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Review

Molecular mechanisms of epithelial to mesenchymal transition in tumor metastasis*

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Epithelial to mesenchymal transition (EMT) is a process where cancer cells lose their epithelial features, the cytoskeletal architecture is re-organized, the cell shape changes and cells activate genes that help to define a mesenchymal phenotype, which leads to an increased cell motility and dissemination of tumor to distant metastatic sites. This review describes different signaling networks between microRNAs and proteins that regulate EMT in tumor growth. Activation of EMT is mediated *via* a series of paracrine signaling molecules. WNT, TGF-β, NOTCH and SHH signaling pathways play crucial roles in activation of EMT-related transcription factors, such as SNAIL, SLUG, ZEB1/2 or TWIST. Recent data provide evidence that crosstalk between microRNAs, long non-coding RNAs and EMT-transcription factors is a crucial event in EMT regulation. MicroRNAs also affect the level of proteins responsible for cellular contact, adhesion and cytoskeletal proteins, which induces changes in the epithelial to mesenchymal phenotype transition. Understanding those signaling networks may help to identify novel biomarkers or develop new treatment strategies based on microRNA therapeutics in the future.

Key words: epithelial to mesenchymal transition (EMT), tumor, metastasis, microRNA, transcription factors

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Abbreviations: APC, adenomatous polyposis coli; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; GSK-3β, glycogen synthase kinase 3β; MET, mesenchymal to epithelial transition; RTK, receptor tyrosine kinases; TF, transcription factor; TGF-β, transforming growth factor β

INTRODUCTION

Cancer can be defined as a growth abnormality, characterized by uncontrolled proliferation of abnormal cells that can invade normal tissues and organ boundaries and then eventually spread throughout the body. In the first step of the tumorigenesis process, called "tumor initiation", genetic alteration of a single cell causes abnormal proliferation, which consequently leads to the outgrowth of a population of clonally derived tumor cells. During tumor progression, successive genetic changes confer growth advances (hyperplasia), morphological and functional deviation (dysplasia), altogether driving progressive transformation of the normal human cells into highly malignant derivatives, finally able to invade neighboring tissues and give distant metastases (Cooper, 2000; Hanahan *et al.*, 2000; Ryan & Faupel-Badger, 2016).

These mutations, driving to oncogenesis, are related to two opposing groups of genes: the oncogenes and tumor suppressor genes, mostly connected with regulation of proliferation and cell cycle. Oncogenes are created by a single, dominant mutation event, which has an activating capability and leads to gain of function, while a mutation in the tumor suppressor genes of both gene loci is needed to drive a loss of gene function (Weinberg, 1994).

On the other hand, cancer cells are in some aspect liberated from dependence on signals derived from the normal tissue microenvironment, for example those connected with acquiring the ability of tumor cells to expand in number. This capability can be achieved by an increased rate of cell proliferation: upregulation of signals promoting proliferation and decreased antigrowth signaling, but also by cancer cells resistance to death, mainly apoptosis (Hanahan *et al.*, 2000; Hanahan & Weinberg, 2011).

In order to fuel growth and division of cancer cells more efficiency, tumor development is also connected with adjustment of the energy metabolism. Recent reports claim that this reprograming can be related to a deregulated uptake of glucose and amino acids, opportunistic modes of nutrient acquisition, utility of glycolysis and TCA cycle intermediates, increased nitrogen demand, alterations in metabolite-driven gene regulation, and metabolic interactions with the microenvironment (Pavlova & Thompson, 2016).

To access the unlimited possibility of cell divisions, cancer cells also need to overcome a progressive shortening of telomeres, i.e. sequences responsible for protecting the ends of chromosomal DNA during successive cycles of replication (Hanahan *et al.*, 2000).

When the tumor size is larger than 2–3 mm^3 (Sherwood & Parris, 1971), it requires a new system of blood vessels to provide nutrients and other factors required for further growth. Therefore, tumors appear to activate the process of angiogenesis by changing the balance of expression between angiogenesis inducers and the countervailing inhibitors (Hanahan & Weinberg, 2011).

Another, worth mentioning aspect of cancer progression, is interaction of tumor with the immune system. During the early stages of tumor development, the effector immune cells eliminate immunogenic cancer cells

which leads to a continuous selection and promotion of more aggressive clones with reduced immunogenicity or those able to secrete immunosuppressive factors (Fouad & Aanei, 2017). Most tumors progress to a state of chronic inflammation, stimulating cancer cell proliferation and metastasis by: genomic instability, epigenetic modification, induction of cancer cell proliferation, enhancement of cancer anti-apoptotic pathways, stimulation of angiogenesis, and eventually, cancer dissemination (Gonzalez *et al.*, 2018).

Taken together, we can describe essential alterations in the cell physiology that collectively dictate the malignant growth in eight "hallmarks", described previously in influential reviews of Hanahan and Weinberg, which include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, inducing angiogenesis, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan *et al.*, 2000), reprogramming of energy metabolism, and evading the immune destruction (Hanahan & Weinberg, 2011).

Despite the described pre-invasive cancer features, an ability to spread throughout the body is inherently connected with cancer. The abilities of invasion and metastasis of cancer cells can be achieved by the epithelial to mesenchymal transition. Understanding the signaling networks and cross-talks between proteins and microRNAs in that process may enable to develop new treatment strategies in the future and may help to identify novel biomarkers of malignant tumors. This review discusses different signaling networks regulating the epithelial to mesenchymal transition (EMT) process.

REGULATION OF EMT BY PROTEIN SIGNALING PATHWAYS

Epithelial to mesenchymal transition is a process responsible for tumor metastasis during which epithelial cells gradually transform into mesenchymal-like cells, and lose their epithelial functionality and characteristic molecular features. The first observations of EMT phenotype changes were described with a primitive streak of chick embryos by Elizabeth Hay in the early 1980s (Hay ED., 1995). These findings have opened an entire field of research, which associates EMT's role in both, the physiological and pathological processes. Based on the biological/physiological context, it can be classified into three subtypes: 1) embryogenesis, organogenesis, 2) tissue homeostasis, repairing and fibrosis or 3) cancer progression and metastasis (Pei *et al.*, 2019; Prieto-García *et al.*, 2017; Stone *et al.*, 2016). The transition of epithelial cells into mesenchymal cells follows a common and conserved program with several hallmarks. The key events in EMT are the loss of polarized organization of the epithelial tissue, the dissolution of the epithelial cell-cell junctions, reorganization of the cytoskeletal architecture and changes in cell shape, downregulation of signature epithelial gene expression, and activation of genes that help to define the mesenchymal phenotype, increased cell motility and ability to degrade extracellular matrix (ECM) proteins to enable invasive behavior (Lamouille *et al.*, 2014). Triggering of the EMT process allows cancer cells to disseminate from the primary tumor site, invade adjacent tissues and generate metastasis by colonizing distant sites through the bloodstream and lymphatic system. Once a new metastatic niche has been reached, cells can revert through an opposite process called MET, to re-acquire the initial epithelial characteristics, similar to those in the primary tumor. This step is necessary to allow metastatic colonization (Prieto-García *et al.*, 2017).

The earliest event in EMT is the loss of cell polarity, following dissolution of tight junctions between the cells. It is caused by the loss of epithelial markers, such as the E-cadherin. Downregulation of the E-cadherin level causes breakdown of adherent junctions between cells and loss of cell polarity, leading to acquisition of the mesenchymal phenotype with migratory abilities. This dynamic process can be caused by a complex interplay of several inducers, such as the transforming growth factor (TGF-β) or fibroblast growth factor (FGF), several receptor tyrosine kinases (RTKs), WNT/β-catenin, NOTCH, activation of EMT-inducing transcription factors (TFs), microRNAs, epigenetic and post-translational modifications (Ghahhari & Babashah, 2015; Serrano-Gomez *et al.*, 2016). These signals regulate the E-cadherin activity and morphogenetic changes. Moreover, loss of the E-cadherin protein expression at the cancer cell surface can be caused by mutations in the *E-cadherin* gene (Petrova *et al.*, 2016).

Under normal, physiological conditions, the EMT program is activated in epithelial cells through signals that they receive from their neighborhood. In case of carcinoma pathogenesis, these signals are acquired from the recruited cells that form the stroma of tumors, known as the tumor microenvironment. This process is mediated *via* a series of paracrine cell-cell signaling molecules, among which WNT, TGF-β and NOTCH ligands play the main role (Gonzalez & Medici, 2014).

The WNT signaling pathway (Fig. 1) involves a lot of components, but a major effector is the transcription factor (TF) β-catenin. WNT signals are transduced across the plasma membrane by the Frizzled and low-density lipoprotein receptor–related protein (LRP) receptors. In the absence of signaling, β-catenin is phosphorylated by a complex of GSK-3β, axin and the tumor suppressor adenomatous polyposis coli (APC), which sequesters β-catenin in the cytoplasm and marks it for proteasomal degradation. Binding of the WNT ligand to a Frizzled/LRP-5/6 receptor complex causes inhibi-

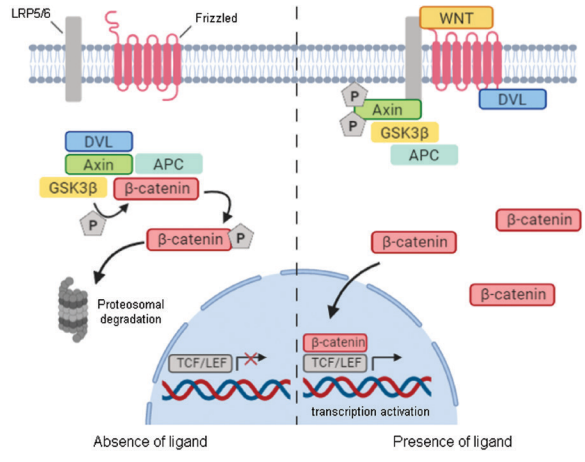

Figure 1. The WNT signaling pathway.
In the absence of the WNT ligand, β-catenin is phosphorylated by a complex of GSK-3β, axin and the tumor suppressor adenomatous polyposis coli (APC), which marks β-catenin for proteasomal degradation. Binding of the WNT ligand to a Frizzled/LRP-5/6 receptor complex causes inhibition the APC/Axin/GSK-3β destruction complex, which leads to stabilization of β-catenin and its translocation to the nucleus for transcription activation.

tion the APC/Axin/ GSK-3β destruction complex, leading to stabilization of β-catenin and its translocation to the nucleus to activate transcription (Eisenmann, 2005). In the past years the role of WNT signaling has been described in many research papers, mostly in colorectal cancer (Bienz & Clevers, 2000; Li *et al*., 2019; Qi *et al*., 2016; Schwab *et al*., 2018). Nevertheless, WNT signaling is observed in many other cancers, such as the breast cancer (Green *et al*., 2013), liver cancer (Takigawa & Brown, 2008), head and neck squamous cancer (Le *et al*., 2019), and renal cell carcinoma (Xu *et al*., 2016; Zhan *et al*., 2017). Mutations of the WNT signaling components are the major cause of various cancer types. In numerous cancers, WNT signaling is inappropriately active and directly induces SNAIL (SNAI1) and SLUG (SNAI2) transcription factors expression (Behrens, 2005). It was recently shown that upregulation of Axin2 by WNT signaling increases SNAIL levels, leading to EMT (Yook *et al*., 2006). SNAIL can also activate WNT signaling by binding to β-catenin, establishing a positive feedback loop for WNT-dependent transcription (Stemmer *et al*., 2008). SLUG is also stabilized by inhibiting the GSK-3β kinase activity and initiates EMT transcriptional programs in different tumor types, including breast cancer cells (Wu *et al*., 2012). WNT-mediated induction of EMT through SLUG is consistent with other reports of decreased E-cadherin and increased fibronectin levels, after accumulation of β-catenin in the nucleus (Brabletz *et al*., 2001). WNT has been also linked to an increased expression of the TWIST transcription factor in mammary epithelial cells (Howe *et al*., 1906).

The NOTCH signaling pathway consists of NOTCH receptors, NOTCH ligands and intracellular proteins that function to transmit the NOTCH signal to the nucleus. NOTCH receptors are transmembrane proteins that are composed of an extracellular (NECD), a transmembrane (TM) and an intracellular (NICD) domain. NOTCH ligands are also transmembrane proteins. By binding to the NOTCH NECD they induce proteolytic cleavage and release of NICD, which then enters the cell nucleus to active transcription and modify gene expression (Ehebauer *et al*., 2006; Kopan & Ilagan, 2009). NOTCH is a key regulator in the induction of EMT in several different types of carcinoma, such as the breast cancer, lung and squamous cell carcinoma (Natsuizaka *et al*., 2017; Yuan *et al*., 2014b). Components of the NOTCH pathway are expressed at high levels in the invasive regions of tumors, which typically express vimentin, a mesenchymal marker, which suggests a crucial role for this pathway in EMT regulation (Saad *et al*., 2010). SNAIL and SLUG are mediators of NOTCH-mediated repression of *E-cadherin* and *β-catenin* activation (Saad *et al*., 2010). Interestingly, NOTCH signaling can also cooperate with other pathways, such as TGF-β, to induce the EMT program. The crosstalk between these pathways occurs *via* SMADs, which associate with other transcription factors to regulate expression of genes required for acquisition of the mesenchymal fate. For example, silencing components of the NOTCH pathway prevent TGF-β-induced EMT in keratinocytes (Blokzijl *et al*., 2003).

The Hedgehog family includes the Sonic Hedgehog (SHH), Desert Hedgehog (DHH) and Indian Hedgehog (IHH) proteins. Hedgehog ligands bind to patched homolog 1/2 (PTCH1/2), which inhibits activity of Smoothened (SMO) in the absence of ligand binding. Activation of PTCH1/2 releases SMO and initiates an intracellular cascade that activates the GLI family transcription factors, which promote transcription of the target genes, such as *PTCH*, *WNT* and *SNAIL* (Gonzalez & Medici, 2014). The Hedgehog (HH) pathway signaling can be involved in various stages of carcinogenesis in different tumors. For example, in pancreatic and esophageal cancer, activation of this signaling pathway is found at the early stages of tumor, as well as in the metastatic tumor (Ma *et al*., 2006). In other tumors, such as the gastric cancer and prostate cancer, activation of the HH signaling pathway is associated with tissue invasion and increased metastatic potential (Sheng *et al*., 2004). In context of the EMT regulation, the Farhart's group described that TGF-β-induced SHH may regulate EMT and tumorigenicity in bladder cancer (Islam *et al*., 2016). Moreover, they also observed an elevated expression of *N-cadherin* and *SHH* in high grade and stage tumor samples, and conversely, downregulation of these genes was observed in the low grade and stage tumor samples. Recent results also support the hypothesis that SHH promote EMT by suppressing E-cadherin and enhancing N-cadherin and vimentin (Kitagawa *et al*., 2019).

The EMT process is mediated by several EMT-related transcription factors (EMT-TFs), such as the SNAIL (SNAI1) and SLUG (SNAI2), TWIST1/2, ZEB1/2. Briefly, they repress genes associated with the epithelial phenotype (such as *E-cadherin*, etc.) and induce the expression of the mesenchymal genes (such as *vimentin*, *fibronectin*) (Lu & Kang, 2019; Tsai & Yang, 2013).

Both, SNAIL and SLUG play critical roles in induction of EMT during embryonic development and cancer progression (Aybar *et al*., 2003). In cancer, their expression leads to a decreased E-cadherin level, enhanced tumor cell invasion and metastatic phenotypes in mouse tumor models and cell line studies, which is associated with poor prognosis in patients with the breast, colorectal, and hepatocellular carcinoma *(*Blanco *et al*., 2002; De Craene *et al*., 2005; Shioiri *et al*., 2006; Tran *et al*., 2014; Yook *et al*., 2006). For example, using multiple genetic breast cancer models with inducible *SNAIL* transgene or *SNAIL* conditional knockout, it was demonstrated that the *SNAIL* expression is required for breast tumor metastasis to the lung (Tran *et al*., 2014). SNAIL and SLUG can promote breakdown of the extracellular matrix *via* upregulation of various matrix metalloproteases (MMPs) (Tsai & Yang, 2013). The TGF-β, WNT, and NOTCH pathways, as well as growth factors, can activate SNAIL expression depending on the physiological context (Peinado *et al*., 2007). Moreover, SNAIL and SLUG can also cooperate with other transcription regulators to control gene expression (Lamouille *et al*., 2014).

Similarly to SNAIL and SLUG, TWIST1/2 belongs to the bHLH (basic helix-loop-helix) transcription family that functions as master regulators of a wide array of developmental and pathological processes. In particular, TWIST-induced suppression of *E-cadherin* transcription is indirect and is mediated by its transcriptional activation of SLUG, as SLUG knockdown blocks the ability of TWIST to activate EMT in mammary cells *(Casas et al*., 2011). *TWIST* expression can be activated by hypoxia-inducible factor 1-alpha (HIF-1a) transcription factor under hypoxia conditions to promote EMT and metastasis (Yang *et al*., 2008). Using xenograft and transgenic tumor models, it has been shown that TWIST1 is essential for tumor cell dissemination and metastasis in breast cancer and squamous cell carcinoma (SCC), although turning off its expression is required for formation of metastasis in distant organs (Tsai *et al*., 2012; Xu *et al*., 2017). *TWIST* overexpression also correlates with cancer invasiveness and metastasis in patients (Lee *et al*., 2006; Yang *et al*., 2004).

Similarly to SNAIL, ZEB1 and ZEB2, two members of the ZEB transcription factor family, directly bind to the E-box elements and repress expression of *E-cadherin* (Comijn *et al.*, 2001). They also increase the level of mesenchymal proteins, i.e. vimentin and N-cadherin (Bindels *et al.*, 2006; Vandewalle *et al.*, 2005). Expression of the ZEB proteins can be induced by TGF-β, WNT signaling and other growth factors (Lamouille *et al.*, 2014). It has been shown experimentally in the cancer context, that ZEB1/2 promoted cell migration and invasion in breast cancer and colorectal cancer (Comijn *et al.*, 2001; Vandewalle *et al.*, 2005).

The EMT process is also activated by epigenetic modifications. Epigenetic modifications, such as DNA methylation, histone modification, or nucleosome positioning, alter the structure of chromatin, thereby regulating gene expression. Among epigenetic mechanisms, it is important to highlight modification of the histone tails, global hypomethylation of the genome, and specific hypermethylation of cytosine residues in CpG islands in the DNA promoter regions, for their role in gene repression and heterochromatin formation (Kouzarides, 2007). Inactivation of a promoter by hypermethylation is common event in several human carcinomas (Tamura *et al.,* 2000.; Wijnhoven *et al.*, 2000; Yoshiura *et al.*, 1995). These modifications cause gene inactivation due to transcriptional silencing, as a consequence of impaired TFs binding to their promoters. On the other hand, different types of modifications in the histone core and tails have been described which can affect the chromatin dynamics and gene expression. Hypermethylation of *CDH1* has been reported and associated with EMT and invasiveness (Tamura *et al.*, 2000). Several histone and DNA methyltransferases, and chromatin modifying enzymes related to EMT, have been described. Among them, KDM1A, KDM4B, and KDM6B, causing histone H3 demethylation at the *SNAIL* promoter; MMSET, which binds to the *TWIST* promoter increasing its activation by methylation; LSD1 and SUV39H1, which act by suppressing the *CDH1* transcription; SET8 which interacts with TWIST acting both, as a repressor or inducer of gene expression, and also mediates transcriptional activation of WNT target genes; G9A and PRMT5, interacting with both, *CDH1* and *SNAIL* (McDonald *et al.*, 2011; Serrano-Gomez *et al.*, 2016). Taken together, the epigenetic modifications described in cancer suggest that aberrant methylation might be triggered by EMT-related transcription factors or epigenetic regulators associated with them. Moreover, epigenetic modifications may be interdependent and successive, and work in different combinations to induce activation of the EMT program. On the other hand, reversibility of the methylation/demethylation state can mediate the shift between EMT and MET. These findings open new fields of research using methylation inhibitors, as targeting epigenetic regulators of EMT could be a promising therapeutic option. However, these treatments, as some authors have indicated, could also have negative effects, since just as they would reactivate the tumor suppressor gene expression, they could also involuntarily activate oncogenes (Prieto-García *et al.*, 2017).

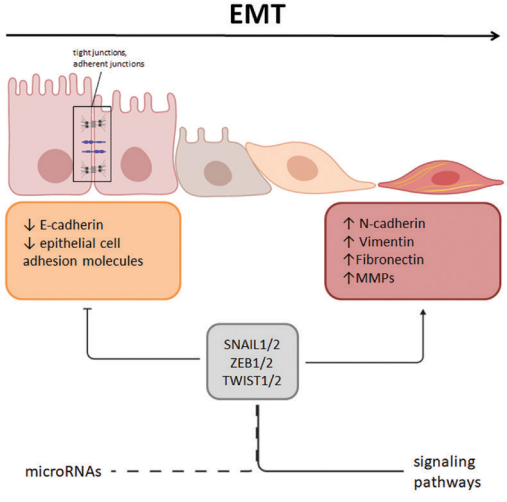

Figure 2. Regulatory network in EMT.
The EMT process can be regulated by many signaling pathways, transcription factors and microRNAs.

To summarize, EMT is regulated by different signaling pathways which lead to acquisition of the mesenchymal phenotype by epithelial cancer cell. The EMT regulatory network is schematically described in Fig. 2.

THE ROLE OF MICRORNA IN CANCER

As mentioned above, EMT is a dynamic process that can be caused by lots of inducers, among which microRNAs seem to be interesting regulators (Serrano-Gomez *et al.*, 2016). MicroRNA (miRNAs) are small non-coding RNAs, with an average length of 22 nucleotides. Most miRNAs are transcribed from DNA sequences into primary miRNAs (pri-miRNAs) and processed into precursor miRNAs (pre-mRNAs), and then into mature miRNAs (Mohr & Mott, 2015).

There are different types (classes) of small regulatory RNAs: 1) small interfering RNAs (siRNAs), and 2) microRNAs (miRNAs), which are generated by the cleavage of double-stranded (ds) RNA precursor molecules by type III ribonuclease Dicer. Although they share some common biogenesis factors, they are very different in terms of their biological role in the cell (Creugny *et al.*, 2018). MicroRNAs can directly repress target genes by inducing cleavage and degradation of their mRNA targets through a high degree of complementarity matching with their 3' untranslated regions (3'UTR). Lower complementarity causes translational repression (Mohr & Mott, 2015).

MicroRNAs were found to downregulate gene expression by base-pairing with 3'UTRs of the target messenger RNA (mRNAs) (Reinhart *et al*, 2000). Each miRNA may regulate many target genes and more than just one miRNA may bind to the same 3' UTR (Ciesla *et al.*, 2011). These discoveries indicated that this class of noncoding RNA molecules may be a new regulatory factor that controls gene expression.

As described previously, cancers are caused by mutations and deregulation of signaling pathways in the cells. The link between microRNA deregulation and cancer was described for the first time by Calin *et al.*'s research results. They found that the miR15a/16-1 cluster (between exon 2 and exon 5 in the Leu2 gene) is frequently deleted in chronic lymphocytic leukemia (CLL), which suggests that these two microRNAs have a tumor suppressor activity (Calin *et al.*, 2002). Another mechanism leading to an aberrant expression of microRNAs, and thus to cancer progression, is the altered expression and function of enzymes involved in the biogenesis of miRNA, like Drosha and Dicer. Their decreased expression has been found in 39% of ovarian cancer patients (Merritt *et al.*, 2008). Transcriptional control is another

important and complex regulation mechanism of miRNA expression. Upregulation of the miR-17/92 cluster modulates an anti-apoptotic action of E2F1, which mediates the MYC proliferative effect (Acunzo *et al*., 2015; O'Donnell *et al*., 2005).

Furthermore, some miRNAs may function as oncogenes or tumor suppressors. miRNAs that are overexpressed in cancer may function as oncogenes and promote tumor development by negative regulation of tumor suppressor genes and genes that control cell differentiation and apoptosis. Underexpressed miRNAs may function as suppressor genes and they inhibit cancer development by regulation of oncogenes and genes that control cell differentiation and apoptosis (Shenouda & Alahari, 2009; Zhang *et al*., 2007).

MICRORNAS AS THE KEY REGULATORS OF EMT

miRNAs may act as regulators of both, EMT facilitating tumor dissemination and its reverse process MET, which promotes metastatic colonization (Tsai & Yang, 2013). Interestingly, attenuation of global biogenesis of miRNAs by miR-103/107 targeting DICER was demonstrated to induce EMT and metastasis in breast cancer (Martello *et al*., 2010). Global miRNA downregulation was shown previously to be a common trait in tumors (Lu *et al*., 2005), whereas poor prognosis of different cancer types is associated with a diminished expression of miRNA processing factors (Sandberg *et al*., 2008).

miRNAs regulate EMT mostly by targeting the main transcription factors involved in this process, such as SNAIL, SLUG, TWIST, ZEB1 and ZEB2. Nevertheless, those factors may also function as regulators of the miRNAs level. Another type of miRNA's role is regulation of the cellular contact, adhesion and cytoskeletal proteins, which induces changes in the epithelial to mesenchymal phenotype (Expósito-Villén *et al*., 2018). Examples of those interactions are described below.

Expression of SNAIL transcription factor may be regulated by different miRNAs with implications in the EMT process. The *SNAIL* 3'UTR acts as a sponge for multiple metastasis-related miRNAs, such as miR-153, miR-199a-5p, miR-203, miR-204, miR-22, and miR-34c (Li *et al*., 2015). The miR-30 family is one of the most widely described SNAIL regulators in several tumor types, including non-small cell lung carcinoma (Kumarswamy *et al*., 2012) or breast cancer (Xiao *et al*., 2018), which leads to EMT regulation in epithelial tumors, or is responsible for non-canonical mechanism of SNAIL effects in the mesenchymal tumors, including rhabdomyosarcoma (Skrzypek *et al*., 2018).

miRNAs can also regulate the SLUG level, such as for example miR-203 in glioblastoma (Liao *et al*., 2015) or breast cancer (Zhang *et al*., 2011).

The TWIST1 level is also affected by several miRNAs, including miR-26b-5p in hepatocellular carcinoma (Wang *et al*., 2016), miR-106b in endometrial cancer (Dong *et al*., 2014), miR-361-5p in glioma cells (Zhang *et al*., 2017) or miR-720 (Li *et al*., 2014b) in breast cancer or miR-300 in gastric cancer (Yu *et al*., 2014).

ZEB1 and ZEB2 expression during EMT is regulated by the largest number of miRNAs. For example, *ZEB1* it is targeted by miR-33 in adenocarcinoma (QU *et al*., 2015), miR-128 in esophageal squamous cell cancer, and miR-200 (Zhao *et al*., 2018) in breast cancer (Bai *et al*., 2014).

Some of the crucial regulators of the ZEB2 level are members of the miR-200 family. They were shown as regulators in different tumor types, including glioma (Li *et al*., 2016), lung cancer (Jiao *et al*., 2016), and gastric carcinoma (Li *et al*., 2014a). *ZEB2* is targeted in lung cancer by miR-132 (You *et al*., 2014) and miR-154 (Lin *et al*., 2016), or in colorectal cancer by miR-132 (Zheng *et al*., 2014).

Nevertheless, besides examples of single miRNAs targeting different transcription factors in EMT, the most interesting are cross-talks between distinct transcription factors and miRNAs. One miRNA may have plenty of targets in different genes to regulate EMT. Therefore, transcription factors may be co-regulated by one miRNA. miR-200 was shown to regulate both SLUG and ZEB1 (Zhang *et al*., 2014), as well as the ZEB1 and SNAIL levels (Díaz-López *et al*., 2015) (Shan *et al*., 2013), miR-218 affects the level of SLUG and ZEB2 (Shi *et al*., 2017), and miR-129 co-regulates TWIST and SNAIL (Yu *et al*., 2015b). These types of regulation demonstrate key roles of miRNAs in regulation of the EMT process.

On the other hand, there are also examples of miRNAs regulated by more than one EMT-associated transcription factor. An interesting example is miR-375, which is regulated by direct binding of SNAIL (Xu *et al*., 2014b) and ZEB1 to its promoter (Selth *et al*., 2017). During EMT, both SNAIL and ZEB1 engage miR-200f epigenetic and transcriptional regulation (Díaz-López *et al*., 2015).

The EMT-related transcription factors may be involved in cross-talks based on their regulation of miRNA expression, with implications in epithelial tumor progression and the role of EMT in this process. SNAIL and SLUG repress miR-101 expression, which is essential for malignant phenotypes and EMT induction of squamous cell carcinoma of the oral tongue (Zheng *et al*., 2015), whereas miR-101 acts as a tumor suppressor by direct *ZEB1* targeting in various cancers, including colorectal cancer (Xiong *et al*., 2018), so there are cross-talks between EMT-related transcription factors *via* miRNAs.

Double negative or positive feedback loops between miRNAs and transcription factors are also described in the literature. Those loops are made of two interactions, so that the EMT-related transcription factors and miRNAs regulate each other. That regulation may either induce or repress expression. The mechanism may involve direct binding of transcription factors to miRNA promoters or direct binding of miRNAs to 3'UTR regions of transcription factors or can be indirect through mediators. Below, several examples of that regulation are described. Positive feed-forward regulatory loop was described for miR-373 that induces the TWIST level, and subsequently TWIST induces miR-373 expression by binding to its promoter (Chen *et al*., 2015). miR-1 and miR-200 can regulate SLUG, and SLUG is also a direct repressor of their action, which forms an interesting negative regulatory loop (Liu *et al*., 2013a). EMT has been also shown to be regulated by the miR-34 and SNAIL double negative feedback loop. miR-34 binds to 3'UTR region of *SNAIL* and thereby represses its expression, whereas SNAIL binds to miR-34 promoter to diminish its level (Siemens *et al*., 2011). That loop regulates EMT in human colon and breast cancer *(Hahn et al*., 2013). That mechanism is dependent on the p53 function. Without the wild-type p53 function, decreased levels of miR-34 result in a SNAIL-dependent EMT (Kim *et al*., 2011), whereas activation of p53 down-regulates the EMT-inducing transcription factor SNAIL *via* induction of the miR-34 genes (Siemens *et al*., 2011).

An interesting negative feedback loop also exists between miR-203 and SLUG (Ding *et al*., 2013), and be-

tween miR-203 and SNAIL that acts as a regulator of the miR-200 expression and its targets, ZEB1 and ZEB2 (Moes *et al.*, 2012).

Certain miRNAs may induce EMT because they act as additional blockers of E-cadherin, besides SNAIL and SLUG. miR-221, which is regulated by SLUG, suppresses the E-cadherin level and thereby promotes breast cancer metastasis (Pan *et al.*, 2016). In breast cancer, miR-210 suppresses *E-cadherin* expression by targeting the open reading frame region of *E-cadherin* mRNA (Tang *et al.*, 2018). Another example is pro-metastatic miR-9 – it also targets mRNA encoding *E-cadherin* (Ma *et al.*, 2010). miR-10b and miR-214 also directly regulate *E-cadherin* by targeting its 3´UTR (Zhang *et al.*, 2015) (Liu *et al.*, 2018). Moreover, multiple miRNAs regulate E-cadherin level indirectly, such as for example members of the miR-200 family (Korpal *et al.*, 2008).

MiRNAs acting as EMT inducers may not be equal functionally. From the set of EMT-associated miRNAs, the most commonly upregulated miRNAs, miR-22 and miR-100, and the most significantly downregulated miRNAs, are capable of inducing EMT in mammary epithelial cells (Martello *et al.*, 2010). MiRNAs inducing EMT may have different functions which depend on their specific target genes.

MiRNAs may regulate not only the epithelial, but also mesenchymal markers, such as vimentin. MiR-22 was shown to affect its level (Xu *et al.*, 2018). What is more, miR-199a modulates the N-cadherin level (Suzuki *et al.*, 2014), whereas miR-27a directly regulates vascular endothelial (VE)-cadherin (Zhao *et al.*, 2016).

The RhoA protein plays a crucial role in re-organization of the actin cytoskeleton and its level is affected by several miRNAs, including miR-122 (Wang *et al.*, 2014), miR-24 (Papadimitriou *et al.*, 2012), and miR-1291 (Xu *et al.*, 2017a).

miR-29b inhibits metastasis by regulating the level of proteins involved in epithelial plasticity and differentiation in breast cancer, such as TGFB1, ITGA6, and ITGB1. Moreover, miR-29b also affects several pro-metastatic proteins levels, such as VEGF, MMP2, MMP9 or PDGF (Chou *et al.*, 2013).

MiRNAs may be also regulated by long non-coding RNAs in the EMT process. In colorectal cancer, long noncoding RNA XIST modulates EMT by competing for miR-200b-3p to modulate the *ZEB1* expression (Chen *et al.*, 2017a). Another example is long non-coding RNA UICLM which promotes colorectal cancer liver metastasis by acting as a competitive endogenous RNA for microRNA-215 to regulate the ZEB2 level (Chen *et al.*, 2017b). What is more, the SLUG level was demonstrated to be regulated by lncRNA UCA1 by interaction with miR-203 (Xiao *et al.*, 2017). Another interesting example is lncRNA CAR10, which directly binds two miRNAs: miR-30 and miR-203, and in that way regulates the level of both, SNAIL and SLUG in EMT (Ge *et al.*, 2019). MALAT1 lncRNA acts as a sponge for miR-126-5p that directly targets *TWIST, SLUG and VEGF* in colorectal cancer, which regulates both metastasis and angiogenesis (Sun *et al.*, 2019). An increasing number of research papers shows several lncRNAs involved in regulation of EMT by modification of the TGF-β (Yuan *et al.*, 2014a) or WNT pathways (Jiang *et al.*, 2018). Usually, lncRNAs act as sponges for miRNAs involved in EMT. Examples of interactions between lncRNAs, miRNAs and EMT-related factors are shown in Fig. 3.

MiRNAs may be also affected by epigenetic modifications. miRNAs' promoters may be hypermethylated or demethylated, which affects their expression. This

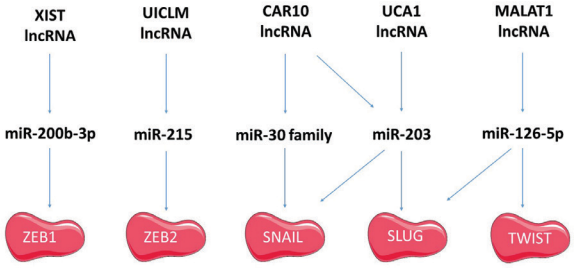

Figure 3. Selected examples of crosstalks between lncRNAs, miRNAs and EMT-related factors.

mechanism was described in different cell types for several miRNAs regulating EMT, such as for example miR-200 (Pieraccioli *et al.*, 2013) and miR-203 (Taube *et al.*, 2013). On the other hand, an opposite mechanism also regulates EMT. Impaired EMT process may be a result of targeting epigenetic modulators, such as *DNMT* by miR-152 (Yu *et al.*, 2015a).

MiRNAs associated with EMT-related transcription factors in epithelial tumors may also play crucial roles in progression of the mesenchymal tumors. An interesting example is regulation of rhabdomyosarcoma growth – in that tumor, SNAIL regulates expression of myogenic associated miRNAs, such as miR-1, miR-206, and miR-378, to affect tumor growth involving other mechanisms than EMT (Skrzypek *et al.*, 2018). miR-1 was previously shown to inhibit EMT *via* SLUG-dependent and *via* SLUG-independent mechanisms (Liu *et al.*, 2013b). Moreover, tumor suppressor miR-1 restrains the epithelial-mesenchymal transition and metastasis of colorectal carcinoma *via* the MAPK and PI3K/AKT pathways (Xu *et al.*, 2014a). miR-206 is a regulator of SLUG and MET in different tumor types. For example, in epithelial tumors, miR-206 regulates EMT in human lung adenocarcinoma cells partly by targeting MET (Chen *et al.*, 2016). That mechanism of regulation is also important in metastasis of mesenchymal tumors, such as rhabdomyosarcoma (Yan *et al.*, 2009; Szewczyk *et al.*, 2017). Those results suggest that miRNAs identified to regulate EMT in epithelial tumors may be also significant in metastasis of the mesenchymal tumor types.

MICRORNAS AS BIOMARKERS AND THERAPEUTICS IN CANCER

The main cause of death in cancer patients is metastasis of tumor cells to distant organs from the primary epithelial tumor. For this reason, understanding the cellular mechanisms that lead to metastasis is critical in the fight against cancer.

miRNAs may have clinical relevance as biomarkers. These biomarkers can be used to indicate presence of a given cancer and predict its stage, progression or drug efficiency (Armand-Labit & Pradines, 2017; Ciesla *et al.*, 2011).

The use of circulating miRNAs as biomarkers in different cancer types is a rapidly developing field. Tumor cells can release miRNAs that can be stabilized by incorporation into microvesicles which have shown stability in the circulation, following multiple freeze-thaw cycles and prolonged exposure to room temperature. miRNAs have also shown stability in other body fluids, but most of the studies focused on serum miRNAs as biomarkers. Moreover, the circulating miRNAs show constant level in the blood of healthy individuals. In cancer patients, most of

Table 1. Examples of clinical trials with microRNA as clinical biomarkers in cancer (clinicaltrials.gov).

miRNA gene	Trial	Clinical trial number; phase status	Cancer type investigated	References
miR-31-3p miR-31-5p;	Expression of microRNA biomarkers as prognostic of patient outcomes or predictive of the benefit from anti-EGFR therapy in stage III colon cancer	NCT03362684; phase 3 (completed)	Colorectal cancer (CRC)	(Taieb *et al.*, 2014)
Numerous miRNAs	MicroRNA Profile in early-stage cervical cancer	NCT04087785 (completed)	Cervical cancer	–
Numerous miRNAs	MicroRNA profile of early cardiotoxicity in breast cancer patients treated with anthracyclines	NCT02065908 (completed)	Breast cancer	–
miR-10b	Expression levels as biomarkers of tumor grade, survival and genetic variation	NCT01849952; (recruiting)	Glioma	–
miR-29a	Exploration of prognostic value of miR-29b in tissue, blood and saliva	NCT02009852	Oral Squamous cell carcinoma	–
Numerous miRNAs	Identifying biomarkers for patient stratification in tissue samples	NCT01828918 (unknown)	Colorectal cancer	–
Numerous miRNAs	Analysis of microRNA expression in basal cell carcinoma	NCT01498250 (completed)	Basal cell carcinoma	–
miR-29 family	Investigate the role of microRNA in Twist1--mediated cancer metastasis	NCT01927354; (unknown)	Head and neck squamous cell carcinoma	–
Circulating secret miRNAs	Biomarker of response to treatment	NCT01391351; (completed)	Breast cancer	–
Numerous miRNAs	MicroRNAs Expression profiles in initiation, progression and treatment response	NCT01108159; (completed)	Hematologic cancer	–

the circulating miRNAs are directly delivered from the tumor tissue (Armand-Labit and Pradines, 2017). On the other hand, circulating miRNAs can occur as a result of treatment, diet or other factors, which increases the noise level in these assays (Hayes *et al.*, 2014). The most current clinical trials for the use of miRNAs as biomarkers for cancer prognosis and drug efficacy studies were described in Table 1.

Deregulation of miRNA expression plays a key role in cancer development. Modulating of miRNA levels or restoring their function may be a new strategy in the cancer treatment. There are two ways to modulate the level of a particular miRNA: restoration of tumor suppressor miRNA by a straightforward transfection of synthetic mimics or transduction of cells with vectors expressing miRNAs (Miroshnichenko & Patutina, 2019). On the other hand, new technologies have been developed to inhibit functions of oncogenic miRNAs. Such compounds may target the miRNA sequence or interrupt the miRNA regulatory activity through interaction with their mRNA targets (Miroshnichenko & Patutina, 2019). Modulation of the miRNAs' level may be used to directly target tumor cells or it may also enhance other therapies, which has been shown in small cell lung cancer for miR-100 that regulated chemo-resistant properties of cancer cells (Xiao *et al.*, 2014). Another example is the epigenetic silencing of miR-199b-5p in a chemoresistant ovarian cancer (*Liu et al.*, 2014).

The miRNA treatment and therapies are challenged by several obstacles. One is associated with off-target effects, which can lead to unwanted responses in tissues other than the intended ones (Broderick & Zamore, 2011; Chakraborty *et al.*, 2017). Another obstacle is the successful delivery of the therapeutic agent to the target tissues. Therapeutics must overcome problems associated with oligonucleotides, such as degradation by nucleases, renal clearance, failure to cross the capillary endothelium, ineffective endocytosis by target cells, or ineffective endosome release (Kim & Rossi, 2007). Different delivery systems are used for better bioavailability, including PEGylated liposomes, lipidoids, and biodegradable polymers. Vesicles with diameters between 50 and 500 nm have been used to deliver therapeutic miRNAs and siRNAs. These vesicles prevent the drugs from being filtered by the kidneys and improve intracellular delivery (Broderick & Zamore, 2011).

Furthermore, while local delivery into the eye or skin has been shown to improve bioavailability in the targeted sites, systemically delivered miRNA formulations and RNA-based miRNA targeting agents might be negatively impacted by the host immune system, since macrophages and monocytes can remove complexed RNAs from the extracellular spaces (Broderick & Zamore, 2011). For instance, 21 base pair or longer dsRNAs can lead to a sequence-independent interferon response (Pai *et al.*, 2006). An additional challenge is represented by the release of RNA-based therapeutics formulated in complexes larger than 5 nm in diameter, from the blood to the target tissue through the capillary endothelium (Whitehead *et al.*, 2009). Another challenge is the safety evaluation of miRNA-based therapeutics, such as the mentioned above potential immune response against the delivery system, toxicity caused by the chemical modification or unexpected off-target effects that are likely to occur, because each miRNA can affect hundreds of target genes. Another obstacle for anti-miR therapeutics is the complexity of the assessment of anti-miR efficacy. This is because anti-miR treatment may not always reduce the miRNA expression levels. High throughput profiling of global mRNA and protein changes in samples could provide more comprehensive information regarding the specificity and effectiveness of a particular anti-miR treatment (Chakraborty *et al.*, 2017).

Clinical trials were established for miRNA mimics (to overexpress the transcript), as well as repressors (to silence the transcript function). The first recently completed phase I trial based on a new technology termed „TargomiR" exhibited encouraging results in patients

Table 2. Interventional clinical trials for microRNAs as therapeutic target (clinicaltrials.gov).

miRNA gene; drug name	Clinical trial number; Phase status	Cancer type investigated	References
miRNA-34a; MRX34	NCT01829971; Phase I (terminated)	Hepatocellular carcinoma (HCC)	(Beg *et al*., 2017)
miR-16; TargomiRs	NCT02369198; Phase I (completed)	Malignant pleural mesothelioma non-small cell lung cancer (NSCLC)	(Reid *et al*., 2013; van Zandwijk *et al*., 2017)

with malignant pleural mesothelioma or non-small cell lung cancer. Briefly, TargomiR delivery vehicles contain a miRNA mimic, the drug delivery vehicle – EDVs that are nonliving bacterial minicells (nanoparticles), and a targeting moiety (i.e. a specific antibody that recognizes a protein on a target cells). In that first human trial of TargomiR drug, the miRNA mimic for miR-16 and a bispecific antibody to the epidermal growth factor receptor (EGFR) were used (Reid *et al*., 2013; van Zandwijk *et al*., 2017). Other clinical studies of miRNAs are described in Table 2. They give hope for an interesting future for miRNA drugs in cancer.

CONCLUSIONS AND PERSPECTIVES

The level of different proteins regulating EMT may be affected by non-coding RNAs, such as miRNAs. MiRNAs may regulate EMT-related transcription factors by direct binding to the 3'UTR region of their mRNA or indirectly, as well as those transcription factors may regulate their level. Sometimes there are crosstalks between several miRNAs and more than one transcription factor, as well as feedback loops are described in the literature. MiRNAs regulate not only the EMT-related transcription factors, but they also affect the level of epithelial and mesenchymal markers and proteins associated with reorganization of the cytoskeleton. Long non-coding RNAs usually act as sponges for miRNAs involved in EMT regulation. Understanding of the complicated signaling networks regulating EMT may help to identify novel biomarkers or develop new treatment strategies. Some of the miRNAs described above have already been enrolled in clinical trials as miRNA based therapeutics. For example miR-34 and miR-29 based therapeutics are under investigation in phase 1 clinical trials (Hanna *et al*., 2019). In the future, there are perspectives for more miRNAs to be used in the therapeutic approaches.

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