Minireview *Highlight article*

Role of E2Fs and mitotic regulators controlled by E2Fs in the epithelial to mesenchymal transition

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Impact statement

The study of the epithelial to mesenchymal transition (EMT) is an active area of research since it is one of the early intermediates to invasion and metastasis—a state of the cancer cells that ultimately kills many cancer patients. We will present in this review that besides their canonical roles as regulators of proliferation, unregulated expression of the E2F transcription factors may contribute to cancer initiation and progression to metastasis by signaling centrosome amplification, chromosome instability, and EMT. Since our discovery that the E2F activators control centrosome amplification and mitosis in cancer cells, we have identified centrosome and mitotic regulators that may represent actionable targets against EMT and metastasis in cancer cells. This is impactful to all of the cancer patients in which the Cdk/Rb/E2F pathway is deregulated, which has been estimated to be most cancer patients with solid tumors.

Abstract

The epithelial-to-mesenchymal transition (EMT) is a complex cellular process in which epithelial cells acquire mesenchymal properties. EMT occurs in three biological settings: development, wound healing and fibrosis, and tumor progression. Despite occurring in three independent biological settings, EMT signaling shares some molecular mechanisms that allow epithelial cells to de-differentiate and acquire mesenchymal characteristics that confer cells invasive and migratory capacity to distant sites. Here we summarize the molecular mechanism that delineates EMT and we will focus on the role of E2 promoter binding factors (E2Fs) in EMT during tumor progression. Since the E2Fs are presently undruggable due to their control in numerous pivotal cellular functions and due to the lack of selectivity against individual E2Fs, we will also discuss the role of three mitotic regulators and/or mitotic kinases controlled by the E2Fs (NEK2, Mps1/TTK, and SGO1) in EMT that can be useful as drug targets.

Keywords: Centrosome amplification, E2Fs, cell cycle, cancer, the epithelial to mesenchymal transition, metastasis

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Introduction

The concept of epithelial to mesenchymal transformation, now referred to as the epithelial-to-mesenchymal transition (EMT), was first coined by Elizabeth Hay[1] when she observed that epithelial cells undergo complex cellular processes to acquire mesenchymal characteristics in order to invade and migrate during primitive streak development in chick embryos. EMT initiates by the loss of epithelial junctions and apical-basal polarity.[2–4] Loss of epithelial junctions and cell polarity results in cytoskeleton reorganization and morphological changes.

These molecular changes are accompanied by changes in protein expression, such as the downregulation of proteins that compose the junction complexes and epithelial markers, as well as the upregulation of mesenchymal markers.[5,6] The loss of E-cadherin is considered a hallmark of EMT.[7] Typically, the loss of E-cadherin results in expression shift with another cadherin isoform, a process known as "cadherin switching." As discussed in Wheelock *et al.*,[7] this cadherin switch is not restricted to E-cadherin to N-cadherin and is context-dependent. Other molecular changes include changes in integrin expression, which correlates with increased expression of matrix metalloproteinases (MMPs) that contribute to the loss of epithelial junctions, enhance ECM degradation, and enable cell invasion.[8,9] EMT can also be regulated at the RNA level either by alternative splicing that generates alternative isoforms of pivotal EMT-related proteins or non-coding miRNA that

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bind to mRNAs coding for epithelial markers and inhibit their translation or promote their degradation. Most molecular changes are mainly orchestrated by EMT transcription factor families such as SNAIL,[10] bHLH (which includes TWIST1),[6,11] ZEB,[6,12] and many others. Several signaling pathways are involved in EMT regulation and can be further reviewed in Lamouille *et al*.[4] Ultimately, all the processes described above result in the acquisition of mesenchymal features that allow the cells to invade and migrate through the extracellular matrix (ECM).

EMT can occur in three distinct biological settings with clearly delineated functional consequences: Type 1 (associated with embryogenesis and development), Type 2 (associated with wound healing and fibrosis), and Type 3 (associated with cancer progression).[13] The latter is our main interest since EMT is a precursor of cancer metastasis, in which prevention and treatment remain a key clinical challenge. However, EMT does not only promote metastasis, but also promote tumor initiation, tumor stemness, and resistance to therapy.[11,14,15]

EMT has been described as a binary process with two distinct populations: epithelial and mesenchymal. However, recent studies have demonstrated the presence of intermediate states referred to as partial, incomplete, or hybrid EMT states. Pastushenko and Blanpain[16] classified the states as epithelial, early hybrid EMT, late hybrid EMT, and full EMT or mesenchymal. Each transition state is grouped according to their distinct characteristics including cell shape, cell adhesion, expression of surface markers, transcription factors, and EMT-associated genes. They also classified these transition states accordingly to proliferative, invasiveness, plasticity, stemness, and metastatic properties.

Since EMT is a key precursor of cancer metastasis, we will describe the role of E2 promoter binding factors (E2Fs) and a subset of mitotic kinases controlled by E2Fs in EMT and cancer.

E2Fs in cancer, EMT, and metastasis

The E2F family of transcription factors is divided into three major groups: activators (E2F1, E2F2, and E2F3a), canonical repressors (E2f3b-E2F6), and atypical repressors (E2F7 and E2F8) of gene transcription.[17] The E2F activators are silenced throughout G1 phase by physical association with the Rb tumor suppressor and are activated by the sequential phosphorylation of Rb first by the G1/S phase CDKs (initially by cyclin D/Cdk4/Cdk6 and then by cyclin E/A/Cdk2); this hyper-phosphorylation results in the occupancy of a plethora of promoters by the E2F activators.[18–24] The E2Fs have many functions such as the regulation of the cell cycle, centriole duplication, cell differentiation, tissue development, DNA repair, genomic integrity, apoptosis, metabolism, and angiogenesis.[17,25–27] Early reports focused on the ability of unregulated E2F activators to trigger hyper-proliferation and apoptosis.[28–33]

Different subsets of E2Fs influence the survival outcomes of several cancers; for example, Huang *et al*.[34] showed that all eight E2Fs are significantly upregulated in hepatocellular carcinoma patients and that overexpression of E2F3, E2F5, and E2F6 correlated with worse overall- and disease-free survival. Studies from our laboratory showed that overexpression of E2F1 and E2F3 strongly correlate with poor overall- and relapse-free overall survival of breast cancer patients, while E2F2 did not.[35] Similarly, Liu *et al*.[36] showed that all E2Fs are overexpressed in breast cancer with the exception of E2F4 and that E2F1, E2F3, E2F7, and E2F8 associated with poor relapse-free survival in breast cancer patients, and similar results were obtained by Li *et al*.[37]

The E2Fs can act as tumor suppressors or oncogenes depending on the tissue context. For example, loss of E2F3 in Rb+/− mice suppressed pituitary tumors but accelerated tumor formation and metastasis of thyroid tumors[38]; in contrast, the ablation of E2F1 in Rb+/− mice suppresses both of these tumor types.[39] Likewise, increased expression of E2F1 driven by the K5 promoter in older transgenic mice resulted in a variety of spontaneous tumors including the skin, but E2F1 expression in the same mouse model blocked skin tumor development in the two-stage induction model.[40] Also, the ablation of E2F1 and E2F3 in an MMTV-neu-driven mouse model of mammary cancer could severely restrict tumor growth.[41] A similar MMTV-neu model showed loss of E2F1 accelerated tumorigenesis and E2F2 could suppress it, but that ablation of E2F1 or E2F2 suppressed metastasis.[42] Also, E2F2 behaves differently than E2F1 or E2F3 in tumors initiated by MMTV-c-Myc, since genetic ablation of E2F2 accelerated metastasis to the lungs of mice, and increased migration and lung-colonization ability when knocked down in triple-negative MDA-MB-231 cells.[43]

While mechanistically the E2Fs could act as tumor suppressors or oncogenes due to their ability to suppress or induce proliferation and apoptosis in different tissue contexts, Saavedra *et al*.[44] demonstrated that genetic ablation of E2F3a and E2F3b in primary mouse fibroblasts resulted in centrosome amplification—defined as the acquisition of three or more centrosomes that result in pseudo bipolar and multipolar mitotic spindles and aneuploidy; neither ablation of E2F1, E2F2, E2F4 or E2F5 could cause these phenotypes. These experiments suggested that loss of repression likely due to the loss of E2F3b was central in suppressing centrosome amplification and chromosome instability. Our group first demonstrated that the overexpression of E2F activators (E2F1, E2F2, and E2F3a) in MCF10A mammary epithelial cells increases centrosome amplification, chromosome instability, and deregulate timing of mitosis/cytokinesis.[45] Moreover, depletion of E2F3a and E2F3b in Her2^{+} breast cancer cells reduced tumor growth and burden by suppressing centrosome amplification and unregulated mitosis.[46] This is an important finding since centrosome amplification and chromosome instability can lead to tumor initiation,[47,48] maintenance,[49–53] and progression[46,54–56]. Centrosome amplification correlates with poor prognostic factors,[56] causes chromosomal instability[49] tumor heterogeneity,[57] and promotes invasion[45] through changes in cell polarity.[58,59] These results suggest that activation or repression by E2Fs plays a major role in maintaining centrosome homeostasis and genomic integrity and may signal

invasion and EMT through centrosome amplification and loss of cell polarity.

It is widely recognized that the E2Fs signal EMT, invasion, and metastasis.[28,60] For example, overexpression of E2F1 in squamous carcinoma cells enhances their ability to invade.[61] Elegant studies from the Chellappan laboratory showed that several MMPs genes are regulated by the E2Fs through an Rb-Raf-1 interaction.[62] They predicted E2F binding sites on all 23 human MMPs gene promoters and showed that MMP9, MMP14, and MMP15 were E2F1-responsive. Furthermore, they demonstrated that the depletion of E2Fs reduced invasion and migration in vitro, and reduced metastasis in tail vein lung metastatic models in mice. Further, several receptor proteins have been identified and associated with E2F1 expression and EMT in breast, bladder, and lung cancers.[65,66] Wang *et al.*[67] showed that E2F1 promotes EMT by the direct regulation of ZEB2 in small cell lung carcinoma. Previous studies from these authors also showed that E2F1 regulates MMP-9 and MMP-16.[68] The E2F1/SP3/STAT6 axis has been shown to increase the expression of ZEB1 and ZEB2, and signal IL-4 induced EMT in colorectal cancer.[69] Also, depletion of E2F1 resulted in decreased expression of DDR1, which in turn decreased migration and invasion of osteosarcoma cells.[70] Further analysis demonstrated that STAT3 signaling was impaired upon E2F1 depletion, resulting in decreased vimentin, MMP2, and MMP9, and increased E-cadherin. Liang *et al.*[71] demonstrated that E2F1 depletion inhibited proliferation, invasion, and migration in prostate cancer cells. Meanwhile, E2F1 depletion in tumor xenograft suppressed tumor growth, invasion, and EMT by increasing E-cadherin and decreasing the expression of vimentin; in this study, CD147 was identified as an interaction partner for E2F1. Further, Knoll *et al.*[72] showed that the E2F1-miR-224/452-TXNIP axis promotes EMT, migration, and invasion in melanoma, and can serve as a predictor of patient survival.

Fewer studies have been devoted to study the role of E2F2 and E2F3 in EMT. One of the few studies done with E2F2 shows that it correlates with elevated levels of vimentin and inversely correlates with miR-99 in lung cancer, that E2F2 inhibition allows miR-99 to repress stemness in lung cancer and that overexpression of E2F2 rescues back the migratory potential of cells overexpressing miR-99.[73] Studies focused on E2F3 also center in non-coding micro RNAs. Studies from Ye *et al.*[74] showed that forced overexpression of miR-363 decreased proliferation, migration, invasion of hepatocellular carcinoma cells, and reversed EMT *in vitro* and *in vivo.* Further analysis revealed that miR-363 targets the 3′ untranslated region of E2F3 and that they are inversely correlated, and that overexpression of E2F3 in cells expressing miR-363 restored colony formation, migration, and invasion capacity. The control of E2F3 by miR-363 has implications for several cancers since the aberrant expression of miR-363 and its role in tumor pathogenesis and progression have already been reported in gallbladder, colorectal, gastric, head and neck, and breast cancers. This is not exclusive to miR-363 since E2F3 has been reported to be regulated in HCC cells by several miRNAs, such as miR-141, miR-144, miR-503, miR-214 miR-424, and miR-217. In another study, it was shown that miR-200c, which is down-regulated in bladder cancer, regulates BMI-1 and E2F3.[75] Mutations in two putative miR-200c-binding sites resulted in reduced cell invasion, migration, proliferation, and increased E-cadherin levels. Other studies show that the E2F activators can contribute to metastasis by influencing the tumor microenvironment. For example, a study from the Leone laboratory evidenced the role of E2F3 in metastasis, although not by inducing EMT. In their study, it was shown that the ablation of E2F3 in tumor-associated macrophages led to decreased expression of extracellular matrix modifiers that resulted in decreased pulmonary metastasis of breast cancer cells.[76] Another study showed in an MMTV-polyomavirus-induced model of mammary tumor metastasis that the ablation of E2F1 and E2F2 reduced metastatic capacity by reducing the expression of several genes involved in angiogenesis, remodeling of the extracellular matrix, tumor cell survival, and tumor-cell interactions with vascular endothelial cells that promote lung metastases.[77]

Some studies have been devoted to study the role of the remaining E2Fs (E2F4-E2F8). To our best knowledge, the only two studies that show any effect of E2F4 in EMT were done by the Chellappan laboratory (briefly discussed above)[62] and one by Baiz *et al.*[78] In this last study bortezomib, an anti-proliferative drug that inhibits the 26S proteasome subunit promotes cell cycle arrest and apoptosis was tested in hepatocellular carcinoma cells and it promoted downregulation of E2F2 and upregulation of E2F4, E2F6, and E2F8. These changes correlated with the expression of some EMT-related genes in a microarray; however, no functional nor other expression analysis was conducted to evaluate how these changes modulate EMT. Another study showed that the treatment of NMuMG cells (mouse mammary cells) with TGFβ1 (a known inducer of EMT) resulted in E2F5 downregulation.[79] Further, a gene expression analysis identified E2F5 as a possible target of LOXL2 (lysyl oxidase-like 2), which stabilizes SNAIL1 and is upregulated in pancreatic cancer.[76] In addition to the study of Baiz *et al.*, a study done in endometrial carcinoma cells showed that miR-424 (which associates with poor predictor factors, cell invasion, and migration) negatively regulates E2F6[63]; this was evidenced by dual-luciferase reporter system and expression assays. Inhibition of miR-424 with an antisense oligonucleotide sequence increased the expression levels of E2F6. Lastly, it was shown that miR-30a-5p decreased EMT and metastasis in gallbladder carcinoma cells by targeting E2F7.[64] Inhibition of miR-30a-5p resulted in increased cell proliferation, migration, invasion, and nuclear translocation of β-catenin. Meanwhile, NOZ (a gallbladder carcinoma cell line) transfected with miR-30a-5p and transplanted in nude mice resulted in slower tumor growth and reduced hepatic metastasis. E2F7 and E2F8 have been shown to also induce angiogenesis, which facilitates metastasis, by interacting with HIF1and regulating VEGFA.[80]

In summary, these studies together with the scarce research focus on E2F2-8 and EMT accentuate the need to redirect our attention to the study of E2F2-E2F8 because

even though they have redundant functions, they have unique functions, expression, and prognostic values.

Current therapies against E2Fs, mitotic regulators, and EMT

Despite their significant contribution to tumorigenesis through cell-cycle deregulation, centrosome amplification, and chromosome instability, and their apparent role in EMT, invasion, and metastasis, E2F activators are virtually undruggable.[81,82] Current therapies directed against E2Fs in cancer include inhibition of upstream regulators using CDK4/6 inhibitors (which prevent the initial phosphorylation of Rb and keep E2Fs in a repressed state), use of truncated E2F1 protein, use of small molecules or peptides to inhibit E2F activity, and the use of E2F-driven oncolytic viruses. The Leone[83] and Knudsen[84] groups reviewed in detail these therapies and their advantages and disadvantages. Out of these four possible therapies directed to E2Fs, CDK4/6 inhibitors and E2F-driven oncolytic viruses are in clinical trials.[85,86] The major disadvantages of the CDK4/6 inhibitors are that they do not work if RB is not intact or if there is E2F amplification.[84,87] On the other hand, E2F-driven oncolytic virus treatment has shown promising results. One example is ICOVIR-7, which features an RGD-4C modification of the fiber HI-loop of serotype 5 adenoviruses for enhanced entry into tumor cells and has been tested clinically.[86] Treatment with ICOVIR-7 showed anti-tumoral efficiency in 9 out of 17 patients, while 5 out of 12 exhibited stabilization or reduced tumor size. The remaining two target therapies mentioned above are not yet in clinical trials and major complications include the delivery method, specificity against individual E2Fs, and potential side effects such as mounting a general autoimmune response against viruses.

Despite E2Fs not currently being ideal targets for cancer therapy because they are transcription factors that regulate numerous essential normal cell processes, and due to the lack of selectivity against specific E2Fs, E2F-regulated mitotic kinases and regulators, including NEK2, Mps1/TTK, BUB1, BUBR1, and SGO1 can serve as potential drug targets. We recently demonstrated that individual expression of E2F1 or E2F3 in mammary epithelial cells elevates the levels of multiple regulators of mitosis and/or the centrosome, including NEK2, Mps1/TTK, SGO1, HEC1, BUBR1, and PP2AC.[35] Some of these centrosome/mitotic regulators have effects on EMT and this notion was first explored by investigating the effect of another gene, Aurora-A. Early studies from Wan *et al.*[88] showed that Aurora-A, a mitotic kinase involved in centrosome segregation and spindle assembly, mediates cell invasion and EMT (denoted by changes in E-cadherin and β-catenin) in nasopharyngeal carcinoma through the MAPK pathway. This was the first report that linked a mitotic regulator with EMT induction. Here, we will discuss briefly the role of these E2F targets in EMT. However, bear in mind that E2F regulates many targets that can promote EMT through pathways not yet consider here—many of which have been discussed in the previous section.

NEK2

NEK2 (never-in-mitosis-a-related kinase 2) is a serine/threonine that localizes in the centrosome and has multiple roles in the cell cycle. NEK2 belongs to the NEK family (which has 11 other members). However, NEK2 has received special attention in cancer due to its numerous functions in normal and oncogenic states. The *NEK2* gene has three splice variants (*NEK2A*, *NEK2B*, *NEK2C*), being NEK2A the full-length protein.[89–91] The N-terminal of NEK2 is the catalytic kinase domain, while the C-terminal is the regulatory domain. NEK2 expression peaks in S and G2 phase.[90] It has been shown that E2F4 binds to the NEK2 promoter during the G1 phase serving as a transcriptional repressor.[92] Our lab also showed that NEK2 is under the control of the Cdk4 pathway and the E2Fs and that NEK2 can rescue back centrosome amplification in Her2+ breast cancer cells silenced for Cdk4 or E2F3.[35,93–95] NEK2 is also repressed by p53 and miRNA-128.[96,97] On the other hand, NEK2 is positively regulated by FoxM1.[98,99] NEK2 is degraded by the ubiquitin-proteosome complex and activated by auto-phosphorylation at several residues—reviewed in Fang and Zhang.[100] NEK2 participates in centrosome duplication and centrosome separation by phosphorylating c-Nap1, Rootletin, and Cep68.[101–103] Moreover, NEK2 stabilizes microtubules through phosphorylation of Nlp and centrobin during interphase.[104] Also, NEK2 phosphorylates Hec1 to control kinetochore attachment and spindle assembly, specifically chromosome alignment by direct interaction with MAD1 or phosphorylation of Hec1 and SGO1.[105–107] NEK2 also mediates chromosome separation.[100,108] Apart from these roles in the cell cycle, NEK2 is a novel regulator of B cell development and immune response.[109]

NEK2 is clinically relevant for cancer treatment since it has been shown to be upregulated in diverse cancer cells and cancer types.[100,110] We demonstrated that Nek2 may represent an important target against mammary cancers since it can significantly suppress centrosome amplification and chromosome instability triggered by Her2, H-Ras^{G12V}, or H-Ras^{G12V} and c-Myc.[35,93–95] Xenograft studies have demonstrated that NEK2 depletion results in reduced tumor growth.[111] Furthermore, studies have shown that uncontrolled NEK2 activity can lead to chromosomal instability,[112] which can have an effect on tumorigenesis and tumor progression.[57] Our studies showed that NEK2 overexpression in Her2+ breast cancer cells downregulated for E2F3 generates pre-invasive protrusions in 3D cell culture models (mammospheres).[45] A recent study shows that NEK2 correlates with migration, invasion, and EMT in hepatocellular cells.[113] Some of the genes that were modulated by Nek2 were E-cadherin, N-cadherin, and vimentin, both in cancer cell lines as well as in patient tissues. Gene microarray identified other potential EMT-related genes that might be modulated. Another study correlated NEK2 expression with hepatoma metastasis and showed that NEK2 modulates E-cadherin and MMP9.[114] Das *et al.*[115] showed that NEK2 overexpression leads to upregulation of the Wnt orthologue wingless and alteration of E-cadherin, β-catenin, and activation of the

Akt pathway; Nek2 cooperates mitogenic pathways (Src or Ras) to cause distant metastasis in *Drosophila melanogaster*; in contrast, such cooperation was abrogated by PI3K inhibitors. Studies from Mbom *et al.*[116] further investigated how NEK2 regulates β-catenin and found that NEK2 (under the regulation of Plk1) binds and phosphorylates the N-terminus of β-catenin (independently of GSK3β). Phosphorylation of β-catenin prevents ubiquitination and degradation; thus, β-catenin accumulates at centrosomes during mitosis. Moreover, NEK2 has been shown to contribute to drug resistance. One study showed that NEK2 induces drug resistance through the activation of efflux drug pumps in myeloma.[117]

Surprisingly, there is not any current inhibitor for NEK2 in clinical trials; however, some studies have tested the effects of pharmacological inhibition of NEK2 or downregulation of NEK2 by RNAi in cellular and animal models. Other strategies include blocking of ATP-binding site and interruption of protein interaction. Studies from Kaowinn *et al.*[118] tested the efficacy of CGK062, a small chemical molecule that inhibits CUG2, which is a gene that interacts with NEK2. Findings from this study showed that CGK062 promotes phosphorylation of β-catenin at Ser33/Ser37 and induced its degradation; thus, posing a potential therapeutic use in lung cancer. Another study showed that the treatment of non-small cell lung cancer cells with deguelin, a rotenoid extracted from *Mundulea sericea* resulted in lower Nek2 levels, reduced migration, and invasion.[119] Ectopic expression of Nek2 negated deguelin function by preventing elevation of mRNAs coding for E-cadherin and the downregulation of vimentin mRNA. Another approach currently tested is NEK2 siRNA therapy using a portal venous port-catheter system for liver metastasis in pancreatic cancer.[120] In their study, NEK2 siRNA inhibited tumor growth in a subcutaneous xenograft mouse model, prolonged survival time, and prevented liver metastasis. The main limitation of RNAi inhibition is the delivery method. Regarding ATP-binding, site blocking is the poor selectivity. The most promising approach is the interruption of protein inhibition. Notably, INH1, which inhibits the NEK2/Hec1 interaction[121] has shown antitumoral activity by reducing tumor growth in breast cancer nude mice models. A series of analogs of INH1 have been synthesized to improve its efficacy.

Mps1/TTK

Monopolar spindle 1 kinase (Mps1) or TTK is a serine, threonine, and tyrosine kinase that has multiple roles in mitosis and meiosis.[122] TTK is widely distributed in most eukaryotes and has alternative splice forms in humans and mice.[122] TTK is found in centrosomes and is required for the assembly of the spindle pole body in yeast and contributes to centrosome duplication in various eukaryotes—reviewed in Pike and Fisk[123]— albeit its role in centriole duplication in human cells has been challenged.[124] TTK localizes at kinetochores and ensures control of proper biorientation of sister chromatids on the mitotic spindle at kinetochores in collaboration with AURORA kinase B and Hec1/NDC80 as its substrate.[125,126] Besides the regulation of TTK in the spindle assembly checkpoint, TTK is involved in the exit of mitosis and cytokinesis[127] through the interaction with Mob1 and MIP1.[128,129] Apart from its roles in mitosis, TTK is involved in the genotoxic stress response (reviewed in Liu and Winey[122]) and meiosis I and II.[130] The C-terminal catalytic domain undergoes auto-phosphorylation at serine, threonine, and tyrosine sites (to a lesser extent) to activate itself.[131] Autophosphorylation also helps in subcellular localization. Like NEK2, TTK is controlled at the transcriptional level by the E2Fs, specifically, E2F4 and to a lesser degree E2F1 binds to TTK promoter and represses TTK transcription in cells during interphase.[92] TTK protein levels also rise upon overexpression of E2F1 or E2F3 in human mammary epithelial cells.[35] Meanwhile, IL-2 appears to be an inductor of TTK.[132] The major route of TTK inactivation is through degradation in anaphase by the ubiquitin E3 ligase APC/Cdc20.[133] Another proposed mechanism of degradation is de-phosphorylation, however; to this date, there has not been any phosphatase identified yet.

TTK is deregulated in several human tumors. Elevated levels of TTK have been reported in thyroid papillary carcinoma, breast cancer, gastric cancer, lung cancer, and many more and its elevation correlates with poor prognosis and tumor aggressiveness—reviewed in Liu and Winey.[122] TTK is often overexpressed in hepatocellular carcinomas and promotes proliferation, anchorage-independent growth, and cell migration.[134] Previously, we showed that TTK is overexpressed in Her2+ cells that also display elevated levels of centrosome amplification and that its silencing reduces that process in Her2+ cells.[135] Recently, we showed the highly novel finding that mitotic kinases may be involved in the early stages of metastasis by showing that pharmacologic inhibition and silencing of TTK reversed EMT, suppressed invasion, and increased the expression of KLF5, an effector of TGF-β signaling; further, TTK inhibition decreased miR-21 but increased miR-200 expression and suppressed TGF-β signaling.[136] Thus, TTK may be an excellent target to suppress cancer cell survival and progression to metastasis. Currently, there is an Mps1/TTK inhibitor in clinical trial Phase I (CFI-402257), in which MPS1 inhibitor is administered along with paclitaxel in patients with advanced/metastatic Her2—breast cancer (NCT03568422) to induce tumor death by increasing chromosome mis-segregation—reviewed in Jusino et al.[57] Also, this inhibitor (CFI-402257) is used alone to treat advanced solid tumors from patients enrolled in another clinical trial Phase I (NCT02792465).

SGO1

SGO1 (Shugoshin 1) plays a role in mediating the centromeric cohesion of sister chromatids, preventing their premature dissociation during cell division.[137] SGO1 is also known as a guardian of the genome due to its role in suppressing chromosomal instability during cell division. SGO1 has several splicing variants and along with SGO2, they conform to the Shugoshin family.[138] Here we will focus only on SGO1 due to its novel role in EMT. SGO1 N-terminal homo-dimerizes and interacts with other

proteins, while the C-terminal contributes to chromosomal localization.[138,139] SGO1 is widely distributed in eukaryotes such as yeast, invertebrates (i.e. *Drosophila melanogaster*), plants, mice, etc., and induces similar functions to human SGO1 when expressed in these species.[139] SGO1 levels are low during G1, gradually increases during S phase, accumulates at G2, and peaks in mitosis (during prometaphase and metaphase), and are followed by a rapid decline upon exiting from mitosis due to proteolytic cleavage dependent on the APC/C complex through removal of Ken box or D box (destruction box) sequences.[140–143] SGO1 has nuclear localization sequences and phosphorylation sites for CDKs,[137] Plk1,[144] AURORA B,[137] BUB1,[145,146] and NEK2A.[107] In early mitosis, SGO1 co-localizes at the inner centromeres with Cohesin, and a small fraction localizes at the kinetochores at metaphase.[147] SGO1 forms a complex with PP2Ato prevent premature dissociation of Cohesin from sister chromatids preventing phosphorylation of SCC3 by Plk1.[148] BUB1 and AURORA B are important for localization of SGO1 in centromeres.[145,146] On the other hand, phosphorylation by NEK2A is not required for SGO1 assembly but for chromosome congression.[107]

Until recently, research on SGO1 was focused on cell embryology; however, some studies have shown the role of SGO1 on tumorigenesis of intestinal cancer,[149] liver cancer,[150,151] neuroblastoma,[152] lung cancer (through Wnt activation),[153] and colon cancer.[154] An interesting study from Mu *et al.*[155] showed that SGO1 overexpression correlates with poor prognostic factors in prostate cancer. Furthermore, the SGO1 knockdown resulted in the inhibition of prostate cancer cell proliferation, migration, invasion, and changes in EMT (through the rescue of E-cadherin protein levels and decrease in vimentin protein levels). Other studies and ours have shown the role of SGO1 in the suppression of chromosome instability.[35,156–158]

Particularly, our studies show that overexpression of E2F1 and E2F3 leads to overexpression of SGO1 in mammary epithelial cells and that SGO1 guard against centrosome amplification and chromosome instability in these cells[35] and that silencing of SGO1 suppresses centrosome amplification in Her2+ cells.[135] Since SGO1 is essential for proper spindle assembly checkpoint functioning and chromosome cohesion,[137,146,159] and our studies have shown its role in centrosome amplification and chromosome instability,[35,135] SGO1 represents a novel drug target for cancer. Furthermore, SGO1 localization to chromosome arms and centromeres is controlled by phosphorylation by the kinases Aurora B[160] and BUB1.[146] In turn, SGO1 brings the kinase PLK1 to centromeres.[161,162] Therefore, Sgo1 is upstream and downstream of proteins currently in clinical trials and its levels and function may affect outcomes of these patients—currently in clinical trials. However, far more research needs to be done to elucidate the mechanism of SGO1 in EMT and how can be targeted, and should be used as a cancer target with care, because it can suppress or induce chromosome instability and centrosome amplification, depending on cell context.

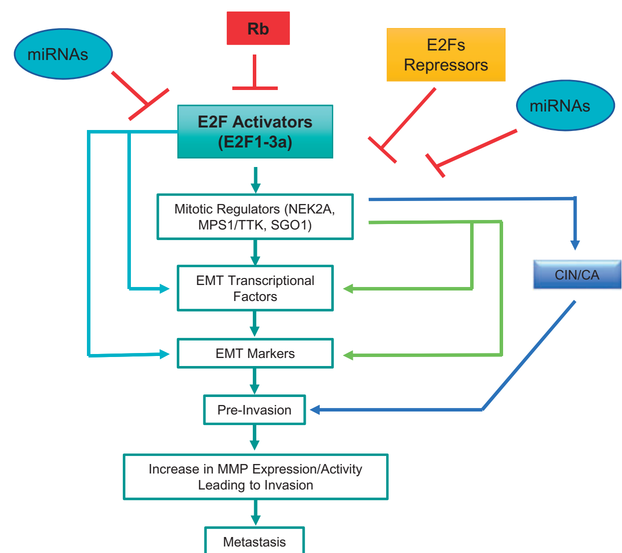

Figure 1. Model depicting how the E2F transcriptional activators and mitotic kinases under their control may control EMT in cancer. Overexpression of E2F activators (E2F1-3) or downregulation of E2Fs repressors (E2F4-8) leads to mitotic regulator deregulation (NEK2, MPS1/TTK, SGO1), which in turn lead to a cascade of events that ultimately leads to metastasis. Alternative models suggest miRNA may mediate these changes through controlling the E2Fs activators or mitotic regulators, that the E2Fs may regulate EMT independently of these mitotic regulators by regulating EMT transcription factors or markers, or that these mitotic regulators signal EMT and/or lead to metastasis through chromosome instability (CIN), centrosome amplification (CA), or independently of E2Fs. (A color version of this figure is available in the online journal.)

Conclusion

As discussed above, both E2Fs and E2F targets exert a role in EMT. However, since E2Fs are presently undruggable, due to their numerous important cellular functions and the lack of selectivity against individual E2Fs, we propose that E2F targets, such as NEK2, TTK, or SGO1 are further investigated in order to design clinical small molecules that inhibit them and prevent EMT. There is a relatively new classification of EMT. This sub-classification is relatively new and there are no publications that correlate E2Fs with any phase. The article published by Pastushenko *et al.*[16] makes a distinction based on EMT gene expression, cell adhesion, invasiveness, plasticity, stemness, and metastasis. Rather than focusing on pathways that mediate these changes, the author focuses on what type of cancer exemplifies these phases. For example, breast cancer grouped into the Hybrid EMT state category. Since we study E2Fs in the context of breast cancer, our data are consistent with this classification. We have observed that E2Fs associated with the hybrid EMT state, characterized by the expression of certain mesenchymal genes like vimentin and N-cadherin and certain EMT transcription factors while not influencing the expression of other genes such as E-cadherin (Jusino and Saavedra, unpublished). The E2Fs may also help in the late stages of metastasis, where cells can undergo the mesenchymal to epithelial transition (MET) to be able to grow in the metastatic site. However, this is all speculation at this point.

We propose the model that that overexpression of E2F activators (E2F1-3) or downregulation of E2Fs repressors

(E2F4-E2F8) leads to the deregulation of mitotic kinases such as NEK2A and Mps1/TTK and/or mitotic regulators such as SGO1 (Figure 1). In turn, these mitotic regulators signal EMT transcription factors and EMT markers (and plenty of studies have been published showing that these mitotic regulators signal EMT). EMT modulation, in turn, leads to invasion and metastasis. As discussed previously, EMT is a complex cellular process that facilitates invasion and migration to distant sites through cytoskeleton remodeling and increased expression and activity of metalloproteases (MMP) that facilitate metastasis. Likewise, we cited several studies that have shown that miRNAs promote EMT through the E2Fs. Therefore, an alternative model is that E2Fs signal EMT independently of these mitotic regulators. Similarly, it is possible that these mitotic regulators signal EMT independently of the E2Fs. In our model, we present two alternatives. The first is that these mitotic regulators signal EMT transcription factors or EMT markers independently of E2Fs or under the regulation of other genes not considered here. The second is that these mitotic regulators lead to chromosome instability and centrosome amplification leading to the formation of pre-invasive structures and eventually metastasis.

Thus, it remains some questions to be answered in order to elucidate the mechanism by which E2Fs, mitotic regulators, and EMT intertwine to lead to metastasis. EMT also leads to therapy resistance, stemness, and tumor heterogeneity; therefore, understanding the molecular mechanisms that underline this process is critical to improving current cancer therapies.

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DECLARATION OF CONFLICTING INTERESTS

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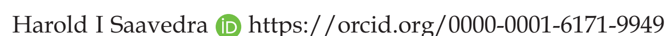
Harold I Saavedra  https://orcid.org/0000-0001-6171-9949

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