Review

TGFβ and matrix-regulated epithelial to mesenchymal transition ☆

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ABSTRACT

Background: The progression of cancer through stages that guide a benign hyperplastic epithelial tissue towards a fully malignant and metastatic carcinoma, is driven by genetic and microenvironmental factors that remodel the tissue architecture. The concept of epithelial–mesenchymal transition (EMT) has evolved to emphasize the importance of plastic changes in tissue architecture, and the cross-communication of tumor cells with various cells in the stroma and with specific molecules in the extracellular matrix (ECM).
Scope of the review: Among the multitude of ECM-embedded cytokines and the regulatory potential of ECM molecules, this article focuses on the cytokine transforming growth factor β (TGFβ) and the glycosaminoglycan hyaluronan, and their roles in cancer biology and EMT. For brevity, we concentrate our effort on breast cancer.
Major conclusions: Both normal and abnormal TGFβ signaling can be detected in carcinoma and stromal cells, and TGFβ-induced EMT requires the expression of hyaluronan synthase 2 (HAS2). Correspondingly, hyaluronan is a major constituent of tumor ECM and aberrant levels of both hyaluronan and TGFβ are thought to promote a wounding reaction to the local tissue homeostasis. The link between EMT and metastasis also involves the mesenchymal–epithelial transition (MET). ECM components, signaling networks, regulatory non-coding RNAs and epigenetic mechanisms form the network of regulation during EMT-MET.
General significance: Understanding the mechanism that controls epithelial plasticity in the mammary gland promises the development of valuable biomarkers for the prognosis of breast cancer progression and even provides new ideas for a more integrative therapeutic approach against disease. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

It has long been understood that epithelial tumors lose their tissue organization, become dedifferentiated and secrete abnormal quantities of extracellular matrix (ECM) in a process that resembles wound healing, and which connects to the invasive and metastatic capacity of the primary tumor [1]. Related to these events is the process of epithelial–mesenchymal transition (EMT), which has biological relevance during early embryonic development and later organogenesis, and which can also be activated during wound healing in fibrotic or cancer tissues that experience chronic inflammatory stress [2]. The term "transition" in EMT, emphasizes the transient and reversible nature of the process, so that under specific conditions within the tissue, mesenchymal cells can also undergo the inverse plastic change that generates epithelial tissue, so called, mesenchymal–epithelial transition (MET). Key cellular alterations that occur during EMT are the loss of cell–cell adhesions, the change in supporting cellular polarity so that the apico-basal differentiation of epithelial cell membranes is destroyed, the new interactions between new plasma membrane receptors and remodeled constituents of the ECM, all being important changes that support collective or even individual cell migration and a more plastic, so-called mesenchymal overall cell identity [3]. Developmental examples of EMT are the morphogenetic processes of gastrulation and neural crest formation, lung organogenesis and specialized tissue formation such as the heart valve cushions [3]. During chronic inflammation, tissues can become either fibrotic, exhibiting excessive synthesis and deposition of ECM, or cancerous, exhibiting again remodeled matrix and enhanced capacity for cell motility [3,4]. Furthermore, EMT is thought to facilitate the extracellular microenvironment that fosters stem cell proliferation and maintenance in the context of cancer development [5]; EMT endows cancer cells with resistance to oncogene-induced senescence and chemo- or radiotherapeutic regimes, thus contributing to the generation and propagation of so-called cancer stem cells that are responsible for the long-term maintenance and metastatic dissemination of this disease [6–8]. Many of the key contributions of EMT in cancer progression

have been understood by studying breast cancer in model organisms, in vitro and in human specimens and for this reason our analysis will focus on this specific group of human malignancy.

2. Molecular factors that regulate EMT

It is now well established that alterations in the ECM including interstitial matrix and the specialized basement membrane, are closely correlated to tumor progression. During the progression of breast carcinomas the epithelial cells as well as the cells of the stroma increase the synthesis of ECM components, resulting in changes in the composition and thereby structure of the matrix [9], as illustrated by histological and biochemical studies [10–14]. Notably, the tumor stroma resembles the stroma during embryonic development, which is rich in glycosaminoglycans and promotes cellular proliferation and migration [1,15].

The mammary glands are composed of bilayered ductal structures of outer myoepithelial cells, inner luminal cells and mammary stem cells that reside between these two populations, adipose cells, fibroblasts, endothelial cells, immune cells and ECM. ECM components include collagens, laminins, proteoglycans, fibronectin, tenascins, elastin and glycosaminoglycans [13,16]. The basement membrane is a specialized ECM that separates epithelial, endothelial cells and adipocytes from the stroma [16,17]. During tumor progression the ECM composition and structure are modified by infiltrating mast cells, leukocytes, macrophages and "activated" fibroblasts that release growth factors, cytokines, chemotactic factors and proteolytic enzymes and exhibit increased biosynthetic activity of ECM components. In particular, elevated production of the ECM components hyaluronan, versican and collagen is prominent in breast tumor stroma [18–22]. In breast cancer, the ECM is enriched in many new constituents, mainly proteins such as specific collagen and fibronectin isoforms and secreted growth factors such as transforming growth factor β (TGFβ) and fibroblast growth factors (FGFs) that have an impact on the tumor cells by facilitating the onset and maintenance of EMT. These changes in the microenvironment prepare the evolving tumor tissue so that tumor cells can invade locally [5]; moreover, blood and lymphatic vessels are recruited towards the tumor and some tumor cells manage to intravasate into the recruited vessels [23]. Understanding how the altered composition of the ECM determines the de-regulated mechanisms during breast cancer invasion and metastasis is of utmost importance.

A growing list of molecular and environmental cues can initiate the EMT [3] (Fig. 1). Smoking, hypoxic conditions and ultraviolet radiation are established inducers of EMT, which usually operates by activating or mediating the secretion of a cohort of growth factors, cytokines and chemokines that induce the EMT process via activation of their respective signaling pathways [3]. For example, during breast cancer progression, estrogens inhibit the EMT and promote epithelial differentiation, but when estrogen receptor expression is misregulated, hypoxic
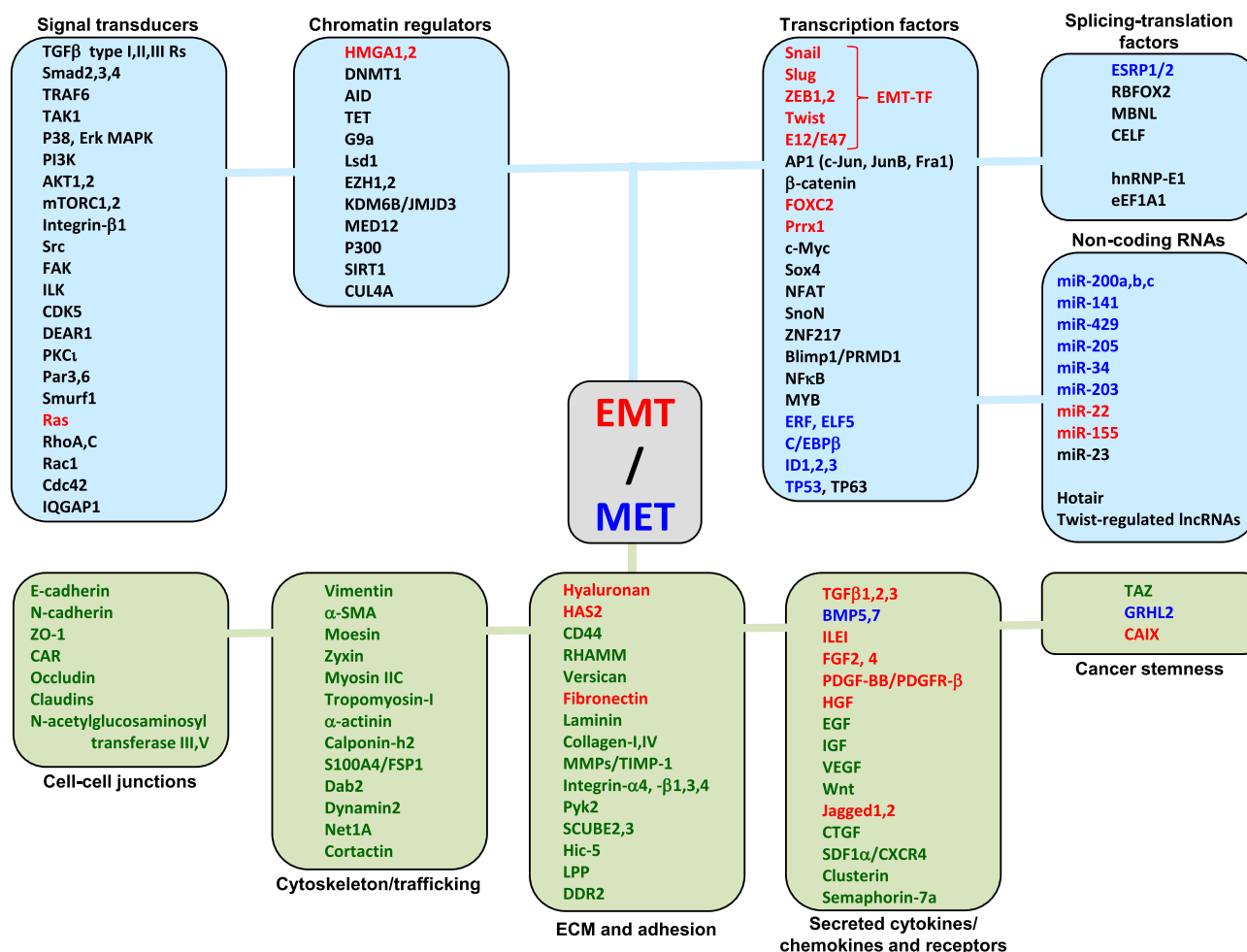
Fig. 1. The EMT–MET program (partial overview). Five regulatory modules that induce or control EMT and MET are shown on top. These modules are signal transducers, chromatin regulators, transcription factors, splicing and translation factors and non-coding RNAs. Below, five distinct but also inter-related functional modules include lists of molecules whose architecture, organization, expression or function changes during EMT–MET and cancer stemness. These modules are the cell–cell junction proteins, the cytoskeleton and trafficking proteins, the ECM and adhesion molecules, the secreted cytokines, chemokines and receptors, and the cancer stemness regulators. In each module, molecular players that are discussed in this review are listed. Factors that induce EMT are shown in red whereas factors that induce MET are shown in blue; other factors that may regulate either process are listed in black or green.

conditions stabilize and induce secretion of TGFβ, which then induces expression of, and mediates crosstalk with, components of the pathways of Notch, insulin-like growth factor, interleukin-like EMT inducer (ILEI) and vascular endothelial growth factor, promoting a sustained EMT that fosters cancer metastasis [24].

Many of these signaling pathways, e.g. TGFβ, FGF and Notch lead to the upregulation of specific embryonic transcription factors, whose normal action is not only to promote the EMT during embryonic development, but also to facilitate EMT and invasiveness in breast cancer. The so-called EMT transcription factors (EMT-TFs) are zinc finger proteins, such as the transcriptional repressors Snail (SNAI1) [25,26] and Slug (SNAI2) [27,28]; zinc finger and homeobox domain proteins, such as δEF1/ZEB1 [29] and SIP1/ZEB2 [30]; basic helix–loop–helix (bHLH) proteins such as E47 [31] and Twist1 [32] transcription factors. In addition, the chromatin protein high-mobility group A2 (HMGA2) integrates EMT signals downstream of TGFβ in breast cancer cell lines, and coordinates the transcriptional induction of Snail, Slug, Twist1 and the repression of the inhibitor of differentiation ID2 [33–35]. The molecular networks that coordinate the expression and function of the EMT-TFs in breast cancer are being understood at increasingly deeper levels [36], and specific examples will be listed below under the action of TGFβ.

3. TGFβ induces mammary EMT

The mechanisms and pathways that mediate signaling by TGFβ and initiate or maintain the EMT program have been studied over the years [37,38]. Induction of EMT by TGFβ represents one of the key cell biological processes that mediate pro-tumorigenic actions by this cytokine, while TGFβ is also known to act as a tumor suppressor by promoting epithelial tissue homeostasis and safeguarding tissues from abnormal growth prior to cancer advancement towards the aggressive and invasive stage [39]. Some useful in vitro cell models of mammary epithelial cells that undergo robust EMT in response to TGFβ have been instrumental for our current understanding of signaling mechanisms that contribute to EMT. Among such models, the Namru murine mammary gland (NMuMG) cell line is very sensitive to TGFβ signaling and shows robust and reversible EMT responses [40–42]; the mouse EpH4 cell line, when transformed with oncogenic H-Ras (generating the EpRas model), undergoes a very stable and irreversible EMT that depends on autocrine TGFβ [43]; more recently the mouse Py2T cell line also shows robust and reversible EMT response [44]. The NMuMG cell model has also been exploited to generate the fluorescently labeled Fucci model that allows in vivo monitoring of the phase of the cell cycle [45]; however, NMuMG cells fail to generate xenografts in immunocompromised mice and thus cannot contribute to correlative studies of EMT and cancer metastasis [40,45]. Both EpRas and Py2T cell models are compatible with in vivo studies of cancer progression and metastasis [43,44].

4. Importance of non-Smad signaling in EMT

The TGFβ signaling pathways commonly start by ligand binding to the heteromeric type II/type I receptor serine/threonine kinase complex, and these two receptors subsequently mediate different types of signals that foster the EMT response [46]. We commonly divide the receptor-initiated signals into non-Smad and Smad signaling pathways based on the profound importance of the Smad family of signal transducers that mediate many of the TGFβ signals. We first discuss examples of non-Smad signaling in EMT. TGFβ activates its type II receptor kinase, which phosphorylates the polarity complex protein Par6, leading to recruitment of the ubiquitin ligase Smurf1 that ubiquitylates and degrades the small GTPase RhoA, thus causing local depolymerization of actin microfilaments at the apical junctions of mammary epithelial cells, and subsequent disassembly of the tight and adherens junctions at the plasma membrane, thus initiating the EMT [47]. Recent

deeper evaluation of this mechanism demonstrates that one of the polarity complex enzymes, the atypical protein kinase C ι (PKCι) also makes a complex with the TGFβ receptors and potentiates the phosphorylation of Par6 by the type II receptor kinase [48]. The TGFβ type I receptor also provides non-Smad signals, as for example, upon recruitment of the ubiquitin ligase TRAF6, activation of proteases leads to cleavage of the receptor and translocation of the cytoplasmic kinase domain of this receptor to the nucleus, where it binds to chromatin and together with the transcriptional co-activator p300 induces the expression of EMT-TFs like Snail [49]. In addition, studies in NMuMG cells and in breast cancer cell lines have linked the TGFβ type I receptor with downstream activation of many key signaling enzymes that provide essential signals towards EMT, and include the phosphatidylinositol 3′ kinase (PI3K)/AKT kinase pair [50], the Src tyrosine kinase and the mitogen activated protein kinase (MAPK) p38 [51,52], the focal adhesion kinase (FAK) whose signaling is coupled to activation of the integrin-β1 [53,54], the Rho family of small GTPases [53], and more [55]. A prominent and more recent pathway that mediates EMT signals downstream of TGFβ is the mammalian target of rapamycin (mTOR) kinase pathway, which is directly coupled to the PI3K [56]. Accordingly, TGFβ type I receptor signaling couples to the PI3K leading to activation of the mTOR complex 2 (mTORC2), whose activity is required for EMT-TF activation and breast cancer cell migration. For this reason, inhibition of mTOR kinase activity could effectively block in vivo metastasis of breast cancer cells [56]. The activation of mTORC2 by TGFβ also involves another kinase, the integrin-like kinase (ILK) that forms complexes with mTORC2 in response to TGFβ and inhibition of ILK activity, similar to mTORC2 inhibition, negatively impacts on EMT and cell invasiveness [57]. Interestingly, an RNAi screen revealed that the mTORC1 kinase complex is required for epithelial differentiation of mammary cells, and downregulation of mTORC1 potently promoted EMT-TF (ZEB1, ZEB2) expression and EMT [58]. TGFβ signaling via the PI3K also affects mTORC1 kinase activity and this regulates cell size and overall levels of protein synthesis in mammary epithelial cells whose cell cycle is arrested by TGFβ [59]. Thus, the two mTOR complexes, mTORC1 and mTORC2 are important in the process of EMT during breast cancer progression and mTOR kinase inhibitors may be useful as part of a cocktail against breast cancer metastasis. A final and novel aspect of non-Smad signaling that controls the EMT response to TGFβ that we discuss here is the role of the cyclin-dependent kinase 5 (CDK5) [60]. CDK5 potentiates the TGFβ signal that initiates EMT at least in part by controlling FAK kinase phosphorylation, and thus leading to breast cancer cell migration and invasion. The fact that CDK5 is highly expressed in many breast cancers also correlates with an overactivity of TGFβ signaling in these cancers and offers another druggable target in the panoply of anti-invasive drugs that are currently under development. Another contribution of the PI3K/AKT signaling axis to EMT is the direct phosphorylation of the EMT-TF Twist1 by AKT, which promotes Twist1 transcriptional activity [61]. Interestingly, one of the target genes of the AKT-activated Twist1 is the gene for the TGFβ2 ligand, which is induced and promotes sustained autocrine TGFβ signaling and metastasis [61].

5. Smad-mediated transcriptional control of EMT

As outlined in the previous section, one of the best understood scenarios of EMT downstream of TGFβ signals that operate in breast epithelial cells involves Smad signaling and transcriptional regulation of a cohort of EMT-TFs [38]. Our original finding that TGFβ induces the embryonic chromatin factor HMGA2 via Smad signaling to elicit EMT [33–35] (Fig. 2), has now been confirmed by independent studies also demonstrating in vivo that HMGA2 overexpression causes metastasis of various breast cancer models [62]. We identified HMGA2 as an immediate-early and direct target of TGFβ/Smad signaling, and the study of Morishita et al. also uncovered that HMGA2 could regulate the expression of the TGFβ type II receptor, presumably via a chromatin-
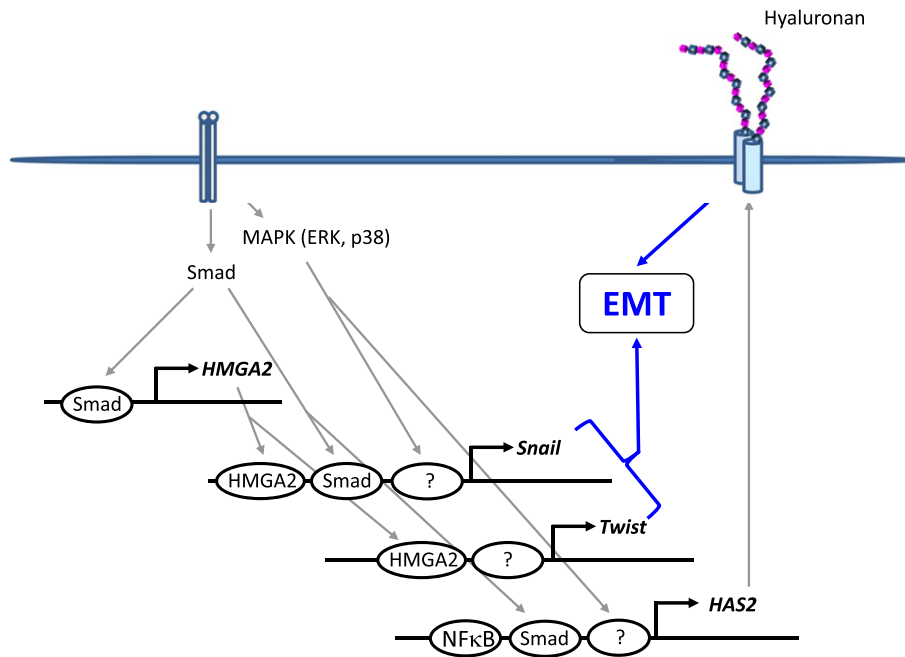
Fig. 2. Suggested mechanisms in TGFβ-induced EMT. TGFβ signals via its heteromeric receptor complex (TβR) to activate Smads, which then leads to direct transcriptional regulation of the *HMGA2* gene. Furthermore, TGFβ-activated Smad and MAPKs (ERK, p38), together with newly synthesized HMGA2 are required for the transcriptional induction of *Snail*, while HMGA2 also contributes to the transcriptional induction of *Twist1*. In addition, TGFβ-dependent induction of HAS2 expression, in a Smad-dependent and Smad-independent manner, is important in TGFβ-induced EMT. Experimentally defined regulatory sequences in the promoters of *HMGA2*, *Snail*, *Twist1* and *HAS2* genes are indicated, while unknown transcription factor binding sites are shown with a question mark. Transcriptional activation of the *HAS2* gene leads to enhanced HAS2 protein expression and hyaluronan production. Upon their induction by TGFβ, the Snail and Twist transcription factors as well as HAS2 molecules contribute to the EMT response of mammary epithelial cells.

dependent transcriptional mechanism, thus generating a double positive regulatory loop between TGFβ, HMGA2 and one of the major TGFβ receptors [62]. Earlier evidence that the related protein HMGA1 could promote mesenchymal differentiation and invasiveness in breast cancer cells [63], was recently expanded to several examples of basal-like breast cancer cell models, emphasizing the role of HMGA1 as a key mediator of the mesenchymal state and of associated stem-like and self-renewal features (as will be discussed later) [64]. One of the direct targets of TGFβ/Smad signaling and of HMGA2 transcriptional regulation is the *Snail* gene [34].

An emerging area in the EMT field is the necessity of epigenetic regulation at a genome-wide level for EMT to be elicited and for the EMT-TFs to properly function, a fact that collectively is now referred to as nuclear reprogramming during EMT [65]. Accordingly, transcriptional repression by Snail requires the direct function of the interacting histone tail methyltransferase G9a and cooperating DNA methyltransferases in a large set of breast cancer cell lines and primary breast cancer samples [66]. Interestingly, one of the direct gene targets of Snail and chromatin-based co-repressor complexes is the glycolytic gene *fructose-1,6-biphosphatase 1* (*FBP1*) [67]. Snail-mediated repression of FBP1 during EMT in basal-like breast cancers mediates in part the well known Warburg effect and thus promotes metabolic switch and stem-like growth properties to cancer cells. In a similar manner, mammary epithelial cells that exhibit robust EMT due to Twist1 overexpression also exhibit dramatic genome-wide changes in the methylation pattern of histone tails and of DNA, in a locus-specific manner [68]. Epigenetic control of the breast cancer genome by Twist1 appears to require the cooperation of the EZH1 and EZH2 histone tail methyltransferases. Regulation of histone tail modifications by EMT-TFs is very dynamic and gene-specific, as exemplified by the involvement of the histone demethylase KDM6B/JMJD3, which positively contributes to TGFβ-induced EMT in mammary epithelial cells, in part by facilitating the transcriptional induction of Snail [69]. Demethylation operates not only on histone tails, but also at the DNA level and the activation-induced cytidine deaminase (AID) that demethylates methylated

cytidines, plays positive roles in the induction of EMT in immortalized mammary epithelial and breast cancer cells by supporting the transcriptional induction of several EMT-TFs [70].

More proximal to the core components of TGFβ signaling, the mediator subunit MED12 was found to be frequently mutated and inactivated in human cancers, including breast cancer [71]. Loss of MED12 derepresses TGFβ type II receptor expression via a cytoplasmic mechanism where MED12 sequesters the receptor, and enhanced TGFβ signaling contributes to EMT and drug resistance, a common feature of cancer stem cells. This mechanism strongly proposed that combinatorial therapy against the TGFβ receptor and another pathway in cancer cells has synergistically beneficial effects at least in vitro. In a functionally similar but distinct mechanism, the nicotinamide–adenine–dinucleotide-dependent deacetylase SIRT1 protects mammary epithelial cells and breast cancer cells from EMT and metastasis by deacetylating Smad4, and thus limiting the transcriptional activity of this major mediator of TGFβ signaling [72]. A similar mechanism targets Smad3, another signaling mediator of TGFβ pathways, and involves the ubiquitin ligase DEAR1/TRIM62, which ubiquitylates and degrades Smad3 [73]. In breast and other types of cancer, the *DEAR1* gene is mutated by loss-of-function mutation, leading to oncogenic progression, enhanced TGFβ signaling (due to loss of negative regulation of Smad3) and subsequent EMT, invasiveness and metastasis in vivo. A *DEAR1–Slug* gene duo was shown to serve as a faithful predictor of poor survival in invasive breast carcinoma [73]. Using a different mechanism of positive feedback towards TGFβ ligand expression, the Krüppel-like zinc finger transcription factor ZNF217 is oncogenically mutated in invasive and metastatic breast cancer and causes EMT and migration upon overexpression in mammary epithelial cells [74]. ZNF217 causes misexpression of TGFβ, and abnormal activation of its signaling pathway; for this reason, TGFβ receptor inhibition can effectively block the pro-invasive and metastatic effects of oncogenic ZNF217.

However, it should be made clear that in addition to the major EMT-TFs and direct modulation of the TGFβ–Smad function, a large cohort of transcription factors cooperates with Smads or gets induced by TGFβ

and participates in regulation of specific genes that impact on the EMT program in mammary epithelial cells. Such a recent prominent example is the homeobox transcription factor Sox4, which is highly expressed in a subset of triple-negative breast cancers and causes activation of TGFβ signaling, while TGFβ induces Sox4 expression [75]. The end result is mesenchymal differentiation and breast cancer stem cell accumulation, which relates with enhanced tumor progression in vivo and enhanced cell invasiveness. Interestingly, the transcriptional program regulated by Sox4 during EMT appears to include only mesenchymal genes, such as *vimentin*, *fibronectin* and *N-cadherin* [76], and the histone methyl-transferase EZH2 that is important for the pro-survival signals generated during EMT [77]. Since EZH2 overexpression in breast cancer cells where Sox4 has been silenced recapitulates the EMT response [77], it is reasonable to suggest that Sox4-induced EZH2 may be required for the full activity of Twist1 in inducing the mesenchymal gene program [68].

Probably the most prominent transcription factor family that participates in the EMT response and mediates transcriptional induction of the mesenchymal and ECM program in breast cancer cells, are members of the activation protein 1 (AP1) family [78]. The AP1 family includes many bHLH members that form heteromeric complexes and regulate a large number of genes, while, many of these family members are immediate-early gene targets of TGFβ signaling and some directly interact with Smads [79]. Possibly, one of the most prominent transcriptional complexes that regulates breast cancer invasiveness and mesenchymal gene expression is the Smad2/3–Fra1 complex [79], a finding that deserves deeper analysis and generalization to a large group of breast cancers. In addition to or possibly in association with AP1, the transcriptional regulator NFAT can also contribute to the regulation of invasiveness and matrix degradation during EMT [80]. In this breast cancer model, NFAT induction has an impact on TGFβ tumor suppressor activity and promotes the tumor promoting effects by TGFβ, connected with a nuclear accumulation of the SnoN transcriptional co-repressor that blocks Smad nuclear activity in a gene-specific manner. In the presence of high NFAT levels, Smad3 was trapped to the cytoplasm and this possibly rescued nuclear SnoN from proteasomal degradation, a mechanism known to be induced by Smads [80].

The TGFβ–AP1 transcriptional module can also induce expression of the transcriptional repressor Blimp1/PRMD1, previously studied in mechanisms of breast cancer cell migration after activation of the nuclear factor κ B (NFκB) pathway, which was recently shown to be transcriptionally induced by TGFβ in NMuMG and breast cancer cells and contribute to the EMT and migratory responses of these cells [81]. Blimp1 repressed the TGFβ family member bone morphogenetic protein 5 (BMP5), which promotes mammary epithelial differentiation by actively repressing Snail expression [81]. Thus, the TGFβ–AP1 pathway induces Blimp1, which allows the further transcriptional induction of Snail by TGFβ, as the BMP5 pro-epithelial antagonist of TGFβ is transcriptionally inactivated. This mechanism is reminiscent of earlier studies that explained how BMP signaling counteracts the pro-EMT features of TGFβ by targeting the function of the inhibitors of DNA binding (ID) -1, ID-2 and ID-3 [82,83]. Thus, while BMP signaling upregulates ID protein levels, TGFβ downregulates their expression, which is important for the establishment of EMT and terminal myofibroblastic differentiation. Among all three ID family members, ID2 has been shown to play more key functional roles in the antagonism of TGFβ-mediated EMT induced by BMP signaling [82,83]. Interestingly, TGFβ utilizes the AP1–JunB module to repress ID2 expression in mammary epithelial cells [84]. The molecular action of ID proteins that lack the basic domain but retain their HLH domains and thus fail to directly bind to DNA, is that they pair with DNA-bound bHLH transcription factors and thus inactivate the function of the bHLH factor [85]. In agreement with this general model of ID function, ID1 pairs with Twist1 to modulate its activity during breast cancer metastasis [86] as discussed further in section 11. But surprisingly, ID2 was shown to associate with the N-terminal SNAG domain of Snail and thus inhibit the transcriptional

activity of Snail on the *integrin-β4* gene that is repressed by Snail [87]. The prediction of this model is that during BMP signaling, ID2 levels increase and promote epithelial differentiation in part by inactivating Snail function, thus allowing expression of the pro-epithelial integrin-β4. More recently, another gene target of the BMP–TGFβ antagonism in breast cancer was characterized, the integrin-β3 subunit, which (in contrast to β4) is induced by TGFβ via AP1/JunB complexes [84], and downregulated by BMP signaling, and when integrin-β3 is artificially overexpressed, it bypasses the pro-epithelial and anti-invasive actions of BMP signaling [88]. It is worth noting that the majority of studies where BMPs antagonize TGFβ in the context of EMT and breast cancer metastasis, BMP7 appears to show more potent activity when compared to other BMP family members. Based on this mechanistic understanding it makes good sense that BMP ligands have been successfully utilized to counteract the pro-invasive and pro-metastatic effects of TGFβ and thus have been proven therapeutically important in breast cancer models of metastasis in vivo [89].

A final example of transcriptional mechanisms that preserve the epithelial phenotype and thus counteract the process of EMT, relates to the tumor suppressor protein p53 and makes a link to the regulation of EMT by microRNAs (miRNAs). TP53 directly regulates the expression of the miR-200c, which is a central negative regulator of the EMT-TF ZEB1 [90]. Thus, p53 promotes epithelial differentiation, the MET, and in breast cancers where p53 is mutated and loses normal activity, the loss of proper regulation of miR-200c is important for the loss of epithelial homeostasis and the progression towards EMT. A second miRNA target of p53 is miR-34, whose expression is maintained by wild type p53 in normal epithelial tissues and repressed in cancer with loss-of-function mutation of p53, including breast cancers [91]. One of the direct targets of miR-34 is Snail, whereby Snail expression is silenced in epithelial cells due to the action of miR-34. Loss of p53 leads to downregulation of miR-34 and release of Snail from this post-transcriptional control mechanism [91,92]. The action of p53 is also relevant to one of the favorite EMT models downstream of TGFβ, the NMuMG cells. NMuMG cells express two p53 alleles, a wild type and a point mutant (R277C) p53, and genetic downregulation of both copies of p53 in these cells promotes the TGFβ-mediated EMT, whereas nutlin3-mediated stabilization of p53 counteracts TGFβ's pro-EMT effects [93]. Interestingly, recent studies in p53-null cancer cells demonstrated a novel activity of nutlin-3 that blocked Smad2/3 phosphorylation by the TGFβ type I receptor, causing overall weaker TGFβ signaling and correspondingly weaker transcriptional upregulation of Snail and Slug, thus blocking the EMT response in a p53-independent manner [94]. The direct target of the nutlin-3 inhibitory mechanism uncovered by this study remains to be determined. Furthermore, p53 null breast cancers, in addition to their EMT features are classified as claudin-low breast cancers and are characteristically enriched in cancer stem cells [95]. Thus, p53-mediated mechanisms that preserve epithelial homeostasis center on the control of mammary epithelial differentiation at the level of miRNAs.

6. Post-transcriptional and translational networks control the EMT program downstream of TGFβ

The key role of miRNAs in controlling the EMT–MET processes in mammary cells were briefly discussed above. Since this topic has been reviewed extensively [96,97], we will only highlight some more key elements of the process and then focus on more recently understood examples that expand the importance of post-transcriptional mechanisms in the control of EMT and their relationship to TGFβ signaling. The most prominent miRNA gene family linked to the control of EMT-MET is the miR-200 that includes the five members miR-200a, -200b, -200c, -141, -429, and the miR-205 [96,97]. Expression of these miRNAs is repressed by TGFβ signaling during the onset of EMT. This makes sense as two of the most potent targets of miR-200 are the 3′-UTRs of the mRNAs for the EMT-TFs, ZEB1 and ZEB2, which are downregulated by the miRNAs

in the epithelial state. When TGFβ signaling downregulates the miR-200 family, conditions for derepression of ZEB1 and ZEB2 are generated. In addition, TGFβ must send signals to transcriptionally induce expression of new ZEB1/ZEB2 mRNA levels. The newly identified mechanism by which the ubiquitin ligase CUL4A regulates chromatin remodeling at the ZEB1 promoter in breast cancer cells [98], may be relevant to the complete understanding of ZEB1/2 transcriptional and post-transcriptional regulation. ZEB1 and ZEB2 then repress miR-200 family gene expression, thus establishing a double negative regulatory loop [97]. Interestingly, for a prolonged EMT to persist in breast cancer, sustained autocrine TGFβ ligand production is necessary, causing among other things methylation of the gene locus of the *miR-200* family leading to stable repression of the gene via the action of ZEB1 and ZEB2 [99]. Sustained autocrine TGFβ is not only important for the prolonged establishment of EMT but also for the gain of stem-like features by breast cancer cells as discussed later [40]. Interestingly, prolonged auto-crine TGFβ signaling is sufficient to bypass the growth inhibitory effects in mammary epithelial cells and to promote the EMT and invasiveness effects [40]; obviously such effects of prolonged autocrine TGFβ are eas-ier to occur in the presence of oncogenic activities such as that of mutant Ras [43]. The latter can be established in part by at least two comple-mentary mechanisms: a) ZEB1 directly represses the pro-oncogenic transcription factor MYB, and in doing so limits the proliferative poten-tial of breast cancer cells undergoing EMT [100]; b) during mammary EMT induced by TGFβ, Smad complexes cooperate with the oncogenic transcription factor Myc and bind together to Snail promoter sequences, leading to Snail induction and EMT [101]. Snail induction by Myc pre-cedes the robust downregulation of Myc by TGFβ–Smad signaling that is necessary for the concomitant cell growth arrest that accompanies the EMT process.

In addition to the miR-200 family, other miRNAs have been linked to the EMT process. miR-34 was discussed above and two more examples have recently surfaced: a) similar to the miR-200/ZEB1/2 double negative loop, miR-203 forms a regulatory loop with Slug during TGFβ-induced EMT in breast cancer cells [102]. MiR-203 downregulates post-transcriptionally Slug, and Slug potently represses miR-203 expression by binding to the *miR-203* promoter, thus enforcing a prolonged silencing of miR-203, so that Slug can accumulate and EMT can proceed during sustained TGFβ signaling. b) The differentiation transcription factor C/EBPβ is necessary for the maintenance of mammary epithelia, and its expression is negatively regulated post-transcriptionally by miR-155 [103]. The pro-epithelial function of C/EBPβ is explained by the direct transcriptional induction of epithelial genes, such as *E-cadherin* and *coxsackie virus* and *adenovirus receptor* (*CAR*) that produce proteins critical for adherens and tight junctional complexes, respectively. Low C/EBPβ levels characterize triple negative breast cancers and induction of miR-155 by TGFβ seems to underlie the loss of C/EBPβ in the mammary gland [103]. Regulation of miRNA expression by miRNAs can also contribute to the control of EMT and breast cancer stemness, as is the case of miR-22, which promotes EMT and mammary stemness in vivo followed by enhanced metastasis [104]. This miRNA functions by targeting the chromatin regulator Ten eleven translocation (TET), which is required for cytosine demethyla-tion, and acts specifically on the promoter of the *miR-200* gene. Thus miR-22 suppresses the pro-epithelial miR-200 and in this way promotes the EMT and metastatic potential of breast cancer.

MiRNAs represent only a small proportion of the large collection on non-coding RNAs (ncRNAs) that are abundantly transcribed in all cell types, including breast. Very recent reports implicate for the first time the so-called long non-coding RNAs (lncRNAs) in the process of EMT downstream of TGFβ. A major function of lncRNAs is the regulation of chromatin organization and gene expression. Accordingly, the lncRNA Hotair is transcriptionally induced by TGFβ in breast cancer cells and is required for the EMT and associated cancer stem cell enrichment [105]. Hotair expression is also enriched in metastatic breast cancer; however, the mechanism of action of Hotair to elicit EMT remains

currently uncharacterized. A genome-wide screen for lncRNAs recently identified several of these genes being upregulated by Twist1 in mammary epithelial cells undergoing Twist1-dependent EMT [106]. Future studies are awaited to explain how lncRNAs are implicated in EMT and the process of breast cancer metastasis.

Another molecular mechanism involved in EMT downstream of TGFβ is the regulation of alternative mRNA splicing. The best character-ized case relates to the epithelial splicing regulatory proteins (ESRPs) 1 and 2, which induce the specific splicing pattern of mRNAs, including those of FGF receptors that mediate terminal myofibroblast differentia-tion during TGFβ-induced EMT [107,108]. Interestingly, TGFβ induces ZEB1 and ZEB2 expression, which then repress directly the *ESRP2* gene during EMT [108]. Additional RNA-seq analyses have identified another cohort of regulators of alternative splicing during EMT in breast cancer, including the proteins RBFOX, MBNL, CELF and more [109]. In contrast to ESRPs, which are downregulated during EMT, RBFOX2 is upregulated during EMT and leads to new splicing variants of mRNAs encoding for the polarity protein Par3, the cytoskeletal protein cortactin and the trafficking regulator dynamin-2 [110]. The function of RBFOX2 appears so far to link more with breast cancer invasiveness than with the onset of EMT per se. This area of EMT research is still wide open and promises interesting new mechanistic insight into the complexity of the program that leads to invasive mesenchymal cells in breast cancer.

Further into post-transcriptional mechanisms of EMT, analysis of ribosome-bound mRNAs revealed a number of years ago the induction of synthesis of proteins such as interleukin-like EMT inducer (ILEI) and the trafficking adaptor Dab2 by TGFβ signaling in breast cancer cell models undergoing EMT [111,112]. TGFβ signaling via the non-Smad AKT2 pathway causes phosphorylation of the heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), which then dissociates from a specific motif located in the 3′-UTR of the ILEI and Dab2 mRNAs, thus causing translational derepression and induction of de novo synthesis of these two critical for the EMT program proteins [112]. HnRNP E1 represses mRNA translation by sequestering the activity of the translational regulator eukaryotic elongation factor 1 A1 (eEF1-A1) and thus stalling ribosomal elongation at the 3′-UTR prior to completion of protein synthesis [113]. More recent genome-wide RNA immunoprecipitation followed by microarray analysis revealed several additional mRNAs that are translationally regulated in a similar manner as ILEI and Dab2 [114]. Interestingly, the HMGA2 and ZEB2 EMT-TFs are included in this cohort of mRNAs. All the above mechanistic examples emphasize the fact that EMT is a global process of change in cellular organization that includes modulation of essentially every step in the transfer of molecular information from genes to proteins and vice versa.

7. Effector programs of TGFβ that establish EMT

All previous regulatory mechanisms aim at providing a new pool of proteins and RNAs that coordinate together with metabolic changes the progressive transition of differentiation from the epithelial to the mesenchymal cell phenotype. The functionally necessary protein changes include secreted signaling proteins, matrix constituents, adhesion components and cytoskeletal elements (Fig. 1). In this and the next sections, we discuss selected molecules by emphasizing ECM and adhesion proteins or glycosaminoglycans giving also emphasis to the processes of protein glycation. This topic has recently been reviewed in more detail [115]. TGFβ itself utilizes a potent co-receptor, the type III receptor or β-glycan that is a cell surface proteoglycan with a short intracellular domain [46]. Interestingly, most of the examined breast cancers underexpress β-glycan and the loss of its expression correlates with the stage of the breast cancer so that the more advanced and met-astatic breast cancers almost completely lose expression of β-glycan [116]. Artificial overexpression of β-glycan in human breast cancer cell lines led to a relative decrease in invasive and metastatic potential, which has been explained based on the shedding of the extracellular domain of β-glycan, which then serves as a trap of TGFβ ligand in the

extracellular space, limiting locally the action of TGFβ. In addition, the soluble ectodomain of β-glycan limits TGFβ signaling towards immune cells in the tumor microenvironment, including dendritic cells and regulatory T lymphocytes, and thus the loss of β-glycan expression in advanced breast cancers allows a more robust suppression of anti-tumor immunity and gives an advantage for tumor growth and dissemination [117]. However, membrane-bound β-glycan can also function antagonistically to pro-invasive TGFβ signaling because β-glycan's short C-terminal tail forms a complex with the adaptor protein β-arrestin which then activates the small GTPase Cdc42 and thus blocks breast cancer cell migration [118].

In general, the ECM response during EMT entails major changes in collagen composition and specific upregulation of fibronectin and specific integrin receptors as already outlined in a previous section of this article and reviewed by others [3,5]. In particular fibronectin is considered a hallmark of the mesenchymal phenotype [3,24]. Only recently specific mechanistic clues have been revealed regarding a more direct (and not a bystander) role of fibronectin in the maintenance of EMT [119]. Exposure of immortalized human mammary epithelial MCF-10A cells (another frequent model of EMT studies) to fibronectin or specific fragments of fibronectin, demonstrated a rather faithful induction of the EMT program including adhesion protein remodeling, EMT-TF induction and further fibronectin synthesis [119]. This mammary epithelial response to fibronectin in part utilized as signaling mediators integrins, TGFβ receptors and the intracellular kinases Src and MAPK. It is thus possible, that fibronectin is induced by TGFβ during EMT and upon synthesis and extracellular deposition, this fibronectin further sustains TGFβ signaling by contributing to the activation phase of the extracellular TGFβ ligand.

One of the defining features of cell surface and ECM proteins is their high content of glycation, either in the form of N- or O-linked sugar chains. Dynamic modulation of the state of glycation of proteins during their biosynthesis or secretion is well established, but specific functions of such a dynamic regulation in the context of EMT are sparse and correlative. A recent study focusing on the enzyme N-acetylglucosaminyltransferase III demonstrated that the expression of this transferase is downregulated by TGFβ during mammary EMT, while N-acetylglucosaminyltransferase V expression is upregulated in mesenchymal cells [120]. The functional role of N-acetylglucosaminyltransferase III at least in part is to modulate the glycation state of E-cadherin in epithelial adherens junctions and thus prolonging their stability and the homeostatic maintenance of the epithelial state, thus explaining why TGFβ downregulates this enzyme during EMT, when E-cadherin turnover is enhanced to mediate junctional dissociation. Additional secreted or cell surface proteins play regulatory roles during the EMT process. The membrane associated signal peptide-CUB-EGF domain-containing protein 2 (SCUBE2) which behaves as a breast tumor suppressor possibly by promoting E-cadherin and adherens junction stability, suppressing β-catenin nuclear accumulation and thus blocking migration and breast cancer invasiveness [121]. TGFβ downregulates SCUBE2 by mobilizing DNA methyltransferase I (DNMT1) to the SCUBE2 promoter, thus enforcing methylation and silencing of this gene during EMT. In EpRas cells where EMT depends exclusively on a sustained autocrine TGFβ signal, TGFβ pro-EMT signaling seems to depend on semaphorin-7a, as the depletion of semaphorin-7a makes EpRas cells resistant to EMT despite the continuous autocrine TGFβ signal [122]. Interestingly, an endogenous factor that can control the response of breast cancer cells to TGFβ and semaphorin-7a is the transcription factor Ets2-repressor factor (ERF), which actively represses the *sempahorin-7a* gene and thus enforces stable epithelial differentiation in EpRas cells, fully bypassing the activity of oncogenic Ras and autocrine TGFβ [122]. Similar to ERF, another Ets family transcription factor, ELF5, promotes mammary epithelial differentiation because it transcriptionally represses the *Slug* gene in normal and in breast cancer cells [123]. Thus, ELF5 suppresses EMT and promotes MET and is required for normal mammary gland differentiation in the mouse, while its expression must be downregulated

in advanced and metastatic breast cancers. Overt misexpression of ELF5 in mouse breast cancer models potently suppresses metastatic potential [123].

Associated with the dynamic remodeling of cell–cell junctions is the process of invasion where major changes in cell membrane architecture are supported by association between membrane proteins with either the cytoskeleton intracellularly or the ECM extracellularly. The result is the formation of specific membrane structures that support invasiveness such as invadopodia and filopodium-like protrusions [124,125]. The EMT-TF Twist1 activates a major genetic program that supports the synthesis of new proteins that assemble invadopodia in breast and other cancer cell types [124]. A protein that modulates invadopodial assembly is the focal adhesion scaffolding protein Hic-5, whose expression is upregulated by TGFβ during EMT [126]. Hic-5 upregulation is required for ECM degradation by metalloproteases and invasion of breast epithelial cells upon EMT, and the function of Hic-5 requires Src-dependent tyrosine phosphorylation and cooperation with the small GTPase RhoC. Thus, EMT promoted by TGFβ enhances the levels of a key adaptor protein so that the assembly of novel adhesion complexes in association with RhoC and actin microfilaments can generate the invadopodia that promote breast cancer migration through the ECM. Similar to Hic-5, the mesenchymal protein lipoma preferred partner (LPP) is another adaptor protein of focal adhesions that forms new assemblies with the microfilament regulator α-actinin into focal adhesions upon TGFβ stimulation in breast cancer cells and thus, promotes invasiveness [127]. Misexpression of LPP in breast cancer correlates with the overexpression of the ErbB2 receptor. Another cell surface receptor that signals after binding to ECM components, namely collagen type I, is the receptor tyrosine kinase discoidin domain receptor 2 (DDR2), which then activates the kinases Src and MAPK [128]. The MAPK Erk2 directly phosphorylates Snail causing stabilization and nuclear accumulation of this transcription factor. Collagen I signaling via DDR2 does not initiate the EMT, but is required for sustained EMT via prolonged stabilization of Snail. Thus, mesenchymal cells that undergo EMT and start invading their local matrix, further activate Snail via DDR2 signaling, a process necessary for metastatic dissemination in vivo [128].

To support the newly formed and invasiveness-promoting assemblies of the plasma membrane with the ECM and the cytoskeleton, a profound remodeling of integrins is required during EMT [129]. This remodeling at least in part contributes to the activation of ECM-embedded TGFβ in a sustained manner [130,131], which is critical for the EMT program as explained above. Both integrin α and β subunits are remodeled during EMT and this depends on the specific developmental origin of the epithelial cell type. In breast cancer, transcriptional induction of integrin-α3 has recently been established during EMT as a marker of aggressive breast cancer [132]. Through their extensive studies on this topic, recent work from the Schiemann laboratory has provided an intriguing scenario of integrin subunit exchange during breast cancer EMT, whereby loss of integrin-β1 is compensated by upregulation of integrin-β3 [133]. Integrin-β3 upregulation not only contributes to invasiveness and breast cancer metastasis in the mouse model examined, but also switches the response of breast cancer cells to TGFβ, so that their growth is no longer inhibited and is even stimulated by the action of this cytokine [133]. Not only integrin subunit expression is important during EMT and breast cancer metastasis, but TGFβ can also induce the expression of the adhesion-related kinase Pyk2, a response important for breast cancer cell invasion and metastasis in vivo [134]. The upregulation of Pyk2 in breast cancer cells correlates with the stabilization of integrin-β1, both required for the invasive process of such tumor cells. It becomes therefore increasingly clear, that the contextual and dual roles of TGFβ signaling during breast cancer progression are now rather well understood, and partake significant remodeling of cell surface and ECM components that communicate new signaling inputs to breast epithelial cells as they transit to the more mesenchymal phenotype.

8. Hyaluronan content in breast carcinomas

Hyaluronan is an anionic, non-sulfated linear glycosaminoglycan comprised of repeating disaccharide units of glucuronic acid and N-acetylglucosamine. Due to its high molecular mass and negative charge it binds up to 1000-fold its weight of water forming loose and elastic matrices that promote cell proliferation and migration [135,136]. Elevated amounts of hyaluronan are found whenever there is rapid tissue remodeling, i.e. during embryogenesis, inflammation and cancer. Using immunohistochemistry, a strong correlation was noticed between the presence of hyaluronan in peritumoral stroma and cancer cells, and disease progression, in unselected clinical breast cancer specimen [14]. However, despite this observation no correlation between the presence of hyaluronan and disease outcome was found in node-negative breast cancer, rather, versican alone appeared to be a predictor for node-negative cancer metastasis [22]. Notably, stromal myxoid changes are characterized by high amounts of hyaluronan and are associated with breast cancer invasion and metastasis [137]. Thus, although the ECM components hyaluronan and versican form complexes, their expression levels vary between individual breast tumors affecting differentially breast cancer progression. Furthermore, sonographic examination of infiltrating breast carcinoma in comparison to histologic sections of the same carcinomas stained for hyaluronan or collagen, revealed a correlation between tumor shape and the shape of extracellular hyaluronan, but not collagen [138]. Most studies have been performed on developing or fully developed malignancies, and not on the early phases of the malignant process. During the early phase of the malignant process, most likely epithelial–mesenchymal interactions lead to deregulation of ECM molecules in the breast gland, whose expression levels and later correlation to malignant progression in breast tissue are not fully understood. Among the ECM components, significantly elevated levels of hyaluronan were detected in serum from patients with metastatic carcinoma compared to non-metastatic or benign breast carcinoma [139]. The functional significance of hyaluronan changes in distinct subtypes of breast cancer tissue stroma and blood as a factor promoting malignant transformation remains to be determined.

Genes that contribute to adverse breast cancer pathophysiologies have been classified into the 50-gene signature termed PAM50 [140–142]. Besides this classification, breast cancer can be classified based on the expression of ECM components into four main groups designated ECM1, 2, 3 and 4 [143]. Hyaluronan was predominantly upregulated in tumors characterized by the ECM2 signature and represented one of the most clinically aggressive phenotypes, i.e. transition between the aggressive luminal B and the less aggressive luminal A phenotype [143]. This study further supports the hypothesis that stromal characteristics are related to clinical outcome.

9. Finely tuned regulation of hyaluronan metabolism in breast cancer

In healthy tissue the levels of hyaluronan are regulated by the coordinated action of hyaluronan synthesizing enzymes (termed HAS1, HAS2 and HAS3) and hyaluronidases (termed HYAL1, HYAL2, HYAL3 and PH-20), as well as by its rate of elimination. In general, hyaluronan chains of a molecular mass higher than 400 kDa are found in healthy tissues whereas smaller molecules, resulting from the action of hyaluronidases, are present during inflammation and cancer progression [136,144,145]. Furthermore, in puberty during the growth of ductal structures, the basement membrane at the tip of the duct, i.e. at the invasive front, is enriched in hyaluronan whereas the basement membrane that surrounds ducts is composed of other ECM components including collagen type IV and laminin [146]. In addition, during the formation of chorioallantoic membrane capillaries hyaluronan-rich matrices at the tip were rapidly repressed due the activation of hyaluronidases [147]. These observations support the notion that high molecular mass

hyaluronan suppresses, whereas fragmented hyaluronan, e.g. occurring in chronic inflammatory foci, promotes angiogenesis. These data suggest that a finely tuned regulation of hyaluronan production and degradation can regulate the behavior of epithelial and endothelial cells, e.g. during inflammation and oncogenesis.

Hyaluronan mediates its cellular signaling through interactions with specific cell surface receptors, such as CD44 and RHAMM (receptor for hyaluronan mediated motility). A large number of studies have implicated these receptors in regulation of cell proliferation, migration and differentiation [148–153]. An interesting new study of breast cancer metastasis demonstrated that one of the mechanisms by which mesenchymal stem cells that are recruited to sites of primary breast cancer growth, promote metastasis, is the induction of synthesis of the lysyl oxidase (LOX) by the breast cancer cells that respond to the mesenchymal cells [154]. Induction of metastatic potential by LOX requires the activation of breast cancer cell CD44 by extracellular hyaluronan, leading to cleavage and nuclear translocation of the CD44 intracellular domain, which then associates with the LOX promoter and enhances transcription of the *LOX* gene. Induction of LOX in the breast cancer cells then stimulates Twist1 expression (possibly via stabilization of Snail, although not yet defined), and subsequent EMT of the breast cancer cells [154]. Such EMT of breast cancer cells induced by mesenchymal stem cells could not be linked to the generation of breast cancer stem cells (see below for further discussion).

The specific physiological functions of each one of the three HASes are not yet understood; they differ in enzymatic properties and activities, and respond differently to various exogenous stimuli, such as growth factors and cytokines [155–158]. Recent studies revealed that changes in hyaluronan production are not always due to changes in HAS mRNA expression [158–160]; modulation of the synthetic rate could also be due to posttranslational modifications of HAS molecules, such as phosphorylation [160–162] and ubiquitylation [163], as well as availability of the substrates [164]. All HASes are expressed in breast cancer, but several studies have in particular implicated HAS2 activity to be closely correlated to breast cancer development and progression.

Whereas upregulation of either HAS1 or HAS2 most likely promotes breast cancer, only HAS2 inhibition reduced the tumorigenicity in v-Ha-Ras transformed cell lines [165]. Furthermore, overexpression of HAS2 in ErbB2-induced mammary tumors led to increased hyaluronan production, which in turn induced the production of pro-angiogenic factors accelerating angiogenesis [20]. During EMT, occurring during breast adenocarcinoma progression, HAS2 expression plays a pivotal role. Normal mammary epithelial cells infected with a HAS2 adenovirus undergo EMT through upregulation of vimentin and loss of adhesion proteins at intercellular junctions [166]. Recently, we demonstrated that TGFβ-induced HAS2 in mammary epithelial NMuMG cells is important for the TGFβ-mediated EMT; silencing of HAS2 inhibited the TGFβ-mediated EMT by about 50% and suppressed the expression of the EMT markers ZO-1, fibronectin, ZEB1 and Snail [167] (Fig. 2). Notably, during zebrafish development, HAS2 is crucial for cell migration through activation of the small GTPase Rac1, where HAS2-expressing cells migrate whereas HAS2-negative cells remain stationary [168]. Rac1 activation could be the result of the binding of HAS2-synthesized hyaluronan to CD44 and subsequent interaction with the GTPase activating protein IQGAP1 [169]. Furthermore, HAS2 −/− mouse embryos lacking hyaluronan, die during midgestation because of failure to undergo cardiac morphogenesis and valve formation [170]. HAS2-synthesized hyaluronan in the cardiac jelly induces PI3K and ErbB signaling in a TGFβ-induced EMT [170]. Interestingly, miR-23 activity modulates cardiac valve formation by inhibiting the TGFβ-induced HAS2 expression and hyaluronan production [171]. The molecular mechanisms through which miR-23, in a TGFβ-dependent manner regulates HAS2 expression during mammalian valve formation remains to be elucidated. In addition, overexpression of HAS2 in aggressive mesotheliomas and colon carcinomas increased their malignant and fibroblast-like migratory phenotype [172,173].

The connection between HAS2-mediated hyaluronan production and breast cancer progression has been further established using breast cancer cell lines. Silencing of the *HAS2* gene led to lower proliferative capacity and decreased migratory and invasive capacity of Hs578T cells, thus demonstrating the necessity of HAS2 expression for the malignant phenotype of this invasive breast cancer cell line [174,175]. Most likely, HAS2-induced hyaluronan is rapidly depolymerized into fragments of a size of 10–40 kDa that could promote tumor neovascularization [149,175–177]. The importance of HAS2 for breast cancer metastasis has been demonstrated in an intracardial and mammary fat pad metastasis experimental model, where the decrease of HAS2 reduced organ metastasis [175]. Notably, soluble factors released in breast cancer, such as osteopontin, TGFβ and platelet-derived growth factor (PDGF)-BB, can both influence the regulation of HAS2 and also bind cell surface receptors that in cooperation with the hyaluronan receptor CD44 affect several signaling cascades, leading to e.g. migration and proliferation [151,174,178,179]. Although a comprehensive understanding of the role of HAS2-synthesized hyaluronan and the HAS2 molecule as such in breast cancer progression is not yet fully understood, it is possible that HAS2 enhances breast cancer invasion by suppression of the tissue inhibitor of metalloproteinases 1 (TIMP-1) and by increase of epidermal growth factor (EGF)-mediated induction of the FAK/PI3K/AKT signaling pathway [180]. Furthermore, increased synthesis of hyaluronan might, through its interactions with versican, create hydrated and expanded matrices that facilitate cancer–host interactions and diffusion of nutrients [20], as well as recruitment of immunosuppressive macrophages, decreasing the immune attack on the tumor cells [181], and bypassing the repression of E-cadherin, leading to suppression of EMT [182].

10. EMT and breast cancer stem cells

The pioneering work from the Clarke laboratory first established the concept of breast cancer stem cells (CSCs) that are usually identified as populations of cells expressing high levels of the hyaluronan receptor CD44 and low levels of the surface sialoglycoprotein CD24 [183]. This was followed by the illuminating work of the Weinberg and Puisieux laboratories that demonstrated that breast CSCs could be enriched in populations that underwent EMT due to the high expression of EMT-TFs, like Snail and Twist1 [7,8]. This concept is now verified in many laboratories and is compatible with the process of reprogramming that epithelial cells undergo during EMT [65,184]. Although clues to the link between transcriptional and epigenetic control during EMT and the generation of CSCs was briefly introduced above, we here enlist a few additional reports that attest to the generality of this molecular link. Novel technology has generated the induced pluripotent stem cells (iPSCs) after transfection of differentiated cells with specific transcription factors that promote stemness, Oct4, Klf4, Myc and Sox2 [185]. During the iPSC protocol, time-dependent analysis has revealed that an EMT program is first initiated during induction of pluripotency, marked by the upregulation of Slug and later followed by an MET step that establishes the epithelial differentiation of the pluripotent stem cells [186]. TGFβ signaling during the early EMT phase promotes pluripotency, whereas the MET phase depends on BMP signaling and upregulation of miR-200 family members that enforce the epithelial phenotype [187]. These studies support the notion that reprogramming during EMT is not only relevant to cancer but also has direct links to the more physiological process of induced stemness and the generation of pluripotent stem cells. Furthermore, it appears that during reprogramming of normal stem cells and cancer cells, cycles of EMT and MET are important to occur, suggesting that these processes may provide an adaptive force when cells change their differentiation potential. This is compatible with the findings that major developmental signaling pathways, like TGFβ, BMP and Wnt mediate these transitions and stemness in the context of both normal and tumor states in the breast [188]. The same trend has been characterized for the EMT-TF

FOXC2, which establishes the mesenchymal state of mammary cells and contributes to CSC potential at least in part by inducing the transcription of the PDGF β-receptor [189]. For this reason, a clinically approved PDGF receptor inhibitor, sunitinib, was shown to limit the CSC and metastatic potential of breast cancer cells with high FOXC2 expression. Yet another pathway that is critical for the generation of the breast CSCs is the Hippo pathway and its downstream effector protein TAZ, which when overexpressed and activated by Hippo pathway kinases endows non-CSCs with CSC potential in the breast [190]. One of the molecular functions of TAZ is to inactivate the function of the epithelial polarity protein Scribble, thus causing EMT and promoting stemness in breast cancer cells.

An important factor regulated by TGFβ and Wnt signaling in breast cancer is the transcription factor Grainyhead-like 2 (GRHL2), which associates with homeobox transcription factors like Six1, a positive regulator of ZEB1 expression, to block their function [191]. GRHL2 therefore blocks EMT and limits the breast CSC potential, while sensitizing breast cancer cells to classical chemotherapy; TGFβ and Wnt downregulate GRHL2 promoting breast CSCs. In basal types of breast cancers with gene amplification of the *TP63* gene, the corresponding protein product ΔNp63 directly transactivates the gene for *BMP7*, which induces BMP signaling and contributes to breast cancer stemness [192]. For this reason, a BMP type I receptor inhibitor limits the breast CSC pool. A similar study using the A17 mouse breast cancer model, demonstrated that the BMP type I receptor inhibitor dorsomorphin limited the number of breast CSCs and reverted these cells to a more epithelial state, and provided evidence for a role of mesenchymal stem cells being dependent on active BMP signaling [193]. Although somewhat counterintuitive, these findings may bear on the relevance of EMT–MET cycles as a critical component for cancer stemness, and BMP7 might support the latter transition following EMT. Furthermore, the EMT-TFs, like Twist1 and ZEB1/2, interfere with key pathways, such as the p53, pRb and PP2A phosphatase, thus preventing oncogene-induced senescence and apoptosis and facilitating tumorigenic conversion by oncogenes, such as Ras, to advanced stages that resemble the claudin-low group of breast cancers that have characteristic enrichment in stem-like features [194]. According to these schemes, breast CSCs arise via EMT and changes in differentiation instead from arising after malignant transformation of stem-like progenitor cell types in the mammary gland, and mechanisms of suppression of cell death in a growing tumor tissue are of importance for the generation of cancer cells with stem-like potential [195].

Normal and malignant stem cells are dependent on a tissue microenvironment often called the stem cell niche. Metabolic conditions in the niche often provide the contextual cues that activate key pathways as those described above that support cancer stemness. One of the metabolic conditions that prevail in breast cancer niches is hypoxia. In an interesting experimental protocol, breast cancer cells were exposed to cycles of hypoxia and normoxia and the pool of breast CSCs and their self-renewal capacity was monitored [196]. The cyclic protocol allowed the enrichment of the cell population in CSCs with enhanced tumor-initiating potential in mouse transplantation experiments. Hypoxia affects multiple molecular pathways, one being the Notch ligand Jagged2 that causes EMT and promotes breast CSC growth; consistent with this observation, Jagged2 expression and active Notch signaling have been found to correlate with poor prognosis and metastasis of human breast cancers [197]. In the breast cancer niche, hypoxia causes changes in local pH, in part mediated by the hypoxia-inducible protein carbonic anhydrase IX (CAIX) [198]. The function of CAIX in breast cancer causes activation of mTORC1 signaling leading to EMT and high expression of Jagged1 and Notch1, which contribute to breast CSC proliferation. Such breast CSCs were eradicated from mice treated with an inhibitor of CAIX, and combination therapy with paclitaxel and the CAIX inhibitor potently blocked metastasis in these mice [198]. A pathway related to hypoxia is the mitochondrial retrograde signaling mechanism that involves the phosphatase calcineurin, which

causes breast EMT and generation of CSCs [199]. This pathway provides novel chances for therapeutic intervention of breast cancer metastasis and is initiated by changes in the copy number of mitochondrial DNA, often observed in human breast cancers.

In addition to hypoxia, other factors in the CSC niche influence the proliferation and survival of breast CSCs. The balance between endothelial and mesenchymal progenitors, including adipose stromal cells has an impact on breast cancer progression in transplanted mice, and mediate EMT, as well as tumor-promoting angiogenesis, leading to enhanced metastatic potential [200]. These findings are relevant to the clinical observations whereby obese breast cancer patients exhibit enhanced chances for advanced disease including metastasis and earlier death. Acquisition of CSC potential via the EMT process may also have other important effects that are relevant to cancer progression. For example, breast cancer cells exhibiting EMT showed enhanced resistance to the cytolytic action of cytotoxic T lymphocytes [201]. The resistance was due to the lack of the development of a normal immunogenic synapse between T cells and tumor cells, while breast cancer cells with overt EMT exhibited enhanced autophagic behavior. The autophagy regulator Beclin1 was shown to be important as genetic ablation of this protein allowed cytotoxic T cells to lyse the EMT breast cancer cells because of the lack of the autophagic behavior by the tumor cells [201].

A corollary of the concept of CSCs is the development of technologies to detect them in the blood of breast cancer patients and utilize them as prognostic means to predict disease outcome. Interestingly, one of the first comprehensive analyses of circulating tumor cells in breast cancer patients found a diverse cohort of cells exhibiting both epithelial and mesenchymal features [202]. Circulating mesenchymal cells correlated with worse disease outcome and exhibited active molecular markers of EMT, including, TGFβ signaling intermediates and EMT-TFs. Thus the concept of EMT linking to cancer stemness provides advanced potential for both novel therapeutic intervention and prognostic means in human breast cancer.

11. EMT–MET and the process of metastasis

The correlation between EMT and breast cancer metastasis has already been discussed above. In this section, we aim at emphasizing novel evidence that clarifies that what is most relevant for metastasis is the cyclic EMT–MET transition. EMT–MET provides new epithelial cancer cells that, after undergoing the EMT, obtained enhanced capacities that make them capable of seeding properly to new metastatic sites and promoting the spread of the disease. The homeobox transcription factor Prrx1 is a newly identified inducer of EMT [203]. Despite an enhanced invasive potential by cells that overexpress Prrx1, this factor must be downregulated for cancer cells to achieve metastatic colonization and as a consequence the metastatic cells undergo MET and acquire epithelial features. This is one of the first molecular examples that explain why most metastatic carcinomas appear epithelial and lack the morphological features of mesenchymal cells. In a more convincing experimental set up, the Yang laboratory studied conditional expression of Twist1 in a mouse model of spontaneous skin cancer induced by chemical carcinogens [204]. Using elegant time-dependent activation of the Twist1 transgene, it was shown that while Twist1 expression was required for the induction of EMT and local invasive cancer, efficient metastatic colonization required the silencing of Twist1 to allow reversion of EMT so that blood-borne circulating tumor cells could generate new colonies of epithelial phenotype [204]. Together with the Prrx1 study, this study emphasizes the functional importance of EMT as a transient process that must be followed by MET if metastasis is to succeed. In accordance with the above two studies, a novel role of ID1 has recently been revealed during breast cancer metastatic colonization [86]. In primary breast cancer, TGFβ induces Snail and EMT as previously established. TGFβ can then induce ID1 in mesenchymal cells produced by the EMT process and this ID1 binds and inactivates

Twist1, causing MET and functionally promoting the epithelial colonization at the metastatic site [86]. In this intriguing model, ID1 in the primary breast cancer does not affect the EMT and invasiveness of the cells. The work establishes the ID proteins as key mediators of plasticity at distinct sites of tumor growth. Based on the complexity of the EMT–MET cycle during breast cancer progression and the impact that TGFβ signaling has at multiple steps of this cycle, it is anticipated that therapeutic intervention against breast cancer metastasis by targeting the EMT–MET process or TGFβ signaling will be complicated and possibly unsuccessful [24,205].

12. Conclusions

In the previous sections we presented specific examples and experimental evidence that clearly link cytokine and glycosaminoglycan signaling to the process of EMT in the context of breast cancer. Due to the focus of our own work, emphasis has been given to TGFβ and hyaluronan, however, wherever appropriate we made reference to other relevant regulators of the EMT process. The current state of the art clearly suggests that deeper understanding is required for the complete elucidation of mechanisms by which cytokine signaling crosstalks with major constituents of the ECM to modulate the process of EMT, cell invasiveness and metastasis. In particular the contribution of such mechanisms to the generation and maintenance of breast cancer stem cells is even more poorly understood and deserves further analysis. Finally, the recent studies that establish the necessity of the EMT–MET cycle for both cancer stem cell generation and metastatic dissemination, provide new ground for future investigations that will clarify in a more quantitative and integrative manner the sequential process that leads from primary tumor to the deadly phase of metastatic growth. Based on this scheme, molecular studies of ECM components, signaling pathways, various non-coding RNAs and epigenetic mechanisms most likely will remain at the forefront of the field as experimentalists seek new biomarkers and therapeutic protocols to assist patients with breast cancer.

Conflict of interest

We declare no conflict of interest.

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