Therapeutic Targeting of Epithelial Plasticity Programs: Focus on the Epithelial-Mesenchymal Transition

Reem Malek^a Hailun Wang^a Kekoa Taparra^{a, d} Phuoc T. Tran^{a–d}

Departments of ^aRadiation Oncology and Molecular Radiation Sciences and ^bOncology, Sidney Kimmel Comprehensive Cancer Center, ^cDepartment of Urology, and ^dProgram in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

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Abstract

Mounting data points to epithelial plasticity programs such as the epithelial-mesenchymal transition (EMT) as clinically relevant therapeutic targets for the treatment of malignant tumors. In addition to the widely realized role of EMT in increasing cancer cell invasiveness during cancer metastasis, the EMT has also been implicated in allowing cancer cells to avoid tumor suppressor pathways during early tumorigenesis. In addition, data linking EMT to innate and acquired treatment resistance further points towards the desire to develop pharmacological therapies to target epithelial plasticity in cancer. In this review we organized our discussion on pathways and agents that can be used to target the EMT in cancer into 3 groups: (1) extracellular inducers of EMT, (2) the transcription factors that orchestrate the EMT transcriptome, and (3) the downstream effectors of EMT. We highlight only briefly specific canonical pathways known to be involved in EMT, such as the signal transduction pathways

TGF β , EGFR, and Axl-Gas6. We emphasize in more detail pathways that we believe are emerging novel pathways and therapeutic targets such as epigenetic therapies, glycosylation pathways, and immunotherapy. The heterogeneity of tumors and the dynamic nature of epithelial plasticity in cancer cells make it likely that targeting only 1 EMT-related process will be unsuccessful or only transiently successful. We suggest that with greater understanding of epithelial plasticity regulation, such as with the EMT, a more systematic targeting of multiple EMT regulatory networks will be the best path forward to improve cancer outcomes.

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Introduction

There is an ever-growing body of evidence nominating epithelial plasticity programs such as the epithelial-mesenchymal transition (EMT) as ideal, clinically relevant targets for the treatment of malignant tumors – both primary and metastatic [van Denderen and Thompson,

R.M. and H.W. contributed equally to this work.

Abbreviations used in this paper

5-aza	5-azacytidine or 5-aza-2'-deoxycytidine
5SGlcNAc	5-thioglucoamine
CpG	cytosine-guanine
DNMT	DNA methyltransferase
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
GSK	glycogen synthase kinase
HDACs	histone deacetylases
LSD1	lysine-specific demethylase 1
miRNAs	micro RNAs
MMP	matrix metalloproteinase
O-GlcNAc	O-linked β -N-acetylglucosamine
OGT	O-GlcNAc transferase
OSCC	oral squamous cell carcinoma
STAT3	signal transducer and activator of transcription 3
TF	transcription factor
UCH-L1	ubiquitin C-terminal hydrolase-L1

2013; Davis et al., 2014b]. In addition to the widely realized role of EMT in increasing cancer cell motility and invasiveness during cancer metastasis, the EMT has also been implicated in allowing cancer cells to avoid tumor suppressor pathways of apoptosis, anoikis, and cellular senescence during tumorigenesis [Sanchez-Tillo et al., 2012; Tiwari et al., 2012]. Moreover, the observation that cancer cells surviving treatment are enriched in EMT markers and the preclinical data linking EMT to innate treatment resistance enhances the need to develop new pharmacological therapies to target epithelial plasticity in cancer [Davis et al., 2014b]. Despite advances in the basic and translational understanding of the EMT (many of which are reviewed in the accompanying articles in this current issue), key questions remain regarding the benefit and practicality of targeting EMT in cancer patients. Many of these key issues have been discussed previously and we refer readers to these excellent references: van Denderen and Thompson [2013], Davis et al. [2014b]. In this review we will concentrate primarily on pathways and agents that can be used to target the EMT in cancer.

We have organized our review of this subject into 3 broad groups classified based on their spatial and temporal role during EMT: (1) extracellular inducers of EMT, (2) the transcription factors that orchestrate the EMT transcriptome, and finally (3) the downstream molecular effectors of EMT (Fig. 1). We have also chosen to structure our review so as to highlight only briefly specific canonical pathways known to be involved in EMT and potential therapeutic agents for these canonical pathways

(e.g., signal transduction pathways such TGF β , EGFR, and Axl-Gas6). At the same time, we aimed to introduce in more detail pathways that we believe are emerging novel pathways and therapeutic targets or that in our opinion deserve greater attention, i.e., epigenetic therapies, glycosylation pathways, and immunotherapy.

Targeting Extracellular Inducers That Initiate the EMT Program

Many signals from the tumor microenvironment can induce EMT in cancer cells. These signals include paracrine-autocrine soluble mediators such as growth factors and cytokines, hypoxia, and extracellular matrix (ECM) components and biophysical properties of the ECM, such as stiffness [Lester et al., 2007; Lo et al., 2007; Polyak and Weinberg, 2009; Yadav et al., 2011].

The primary autocrine-paracrine inducers of EMT are from the TGF β , BMP, and other growth factor (EGF, HGF, IGF, and FGF) signal transduction pathway families [Lamouille et al., 2014]. Potential inhibitors of EMT inducers have been identified using high-throughput drug screening. For example, connectivity mapping using global gene expression profiles of TGF β -induced EMT was used to identify rapamycin and 17-AAG as potential inhibitors of EMT [Reka et al., 2011]. Furthermore, inhibitors of ALK5, MEK, and SRC were shown to inhibit EMT downstream of EGF, HGF, and IGF-1 [Reka et al., 2011; Chua et al., 2012]. We will only briefly highlight further examples of molecules targeting these pathways below.

TGF β Pathway

The most well-known inducer of EMT remains TGF β (transforming growth factor beta) and there is considerable interest in the inhibition of TGF β signaling as a strategy to inhibit EMT-induced tumor cell invasion and dissemination. Several inhibitors in the preclinical pipeline have been identified. SB431542 can inhibit TGF β R kinase, thus blocking TGF β -induced EMT in pancreatic cancer cells [Halder et al., 2005]. SD-093 and LY-580276 are competitive inhibitors for the ATP-binding site of TGF β RI kinase. These drugs blocked EMT and cancer cell migration in many cell types [Subramanian et al., 2004; Peng et al., 2005]. More recently, specific TGF β inhibitors, EW-705, EW-7195, and EW-7197, have been shown to disrupt EMT in TGF β -treated breast cancer cells as well as in vivo using the 4T1 orthotopic xenograft mouse model [Nagaraj and Datta, 2010; Park et al., 2011a, b].

Fig. 1. Potential therapeutic targets arranged based on their spatial-temporal roles during the EMT.

Compounds in clinical trials that may interfere with the EMT by blocking the TGF β pathway include LY2157299, a TGF β 1 receptor inhibitor that specifically downregulates the phosphorylation of SMAD2. This compound has shown antitumor activity in animal models of breast, colon, lung, and hepatocellular carcinoma [Bueno et al., 2008; Rodon et al., 2013]. LY2157299 has also shown antitumor effects in patients with glioblastoma and hepatocellular carcinoma [Rodon et al., 2013]. This compound is currently being tested in the following 4 clinical trials: phase Ib/IIa in stages II–IV of unresectable pancreatic cancer of LY2157299 combined with gemcitabine versus gemcitabine plus placebo (NCT01373164); phase II in HCC patients with or without sorafenib (NCT01246986); a phase Ib/IIa study combining LY2157299 with standard temozolomide-based radiochemotherapy in patients with newly diagnosed malignant glioma (NCT01220271), and a phase II study of LY2157299 monohydrate monotherapy or LY2157299 monohydrate plus lomustine therapy compared to lomustine monotherapy in patients with recurrent glioblastoma (NCT01582269). In addition to inhibitors of TGF β , a human anti-TGF β antibody (fresolimumab) has recently undergone a phase I clinical trial in patients with melanoma or renal cell carcinoma. It has demonstrated acceptable safety and toxicity and the maximum dose of 15 mg/kg was established for phase II clinical trials. The antibody is being tested for antitumor activity in a phase II trial – fresolimumab and radiotherapy in metastatic breast cancer (NCT01401062).

EGF Pathway

EGF (epidermal growth factor) can induce EMT via signal transducer and activator of transcription 3 (STAT3) upregulation of the EMT transcription factor (TF), Twist1, in breast cancer cells. The EGFR kinase inhibitor AG1478 has been shown to block this activation in vitro [Lo et al., 2007]. Erlotinib, another EGFR tyrosine kinase inhibitor, is approved for the treatment of advanced NSCLC patients [Shepherd et al., 2005; Capuzzo et al., 2010]. Importantly, the response to erlotinib is inversely correlated with lower levels of E-cadherin and higher levels of vimentin and Zeb1, suggesting that EMT may confer resistance to erlotinib [Witta et al., 2012].

Axl-Gas6 Pathway

The Axl receptor tyrosine kinase and its ligand, Gas6 (growth-arrest specific 6), have been implicated in maintaining the mesenchymal phenotype in some cell lines [Gjerdrum et al., 2010]. Rigel Pharmaceuticals Incorporated has a small molecular inhibitor of Axl kinase, BGB324 (formerly R428), which inhibits breast cancer metastasis alone and synergizes with cisplatin to inhibit liver metastases [Holland et al., 2010]. These studies indicate that the inhibition of some cell surface receptors may have roles beyond the initiation of EMT and may be used to target the mesenchymal cell type at later stages of the metastatic cascade.

Hypoxia

Hypoxia is a well-characterized inducer of EMT in cancer cells [Marie-Egyptienne et al., 2013]. The ubiquitin C-terminal hydrolase-L1 (UCH-L1) is a deubiquitinating enzyme that hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin. UCH-L1 deubiquitinated HIF-1 α promoted protein stability and resulted in the EMT and metastasis of different cancer cells. A specific small molecule inhibitor, LDN57444, targeting UCH-L1, significantly inhibited HIF-1 α activity in the presence of endogenous UCHL1 expression and suppressed EMT, reducing the incidence of distant tumor metastases [Jang et al., 2011; Goto et al., 2015]

Extracellular Matrix

Remodeling of the ECM and changes to cell interactions with the ECM are essential in the initiation and progression of EMT. As epithelial cells differentiate into mesenchymal cells they downregulate some epithelial integrins, but activate the expression of others; some of these newly expressed integrins have key roles in EMT progression [Yilmaz and Christofori, 2009]. Changes to the integrin repertoire during EMT correlate with the increased expression of proteases, such as the matrix metalloproteinases (MMP)2 and MMP9, thus enhancing ECM protein degradation and enabling invasion [Nisticò et al., 2012]. Some proteases and integrins can mediate invasion, such as $\alpha\text{v}\beta 6$, by activating the differentiation factor TGF β that is stored in a latent form in the ECM [Sheppard, 2005]. This exposes the cells to increased TGF β signaling, which promotes EMT and stimulates the expression of ECM proteins, such as collagens and fibronectin, enhancing the remodeling of the ECM into a matrix with a different composition and biophysical properties.

One limitation to targeting the ECM is that several of its components may work together to induce EMT and resistance may emerge quickly if only 1 of these pathways is impaired. Redundancy may be overcome by targeting multiple coacting intracellular signal transduction pathways. Furthermore, an additional strategy may be to target one of the crucial downstream signal transduction pathways that can activate an EMT transcription program. For example, there was compelling preclinical data for the use of MMP inhibitors for cancer therapy; however, most MMP inhibitors failed in clinical trials likely due to the complexity of the metastatic process [Sparano et al., 2004]. In addition to the MMP inhibitors MMP1 and MMP2, a microarray signature for lung-specific metastasis included the EGFR ligand, epiregulin, and COX2.

Administration of a cocktail of inhibitors of these proteins reduced the tumor volume and pulmonary metastases [Gupta et al., 2007].

Targeting Orchestrators of EMT

EMT is regulated by a core group of EMT-TFs, such as Snail1, Snail2 (Slug), a basic helix-loop-helix family (Twist1/2, E47, E2-2), and Zeb1/2 [Mikheeva et al., 2010; Kahlert et al., 2011; Lamouille et al., 2014; Teng and Li, 2014]. In addition, there is some indication that additional noncanonical transcription factors are also capable of inducing EMT in cancer cells, such as the T-box transcription factor Brachyury [Fernando et al., 2010], and thus may also serve as attractive therapeutic targets [Hamilton et al., 2013; Palena and Hamilton, 2015]. The function of these transcription factors in promoting and sustaining the neoplastic phenotype, cancer progression, and therapy resistance has been studied, and they present appealing targets to block EMT in tumors.

Credentialing EMT-TFs as Cancer Therapeutic Targets

There is ample evidence for the effectiveness of inhibiting EMT-TFs in blocking EMT in various normal and cancer cells. Inhibition of Twist1 was first shown to reduce the frequency of lung metastases in the highly metastatic 4T1 mammary carcinoma cell line mouse model of breast cancer [Yang et al., 2004]. We and others have shown that Twist1 downregulation can activate latent tumor suppressor programs of oncogene-induced senescence and apoptosis in oncogene-driven cancer cells in vitro and in vivo [Ansieau et al., 2008; Morel et al., 2012; Tran et al., 2012; Burns et al., 2013]. Twist1 inhibition also reduced the cell invasiveness in metastatic oral squamous cell carcinoma (OSCC) as shown in vitro and in vivo using an orthotropic model of OSCC [da Silva et al., 2014]. Twist1 inhibition similarly reduces prostate cancer cell prometastatic phenotypes and alters sensitivity to both chemotherapy and hormonal therapy [Kwok et al., 2005; Shiota et al., 2010; Gajula et al., 2013; Shiota et al., 2013, 2014]. Many additional examples of credentialing the Snail family and Zeb1/2 EMT-TFs as cancer therapeutic targets are available [Nemeth and Kosz, 1989; Li et al., 2011; Tania et al., 2014].

EMT has also been shown to mediate resistance to traditional therapeutic agents and targeting EMT-TFs could increase cellular sensitivity to these agents. Renewed interest in this approach has come from a study using trans-

genic mouse models of *Kras*^{G12D}-induced pancreatic cancer [Zheng et al., 2015]. The group showed that deletion of the EMT-TFs Twist1 and Snai1 led to an increase in the expression of nucleoside transporters that contributed to enhanced sensitivity to gemcitabine treatment. Similarly, knockdown of Snai1 or Twist1 in lung carcinoma cell lines can restore chemosensitivity to cisplatin [Zhuo et al., 2008a, b]. Silencing Zeb1 in a panel of pancreatic cell lines increased their sensitivity to cancer therapeutics.

One caveat to this approach is the difficulty of identifying EMT-TFs that are active in tumor tissues. Expression of EMT-TFs can be regulated posttranscriptionally and/or posttranslationally [Zheng and Kang, 2014]; thus, their levels as determined by qRT-PCR or IHC may not correlate with activity. Furthermore, the spatial and temporal heterogeneity in expression of the various EMT-TFs make them a challenging therapeutic target [Voulgari and Pintzas, 2009; Iwatsuki et al., 2010].

Targeting Signaling Pathways That Regulate EMT-TFs

Direct inhibition of transcription factors has been chemically challenging and studies reporting direct targeting of EMT-TFs are lacking. Another complimentary approach is to target regulators of EMT-TFs. We will summarize a few of these agents below. The cyclin-dependent kinase 4/6 inhibitor PD0332991 has been shown to downregulate Zeb1 expression in breast cancer cells [Arima et al., 2012]. The transcription factor STAT3 has critical roles in the regulation of EMT-TFs [Balanis et al., 2013; Davis et al., 2014a; Yuan et al., 2015] and several small molecule inhibitors of STAT3 have been developed, including Stattic [Schust et al., 2006], S3I-201 [Siddiquee et al., 2007], OPB-51602 [Ogura et al., 2015], and OPB-31121 [Bendell et al., 2014]. Sulforaphane has been shown to downregulate Twist1 and vimentin, leading to a decrease in stem-like properties in pancreatic cancer cell lines [Srivastava et al., 2011]. Moscatilin (component of the orchid *Dendrobium loddigesii*) suppresses the migration and metastasis of human breast cancer MDA-MB-231 cells by targeting the Akt-Twist-dependent pathway [Pai et al., 2013]. Fucoxanthin (a brown seaweed polysaccharide) was also described to inhibit EMT in breast cancer cell lines such as 4T1 and MDA-MB-231 through decreased Twist1, Snai1, and Snai2 expression [Hsu et al., 2013]. In head and neck cancer-derived sphere cells, quercetin (a major polyphenol and flavonoid, commonly detected in many fruits and vegetables) minimizes migration ability partially by decreasing the production of Twist, N-cadherin, and vimentin [Chang et al., 2013]. Thymoquinone (major active ingredient of the plant

Nigella sativa) increases Twist1 promoter methylation, resulting in Twist1 downregulation and the inhibition of migration, invasion, and upregulation of E-cadherin [Khan et al., 2015]. Imipramine blue promotes degradation of the EMT inducer Twist1 by enhancing FBXL14 (F-box and leucine-rich repeat protein 14) polyubiquitination-mediated destruction of Twist1 [Yang et al., 2016].

We also have our own data with the harmala alkaloids, which we isolated from a bioinformatic-chemical screen for Twist1 pathway inhibitors, demonstrating the post-translational downregulation of the Twist1-E12/E47 heterodimeric complex in lung cancer cells in vitro and in vivo [Burns et al., unpubl. data]. Given our previous data credentialing Twist1 as a therapeutic target in various oncogene-driven lung cancer subtypes [Tran et al., 2012; Burns et al., 2013], we believe the development of this novel class of harmala Twist1 inhibitor compounds could have a significant clinical impact.

Epigenetic Therapy Targeted against EMT-TFs

Epigenetic changes are defined as heritable changes in gene expression that occur without changes in the DNA sequence and include DNA methylation and histone modification. Some consider RNA transcriptional regulation by micro-RNAs (miRNAs) to be another form of epigenetic regulation. DNA methylation is by far the best-studied epigenetic change in cancer cells. DNA methylation, the transfer of a methyl group to the carbon-5 position of cytosines, occurs almost always within the context of cytosine-guanine (CpG) dinucleotides in the promoter regions of genes. In cancer, CpG islands can become hypermethylated, contributing for example to the silencing of tumor suppressor genes like p53 [Baylin, 2005; Soto-Reyes and Recillas-Targa, 2010]. DNA methylation at CpG dinucleotides occurs through the action of DNA methyltransferase (DNMT) enzymes [Bird, 1986; Okano et al., 1998]. Noncoding microRNAs are also critical players in the neoplastic phenotype and have also been shown to have important regulatory roles in EMT [Hayes et al., 2014]. Noncoding miRNAs are small (19–25 nucleotides long), single-stranded RNAs that control gene expression by targeting mRNA transcripts, leading to their translational repression or degradation. Multiple miRNAs have been reported to suppress the EMT process [Gregory et al., 2008b]. The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) plays a critical regulatory role in EMT suppression, mainly through targeting ZEB1/2 [Koutsaki et al., 2014], and is downregulated in cancer cells during EMT [Bracken et al., 2008; Burk et al., 2008; Gregory et al., 2008a, b].

Interestingly, the CpG island near the miRNA-200c transcription start is unmethylated in epithelial cell lines, but is heavily methylated in transformed mesenchymal cells and invasive tumor cells [Davalos et al., 2012]. This epigenetic DNA methylation process can be inhibited through the use of 5-azacytidine or 5-aza-2'-deoxycytidine (5-aza), nucleoside analogs that bind to and inhibit DNMTs, leading to hypomethylation and increased gene expression [Juttermann et al., 1994; Bender et al., 1998; Kurkjian et al., 2008]. In fact, epigenetic therapy in the form of 5-aza treatment has been shown to upregulate the miRNA-200 family and reverse the EMT of mammary epithelial cells [Eades et al., 2011; Bi et al., 2015].

Histone lysine methylation has been extensively linked to both gene activation and gene repression events, depending on which lysine residues in the histones are methylated, and how many methyl groups are present [Lachner and Jenuwein, 2002]. Polycomb group protein complexes PRC1–4 play key roles in transcriptional silencing [Lachner and Jenuwein, 2002]. As shown by Herranz et al. [2008], Snail recruits the PRC2/EZH2 to the promoter of *CDH1*, trimethylates Lys27 on histone H3 (H3K27m3), and mediates the transcriptional repression of *CDH1* [Cao et al., 2008; Herranz et al., 2008]. G9a (also known as KMT1C or EHMT2) is a major euchromatin methyltransferase responsible for H3K9me2 levels [Tachibana et al., 2002]. Recent findings revealed that Snail binds with G9a and recruited G9a and DNMT to the *CDH1* promoter, leading to DNA methylation. Knockdown of G9a restored E-cadherin expression by suppressing H3K9me2 and blocking DNA methylation, and resulted in the suppression of tumor growth and lung colonization with in vivo models of breast cancer metastasis [Dong et al., 2012]. SET8 (also known as PR-Set7/9, SETD8, KMT5A) is a member of the SET domain-containing methyltransferase family specifically targeting H4K20 for monomethylation. Yang et al. [2012] demonstrated that the Twist transcription factor interacts with the monomethyltransferase SET8, which acts as a dual epigenetic modifier on the promoters of E- and N-cadherin to induce the expression of N-cadherin and the repression of E-cadherin. Lysine-specific demethylase 1 (LSD1) was the first histone demethylase identified that catalyzes the removal of mono- and dimethylation marks on histone H3K4 and H3K9 [Shi et al., 2004; Metzger et al., 2005]. As shown by Lin et al. [2010], Snail recruits the histone demethylase LSD1 (KDM1A, AOF2) to dimethylate Lys4 on histone H3 (H3K4m2) and mediates the transcriptional repression of Snail target genes, such as *CDH1*.

Drugs targeting histone methyltransferases and demethylases are still in their infancy. Several compounds have been developed recently, such as the G9a inhibitors UNC0638 and BRD4770 and the EZH2 inhibitor EPZ005687. Those compounds have already demonstrated antitumor activity on breast cancer cells, pancreatic cancer cells, and lymphoma cells in vitro [Vedadi et al., 2011; Knutson et al., 2012; Wagner and Jung, 2012; Yuan et al., 2012]. However, their effects on EMT and migration have yet to be explored.

Targeting Glycosylation Pathways That Regulate EMT-TFs

Glycosylation is a form of cotranslational and post-translational modification that plays important roles during embryogenesis [Haltiwanger and Lowe, 2004]. Aberrant glycosylation has also been associated with the malignant transformation of cells [Kim and Varki, 1997] and has been discovered in many cancers, including melanoma [Pochec et al., 2013], colon [Holst et al., 2015], breast [Guo and Abbott, 2015], lung [Lemjabbar-Alaoui et al., 2015], liver [Mehta et al., 2015], and prostate cancers [Drake et al., 2015]. Two major forms of protein glycosylation are N-glycosylation and O-glycosylation. We will focus on the O-glycosylation known as O-linked β -N-acetylglucosamine (O-GlcNAc), which is conserved among multicellular eukaryotes. This modification regulates diverse cellular processes, including gene expression [Gambetta et al., 2009; Hanover et al., 2012], stress response [Zachara et al., 2004; Ohn et al., 2008], and circadian rhythm [Kim et al., 2012; Li et al., 2013]. The O-GlcNAcylation level was demonstrated to be globally elevated and correlated with aggressiveness in a number of malignancies, including breast [Gu et al., 2010; Krzeslak et al., 2012; Champattanachai et al., 2013], prostate [Gu et al., 2014; Kamigaito et al., 2014], lung [Mi et al., 2011], pancreas [Ma et al., 2013], liver [Zhu et al., 2012], and colon cancers [Mi et al., 2011; Yehezkel et al., 2012; Phueaouan et al., 2013]. While the O-GlcNAcylation modification is analogous to ATP phosphorylation in many ways, unlike the hundreds of kinases, protein O-GlcNAcylation is regulated by only 2 enzymes: O-GlcNAc transferase (OGT) adds O-GlcNAc on proteins [Haltiwanger et al., 1990; Lubas et al., 1997], and O-GlcNAcase removes O-GlcNAc [Gao et al., 2001]. These 2 enzymes act together to dynamically modulate the levels of O-GlcNAc on proteins within cells. The addition of O-GlcNAc generally stabilizes modified proteins and prevents them from degradation by the ubiquitin-proteasome system [Yang et al., 2006; Park et al., 2010; Li et al.,

2013; Ruan et al., 2013; Olivier-Van Stichelen et al., 2014]. One important class of proteins heavily O-GlcNAcylated are TFs. Early O-GlcNAc proteome analyses suggested over 25% of known O-GlcNAcylated proteins were in fact transcription factors [Love and Hanover, 2005]. For the majority of these TFs, O-GlcNAcylation serves as a direct or indirect competitor of key phosphorylation sites. Particularly relevant to EMT is the O-GlcNAcylation of Snail. It is known that Snail activity is regulated by phosphorylation. Upon serial phosphorylation by CK1 and glycogen synthase kinase (GSK)-3 β , Snail is primed for nuclear export, β -TrCP ubiquitination, and subsequent proteosomal degradation [Zhou et al., 2004; Xu et al., 2010]. However, in hyperglycemic conditions, Snail is O-GlcNAcylated to prevent GSK-3 β phosphorylation, thus leading to Snail stabilization, E-cadherin repression, and cancer cells to undergo EMT-mediated migration [Park et al., 2010]. Other than Snail, O-GlcNAcylation occurs on a number of transcription factors generally relevant to EMT including c-Myc [Chou et al., 1995], β -catenin [Olivier-Van Stichelen et al., 2014], and NF- κ B [Yang et al., 2008; Allison et al., 2012].

Several OGT inhibitors have so far been developed. An OGT inhibitor identified in a screen of a small molecule library reduces breast cancer cell hyper-O-GlcNAcylation and blocks anchorage-independent growth [Chou et al., 1995; Caldwell et al., 2010]. However, the potency of the inhibitor is relatively low. 5-thioglucosamine (5SGlcNAc) and its per-O-acetylated analog Ac-5SGlcNAc have been developed as alternative OGT inhibitors [Gloster et al., 2011]. Ac-5SGlcNAc can be converted into UDP-5SGlcNAc via the GlcNAc salvage pathway, thereby competing with UDP-GlcNAc and inhibiting O-GlcNAcylation [Gloster et al., 2011]. It has been shown that Ac-5SGlcNAc is able to reduce pancreatic cancer cell hyper-O-GlcNAcylation and inhibit cell growth in vitro [Ma et al., 2013]. However, the effects of these compounds on EMT were not reported in either of these studies. Other OGT inhibitors, such as ST045849 and Alloxan, have been shown to inhibit migration and proliferation in mouse embryonic stem cells and retinal pericytes, respectively [Gurel et al., 2013; Jeon et al., 2013].

The monosaccharide GlcNAc is the product of the hexosamine biosynthesis pathway which produces UDP-GlcNAc [Hanover et al., 2012]. Therefore, a second reasonable target is to inhibit GFPT2, the rate-limiting enzyme in the hexosamine biosynthesis pathway. Currently, DON and azaserine are commonly used GFPT2

inhibitors [James et al., 2002]. Both compounds demonstrated potent inhibition of cell viability [Catane et al., 1979; Olsen et al., 2015]. DON also exhibit as much as 10 times more cytotoxicity upon 2 types of murine leukemia cell as compared to normal embryonic fibroblasts [Rosenfeld and Roberts, 1981]. Systemic administration of DON in the VM-M3 murine tumor model also led to a profound decrease in tumor proliferation and inhibition of visceral metastases [Shelton et al., 2010]. However, off-target effects are likely with DON as it can inhibit a number of glutamine utilizing enzymes [Thanagavelu et al., 2014].

Immunotherapy Approaches to Target EMT-TFs

When cancer cells undergo EMT, there are profound morphological changes dictated by accompanying gene expression and protein changes, many of which are silenced following embryogenesis. It is possible that the immune system may be able recognize and act on these neoplastic EMT changes. Ardiani et al. [2014] demonstrated that in principal this concept is feasible. They expressed Twist1 in heat-killed yeast and immunized tumor-bearing mice with this recombinant yeast. After vaccination, they confirmed that both CD4+ and CD8+ Twist-specific T-cell responses were induced. Surprisingly, not only did the primary tumor grow much slower in the recombinant Twist1-vaccinated mice, the number of clonogenic metastatic cells was also significantly decreased after Twist1 vaccination. As remarked on previously, the non-canonical EMT-TF Brachyury has also served as an attractive therapeutic target and was used in a similar heat-killed yeast vaccination platform (designated as GI-6301), demonstrating similar immunologic and in vivo tumor responses [Hamilton et al., 2013; Palena and Hamilton, 2015]. Based on these results, a phase I clinical trial of GI-6301 was initiated and accrued recently in patients with advanced tumors (NCT01519817). A follow-up phase II trial in chordoma patients in combination with standard of care radiotherapy is ongoing (NCT02383498). Preclinical results from these seminal studies provide evidence that EMT-TFs can be processed in the cytoplasm and presented on the cell surface by either MHC class I or MHC class II molecules, offering a novel strategy to target EMT-TFs in cancer. These EMT-TF vaccination strategies should be explored further in combination with traditional cancer therapies such as chemotherapy and radiation, but also in combination with other complimentary immunotherapies.

Targeting Effectors of EMT

EMT is frequently characterized by acquisition of a plethora of mesenchymal cell markers such as vimentin, N-cadherin, and fibronectin, and loss of epithelial markers such as E-cadherin and cytokeratins. These changes are associated with increased cell mobility and invasion, and are frequently found in circulating tumor cells and cells from metastatic lesions [Bednarz-Knoll et al., 2012; Yu et al., 2013]. Targeting these proteins may benefit patients with more advanced disease by eliminating existing metastatic cells. In addition, this same approach may target cancers of mesenchymal origins such as sarcomas.

E-Cadherin

It has been shown that transfection of E-cadherin in highly mesenchymal and invasive cells can revert poorly differentiated carcinoma cells into a more differentiated state with a minimal invasive phenotype [Luo et al., 1999; Wong and Gumbiner, 2003; Witta et al., 2006]. E-cadherin has been established as a tumor suppressor in many cancers, including breast cancer [Berx and van Roy, 2001], HCC [Zhai et al., 2008], melanoma [Molina-Ortiz et al., 2009], and esophageal cancer [Ling et al., 2011]. In contrast, E-cadherin is upregulated in ovarian cancer cells that metastasize to the peritoneum and omentum [Köbel et al., 2011]. Additionally, several studies suggest that E-cadherin may promote tumor progression in various epithelial cancers, such as ovarian, breast, or brain cancer [Rodriguez et al., 2012]. Thus, targeting E-cadherin for therapeutic gain is challenging, owing to its multifaceted and context-specific role in carcinogenesis. Despite these challenges, Gupta et al. [2009] used a high-throughput screening approach to identify compounds that may selectively target E-cadherin-negative breast epithelial cells as compared to E-cadherin-positive cells in mouse models. Salinomycin, a potassium ionophore, was identified as having significant toxicity against mesenchymal-type breast cancer cells (independent of the mechanism used to induce EMT in these cells). This is in contrast to treatment with paclitaxel, which significantly increased the enrichment for EMT and stem cell markers in breast cancer cell lines surviving chemotherapy.

Another orthogonal strategy that focuses on E-cadherin is targeting the epigenetic regulatory machinery, as described above. *CDH1* has a large CpG island in the 5'-proximal promoter region, which shows aberrant DNA methylation in many different human cancers and correlates with reduced E-cadherin protein expressions [Graff et al., 1995; Yoshiura et al., 1995]. It was reported

that hypermethylation at the *CDH1* promoter inversely correlated with the expression of E-cadherin in 11 different cancer cell lines, and treatment with 5-aza caused the reexpression of E-cadherin, reversion of spindle-shaped cells to cells with epithelial morphology, and reduced metastasis in these cancer cells [Yoshiura et al., 1995; Nam et al., 2004]. In an in vivo model of breast cancer using the cell-line MDA-MB-435S, 5-aza was able to restore E-cadherin expression and suppress metastasis formation as well as primary growth, possibly through E-cadherin up-regulation [Nam et al., 2004].

Histones, the major components of chromatin, can undergo multiple posttranslational modifications, such as acetylation by histone acetyltransferases and deacetylation by histone deacetylases (HDACs), resulting in chromatin that is permissive for transcriptional activation and repression, respectively [Struhl, 1998; Archer and Hodin, 1999]. Histone deacetylation can be inhibited by HDAC inhibitors, which promotes accumulation of the acetylated form of histone proteins, leading to less condensed chromatin and the reexpression of silenced genes. HDAC inhibitors have been shown in preclinical studies to selectively target cancer cells by inducing apoptosis, cell cycle arrest, suppression of tumor angiogenesis, and metastasis and invasion at least partially through upregulating E-cadherin [Shaker et al., 2004]. The first HDAC inhibitor identified was butyrate, which was shown to induce cell-cycle arrest and to increase cell-cell adhesion in breast cancer cells. Interestingly, this effect has been shown to be reversible upon the addition of E-cadherin antibodies [Kondo et al., 1998]. Butyrate was also found to upregulate E-cadherin expression in many other cancer cells, including colon cancer, liver cancer, and endometrial carcinoma cells [Barshishat et al., 2000; Masuda et al., 2000; Takai et al., 2004]. Other HDAC inhibitors, such as Trichostatin A and SAHA (suberoylanilide hydroxamic acid), are also capable of stimulating E-cadherin expression in endometrial carcinoma cell lines [Takai et al., 2004]. Sodium butyrate is currently being tested in phase I and II trials. SAHA, structurally similar to trichostatin A, was approved by the US FDA in 2006 for the treatment of advanced and refractory primary cutaneous T-cell lymphoma, and is marketed as vorinostat (Zolinza®) [Mann et al., 2007; Ma et al., 2009].

N-Cadherin

Inhibition of N-cadherin has been proposed in several studies. Blocking N-cadherin using the peptide ADH-1 was shown to prevent tumor progression in a mouse model of pancreatic cancer [Shintani et al., 2008]. Targeting N-cadherin using a monoclonal antibody approach has also been

shown to inhibit prostate cancer cell invasion and reduce metastasis formation [Tanaka et al., 2010]. In multiple myeloma cells, the N-cadherin neutralizing antibody impeded the proliferation of cancer cells [Sadler et al., 2013]. Quercetin, a natural polyphenol found in vegetables, reduced the migration ability of head and neck cancer cells partially due to the decreased production of N-cadherin, vimentin, and the EMT-TF, Twist1 [Chang et al., 2013].

Vimentin

Another marker of EMT, and thus a potentially important effector, is vimentin [Satelli and Li, 2011]. There are only a few reports showing the direct inhibition of vimentin using bioactive compounds. Withaferin-A is a compound extracted from *Withania somnifera* that can promote the degradation of vimentin in breast and lung cancer cell lines, leading to the inhibition of cell migration and invasion and apoptosis at higher concentrations and inhibition of metastasis formation in in vivo xenograft studies [Lahat et al., 2010]. Two compounds, silibium and flavonolignan, were shown to inhibit the invasion, motility, and migration of cancer cells through the downregulation of vimentin in cancer cell lines and mouse models [Singh et al., 2008; Wu et al., 2009].

Statins, the cholesterol-reducing drugs, attenuate the growth of mesenchymal-like cancer cells that show an increased expression of vimentin. Exogenous expression of cell surface E-cadherin converts statin-sensitive cells to a partially resistant state, implying that statin resistance is in part dependent on the tumor cells attaining an epithelial phenotype. As metastasizing epithelial tumor cells may undergo EMT during the metastatic cascade, statin therapy may represent an effective approach to targeting the cells most likely to disseminate [Warita et al., 2014].

HoxA9

We have demonstrated previously that Twist1 activates the transcription of *HoxA9*, which contributes to the induction of a Twist1-dependent metastatic phenotype in prostate cancer cells [Gajula et al., 2013]. Twist1 is canonically known as a transcription factor, but recent data suggest an epigenetic role for Twist1 in gene regulation [Wu et al., 2011; Yang et al., 2012]. During embryogenesis, the histone methyltransferase complex MLL-WDR5 and the long noncoding RNA *HOTTIP* regulate the expression of the *HoxA* cluster of genes by H3K4 chromatin methylation [Wang et al., 2011]. Interestingly, prostate cancer whole exome sequencing has shown frequent mutations in *MLL2* [Grasso et al., 2012]. Our unpublished data have demonstrated that Twist1 and *HoxA9* are co-overex-

pressed in the developing mouse prostate and human prostate cancer samples, and that Twist1 forms a complex with the MLL-WDR5-*HOTTIP* (MWH) methyltransferase complex and is found to be co-overexpressed in prostate cancers, particularly in the metastases. We also demonstrated that this Twist1-MWH complex activates *HoxA9* expression by H3K4me3 chromatin modification of the *HoxA9* promoter region, and that the MWH complex is required for full Twist1-induced prometastatic behaviors in prostate cancer. Thus, our data suggest that reactivation of a latent developmental program involving a Twist1-MWH complex drives the expression of *HoxA9*, mediating prostate cancer metastasis. We have also shown that Twist1-induced prometastatic behaviors in prostate cancer cells can be pharmacologically subdued by targeting *HoxA9* indirectly with UNC0646, a small-molecule inhibitor of the methyltransferase G9a [Lehnertz et al., 2014], or directly with a peptide that disrupts *Hoxa9* function [Ando et al., 2014; [Tran et al., unpubl. data].

Conclusion

Our understanding of the consequences of the EMT program in cancer suggests a broad effect on the neoplastic phenotype involving early tumorigenesis, tumor progression, and treatment resistance. Thus, targeting epithelial plasticity programs such as EMT represents a promising strategy to treat the neoplastic phenotype along the full spectrum of cancers. With increased knowledge of EMT signaling and regulation, many specific compounds have been developed that may target EMT, many of which we have discussed in this review. However, the heterogeneity of tumors and the dynamic nature of epithelial plasticity in cancer cells make it likely that targeting only 1 EMT-related process (Fig. 1, 1–3) will be unsuccessful or only transiently successful. Thus, we envision that systematically targeting EMT regulatory networks provides the best option for success. With the development of more specific pathway inhibitors, new pathways to target, such as glycosylation, new combinations of agents, and novel strategies that involve the immune system, we expect to see significant advances in targeting epithelial plasticity programs for cancer treatment.

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Disclosure Statement

The authors declare that they have no competing interests.

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