assessed by immunoblotting. We hypothesized that Smad4 may regulate the expression of claudin-1. We tested this hypothesis by expressing Smad4 in claudin-1-expressing colon-cancer cells that were Smad4 deficient, and we found that expression of Smad4 was sufficient to silence claudin-1 expression in both SW480 and HT29 cells. Smad4 expression in the SW480 cells results in a more epithelial phenotype, increased expression of E-cadherin, redistribution of beta-catenin to the cell membrane, decreased Wnt-specific reporter activity and decreased invasiveness.\textsuperscript{69} Consistent with these findings, Schwartz-Waldhoff et al\textsuperscript{130} and Muller et al\textsuperscript{131} noted that stable Smad4 expression in SW480 cells is correlated with decreased xenograft tumorigenicity and associated E-cadherin induction in vivo. To reiterate, if Smad4 is re-expressed in SW480 cells that are both Smad4 and E-cadherin deficient, the result is the induction and membrane localization of E-cadherin, suppression of Wnt signaling, and suppression of invasiveness and xenograft growth in nude mice.

**SUMMARY**

Metastases account for most fatalities caused by colon cancer. The metastatic phenotype, or the ability of normally polarized epithelial cells to separate from their tissue of origin, invade basement membrane, survive transit through the bloodstream, and recolonize at distal sites recapitulates a developmental process known as EMT. Regulators of EMT in adult epithelial cells, which includes the TGF-β and Wnt signal transduction systems, are important mediators of embryonic tissue development, tissue homeostasis, and wound healing. In addition, they have important roles in tumor cell behaviors that are still being defined. Research into the potential epigenetic and reversible nature of EMT, and the downstream effector signaling pathways that lead to EMT, may enable the identification of novel therapeutic interventions to disrupt the metastatic process. Through a better understanding of the biological mechanisms that contribute to metastasis lies the hope of translating these discoveries into new treatments that will improve the survival of cancer patients.

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