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Javier Vaquero[1,2], Nathalie Guedj[3,4], Audrey Clapéron[1], Thanh Huong Nguyen Ho-Bouldoires[1], Valérie Paradis[3,4], and Laura Fouassier[1]

1-INSERM, Sorbonne Universités, UPMC Univ Paris 06, Centre de Recherche Saint-Antoine (CRSA), F-75012 Paris, France;

2-FONDATION ARC, F-94803 Villejuif, France;

3-Service d'Anatomie Pathologique Hôpital Beaujon, F-92110, Clichy, France;

4-INSERM, UMR 1149, Centre de Recherche sur l'Inflammation, F-75018, Paris, France.

Corresponding author:

Laura Fouassier, Ph.D.

INSERM UMR_S 938, Centre de Recherche Saint-Antoine (CRSA)

184 rue du Faubourg Saint-Antoine

75571 - Paris cedex 12, France

33 6 98 77 40 01 (phone)

laura.fouassier@inserm.fr

Keywords: Cholangiocarcinoma, epithelial-mesenchymal transition, invasiveness, chemoresistance, tumor microenvironment.

Abbreviations: CAF, cancer-associated fibroblasts; CCA, cholangiocarcinoma; CSC, cancer stem cell; dCCA, distal CCA; DFS, disease free survival; eCCA, extrahepatic CCA; EMT, epithelial-mesenchymal transition; EMT-TFs, EMT-inducing transcription factors; HSCs, hepatic stellate cells; OS, overall survival; pCCA, perihilar CCA; TAM, tumor-associated macrophages. **Words**: 5349, **Tables**: 2, **Fi gures**: 3

Conflict of interests: **None to declare.**

Financial support: LF is supported by grants from Fondation de France (N° 2014 00047502) and La Ligue National contre le Cancer (N° RS14/75-112). JV is recipient of two following postdoctoral fellowships from the Spanish Association for the Study of the Liver (AEEH) and the Fondation ARC N° PDF2014601431.

Authors' contributions: JV and AC contributed to outline and drafting of the review, intellectual content, critical revision and figure illustrations. NG and VP supervised content on human part, THHB provided critical revision and LF contributed to the conception of the paper, writing and revision of the manuscript.

Abstract

Cholangiocarcinoma (CCA) is an aggressive tumor with a poor prognosis due to its late clinical presentation and the lack of effective non-surgical therapies. Unfortunately, most of the patients are not eligible for curative surgery owing to the presence of metastases at the time of diagnosis. Therefore, it is important to understand the steps leading to cell dissemination in patients with CCA. To metastasize from the primary site, cancer cells must acquire migratory and invasive properties by a cell plasticity-promoting phenomenon known as epithelial-mesenchymal transition (EMT). EMT is a reversible dynamic process by which epithelial cells gradually adopt structural and functional characteristics of mesenchymal cells, and has lately become a center of attention in the field of metastatic dissemination. In the present review, we aim to provide an extensive overview of the current clinical data and the prognostic value of different EMT markers that have been analyzed in CCA. We summarize all the regulatory networks implicated in EMT from the membrane receptors to the main EMT-inducing transcription factors (SNAIL, TWIST and ZEB). Furthermore, since a tumor is a complex structure not exclusively formed by tumor cells, we also address the prominent role of the main cell types of the desmoplastic stroma that characterizes CCA in the regulation of EMT. Finally, we discuss the therapeutic considerations and difficulties faced to develop an effective anti-EMT treatment due to the redundancies and bypasses among the pathways regulating EMT.

Key Point Box

- EMT is an early event of metastasis that endows tumor cells with invasive properties enabling them to spread toward other territories.
- EMT contributes to CCA progression and chemoresistance.
- The three families of transcription factors that regulate epithelial and mesenchymal marker expression during EMT (SNAIL, TWIST and ZEB) contribute to CCA progression.
- Cells of CCA microenvironment, and not only cancer cells, lead to the activation of EMT.
- Targeting of EMT is challenging due to the redundancies and bypasses among EMT-regulated pathways.

Introduction

Cholangiocarcinoma (CCA) accounts for 3% of all gastrointestinal cancers and it is the second most common primary hepatic tumor after hepatocellular carcinoma [1, 2]. CCA is composed of tumor cells exhibiting a phenotype of biliary epithelial cells, as well as non-tumor cells, essentially myofibroblasts [3], and characterized by an aggressive behavior with early lymphatic and metastatic spread. CCA is subdivided into two main subclasses that differ in their anatomical presentation, natural history and treatment [4]. Intrahepatic CCA (iCCA) arises from small bile ducts and bile ductules whereas extrahepatic CCA (eCCA) originates from the hilum (perihilar CCA (pCCA)) to the distal portion of large bile ducts (dCCA) [4]. The term CCA will be used when studies do not distinguish between iCCA and pCCA/dCCA. While eCCA incidence remains stable, an increase in the incidence of iCCA has been observed, without any clear explanation [5].

Patients with CCA display a poor prognosis due to its late diagnosis and lack of effective non-surgical curative therapies. Surgical resection is the only curative treatment, but it is only available for a small percentage of patients with early-stage disease, and only 20-30% of these patients survive after 5 years due to the high rate of recurrence after surgery [6, 7]. Most of the patients are ineligible for curative surgery because of the presence of metastases at the time of diagnosis [8]. The only option for these patients is to undergo a palliative treatment with a combination of gemcitabine and platinum salt, the reference chemotherapy validated for advanced unresectable CCA [8, 9]. Understanding the steps that lead to cell dissemination in patients with CCA is currently an important issue to be resolved in order to identify new therapeutic targets to prevent cancer progression and recurrence. During the past few years, epithelial-mesenchymal transition (EMT) has gained a lot of attention regarding metastatic dissemination. However, other mechanisms, such as exosomes released by different tumor cell types or the role played by circulating tumor cells, cannot be ruled out.

EMT is a reversible dynamic process during which epithelial cells gradually adopt structural and functional characteristics of mesenchymal cells [10-13]. EMT is fundamental in several physiological processes, such as embryogenesis and wound healing. During the past decade EMT has been proven to be deeply involved in different pathological processes, including fibrosis development and cancer progression. More specifically, EMT is an early event of metastasis that is required for tumor cell migration and invasion from the primary tumor (Figure 1). Major EMT steps comprise modifications of gene expression allowing concurrently epithelial phenotype repression and mesenchymal phenotype activation [14, 15]. The first changes take place at the adherens junctions with a deregulation of two main components, E-cadherin and β-catenin. Tight junction disruption also occurs and leads to a loss of apical-basal polarity. One mechanism by which E-cadherin is down-regulated occurs via EMT-inducing transcription factors (EMT-TFs), which comprise three families: SNAIL, ZEB and TWIST. EMT-TFs primarily regulate E-cadherin expression by repressing its promoter, but they also regulate in a positive manner the expression of genes associated with mesenchymal phenotypes including N-cadherin, vimentin, fibronectin, α-smooth muscle actin (α-SMA) and matrix metalloproteinases (MMPs) [15, 16]. Then, a reorganization of the epithelial actin cytoskeleton takes place with the formation of several migratory structures and the expression of MMPs to degrade the extracellular matrix (ECM). Thus, the acquisition of a mesenchymal-like phenotype endows the tumor cells with invasive properties, enabling them to spread toward other territories.

This review aims to give a complete overview on the knowledge gained so far on EMT in CCA and its regulation. In addition, contribution of the microenvironment cells in the induction of tumor cell plasticity and therapeutic consideration of EMT will be discussed.

Clinical evidence of EMT in cholangiocarcinoma (Table1)

Disruption of intercellular junctions

E-cadherin is a calcium-dependent cell-cell adhesion glycoprotein that constitutes with β-catenin the backbone of adherens junctions and plays a key role in the maintenance of

epithelium integrity [17]. E-cadherin deregulation affects this integrity constituting one of the major hallmarks of EMT. While E-cadherin is localized at the plasma membrane in healthy biliary epithelium, its down-regulation and/or ectopic localization, *i.e.* cytoplasmic internalization, have been reported in malignant cholangiocytes [18-37]. Genetic mutations and epigenetic silencing through promoter hypermethylation of the E-cadherin gene (*CDH1*) are among the mechanisms that account for the down-regulation of E-cadherin [30, 33, 38]. A reduction of E-cadherin immunostaining was observed in 16.5-82.1% of iCCA [21, 24-26, 29, 31, 32, 34-36] and eCCA [18, 37, 39, 40]. Although, down-regulation of E-cadherin in iCCA has been correlated with poor tumor differentiation, tumor size, advanced pTNM stage, intrahepatic metastasis and lymph node metastasis [25, 26, 29, 31, 32, 36], its prognostic value is still controversial. Ryu *et al.*, did not find any impact of E-cadherin expression on overall survival (OS) or disease free survival (DFS) [24] whereas other groups demonstrated that E-cadherin loss was significantly associated with these parameters and was an independent prognostic factor [25, 32, 36]. In eCCA, patients with weak E-cadherin expression displayed lower survival rate than patients with high E-cadherin expression [18] and was also an independent prognostic factor [40].

During EMT, E-cadherin is disordered at the expense of the expression of another cadherin named N-cadherin that is normally expressed by mesenchymal cells. This switch operates in both types of CCA in which an up-regulation of N-cadherin expression has been observed [18, 25, 34, 40, 41]. Increasing N-cadherin expression has been associated with lower OS in both CCA subclasses [25, 40] and is an independent unfavorable prognostic factor in eCCA [40].

β-catenin is retained at the membrane by E-cadherin while in cell cytoplasm β-catenin is recognized by the destruction complex wherein it is phosphorylated by GSK-3β (Glycogen Synthase Kinase-3 β). Thus, GSK-3β prevents β-catenin cytoplasm accumulation and consequently its translocation to the nucleus. When β-catenin reaches the nucleus, it associates with DNA-binding transcription factors to regulate gene expression, including

EMT genes [42]. Mutations in that β-catenin that prevent phosphorylation by GSK-3β, increasing β-catenin accumulation in the cytoplasm, are frequent in other cancers, but they are absent or in a very low frequency in CCA [43-45]. However, increments in β-catenin expression both in cytoplasm and nucleus have been observed in 14.1-82% of iCCA and 12.6% of eCCA, indicating a release of β-catenin from the E-cadherin/β-catenin membranous complex by other mechanisms. These changes were associated with high-grade tumor, tumor size, lymph node metastasis and OS, but not with DFS [20, 21, 24, 31, 32, 44-47].

Intermediate filaments

Cytokeratin 19 (CK19) and vimentin are members of the intermediate filament family that display a differential cell type expression. While CK19 is expressed in epithelial cells, vimentin is detected in mesenchymal cells. CK19 down-regulation has been observed in tumor cells of CCA, and this down-regulation was significantly associated with neural invasion, intrahepatic metastasis, undifferentiated tumor grade and shortened DFS and OS [24]. Several studies confirmed an aberrant staining of vimentin in CCA tumor cells while there was no staining in benign cholangiocytes [24, 25, 32, 35, 40, 48-50]. Vimentin expression was associated with lymph node metastasis, portal vein invasion, tumor size, pTNM stage and poorer OS and DFS [25, 31, 32, 35, 50]. In addition, vimentin was mostly detected in poorly differentiated tumor foci and a multivariate analysis demonstrated that aberrant vimentin expression is an independent adverse prognostic factor in iCCA [50]. As expected, vimentin expression was negatively correlated with the expression of CK19 and E-cadherin in iCCA [24, 25, 35]. Consistently, in eCCA cases (both pCCA and dCCA) expressing SNAIL, CK19 expression was lower and vimentin prevailed [48].

S100 Calcium Binding Protein A4 (S100A4)

S100A4 expression has been associated with transcriptional regulation of MMPs and E-cadherin genes through unidentified mechanisms [51, 52]. Undetectable in normal cholangiocytes, S100A4 immunoreactivity was evidenced in approximately 30-50% of iCCA [24, 53] and 11% of eCCA [40]. In iCCA, S100A4 protein expression was correlated with

aggressive clinical parameters, shortened DFS and OS and was an independent prognostic factor in a multivariate analysis [24, 53]. In eCCA, univariate and multivariate analyses revealed that S100A4 was a significant and an independent prognostic factor [40]. Recently, Fabris *et al*. demonstrated that nuclear S100A4 localization in iCCA and eCCA defines a subclass of CCA associated with decreased OS after resection [54].

EMT-inducing transcription factors (EMT-TFs)

*SNAIL family (*SNAIL*/SNAI1* and SLUG*/SNAI2)* is by far the most studied in CCA. Tumor cells from human iCCA expressing both the mesenchymal marker SNAIL in the nucleus and the biliary epithelial marker CK19 were identified by double immunofluorescence staining, providing conclusive evidence for the presence of EMT traits in malignant cholangiocytes [55]. While not detected in normal biliary epithelium, SNAIL mRNA and protein levels are markedly expressed in CCA, with a sub-cellular localization of the protein in the nuclei of iCCA and eCCA tumor cells [24, 32, 37, 39, 48, 56]. High expression of *SNAIL* transcripts was correlated with the presence of metastasis [56]. Nuclear overexpression of SNAIL protein was associated with aggressive parameters and poor prognosis in both iCCA (28.6-48.6% of cases) and eCCA (38-54% of cases), and predicted worse OS and DFS [24, 32, 39, 48]. Consistently with its repressing function on E-cadherin, increased *SNAIL* mRNA levels were negatively associated with E-cadherin transcripts and protein expression [32, 56]. SLUG is not detected in normal intrahepatic bile ducts and liver parenchyma but is expressed in 72.2% of iCCA, in which it has been associated with lymphovascular invasion, lymph node and distant metastasis [57]. Furthermore, high expression of SLUG was observed in 38.1% and 61.9% of long-term survivors and short-term survivors (short survival time < 12 months), respectively [57]. Described as an independent indicator of poor prognosis, SLUG could be used as a marker for predicting the outcome of patients with iCCA after surgical resection. High levels of *SLUG* mRNA were detected in cases of eCCA that displayed nodal and distant metastasis, portal vein and liver artery invasion, and lymphatic and perineural invasion [37], and also correlated with reduced E-cadherin expression.

*TWIST family (*TWIST1/*TWIST1* and TWIST2/*TWIST2)*. TWIST is overexpressed in CCA tumors and significantly associated with poor prognosis [41]. Moreover, TWIST nuclear expression was significantly correlated with high N-cadherin expression [41]. More particularly, TWIST1 was highly expressed in poorly differentiated and sarcomatous CCA tissues suggesting a close relationship between EMT-TFs and the appearance of a mesenchymal phenotype [58].

*ZEB family (*ZEB1/*ZEB1* and ZEB2/*ZEB2)*. Nuclear ZEB1 was highly expressed in malignant iCCA cells compared to normal non-neoplastic epithelial cells and was associated with aggressive parameters and poorer OS [35, 59]. High expression of ZEB2 was found in 51% of CCA and correlated with metastasis and poor prognosis [60].

Prognostic value of EMT markers in cholangiocarcinoma

As summarized above (Table 1), hallmarks of EMT (*i.e.* disruption of intercellular junctions) as well as expression of the master regulators (EMT-TFs) can be detected in human CCA samples and their presence is associated with poor clinical outcome, both in iCCA and eCCA. However, it should be stressed that if a single marker does not show any correlation with clinicopathological factors and/or patient outcome, the combination of several EMT markers (E-cadherin or β-catenin, two epithelial markers, with vimentin or fibronectin, two mesenchymal markers) could do, as it has been shown in different tumor types, including CCA [24, 31, 61-63]. Thus, consideration of cumulative alterations of EMT-related markers should be taken into account instead of considering either epithelial or mesenchymal markers individually to predict poor outcomes in human CCA patients. Whether EMT is responsible for the poor outcome of the patients or if EMT markers could be used as biomarkers of poor outcome needs further investigations with larger cohorts to deepen the current knowledge and strengthen the existing data.

Functional regulatory networks of EMT in cholangiocarcinoma (Table 2)

Cytokines (Figure 2A)

Transforming-growth factor-β (TGF-β)-dependent signaling is the prototypic inducer of EMT in several cancers including CCA [64]. TGF-β1-3 and their receptors TGF-β RI and RII were found expressed in both CCA tumor cells and surrounding stroma cells [65-70] while no data on TGF-β RIII expression is available. Regarding TGF-β1, it is expressed not only by invading malignant cholangiocytes, but also by stroma cells (*e.g.* fibroblasts, hematopoietic cells and macrophages) [71, 72]. Moreover, TGF-β1 expression was significantly correlated with lymph node metastasis, distant metastasis, and tumor recurrence in iCCA [73] and shorter OS both in iCCA and eCCA [69, 73]. Upon TGF-β1 stimulation, the expression of epithelial markers decreased concomitantly with an increased expression of mesenchymal markers in CCA cells [18, 41, 48, 69, 74-76]. Abrogation of TGF-β-dependent signaling pathway by a soluble TGF-β RII reduced CCA cell invasiveness in a murine CCA xenograft model [48]. Similar results were obtained in a rat model of CCA induced by thioacetamide wherein TGF-β signaling inhibition by a neutralizing TGF-β antibody led to a reduction in number and size of neoplastic ductules [77]. siRNA knockdown of HMGB1 (high–mobility group box 1), a chromatin protein, inhibited TGF-β-induced EMT in CCA cells, suggesting a role of HMGB1 in TGF-β regulation of EMT [78]. Similarly, chloroquine, an autophagy inhibitor, has been described to interfere with EMT induction through TGF-β [79], suggesting a potential link between EMT regulation and autophagy. In contrast, another member of the TGF-β superfamily, BMP-7 (Bone morphogenetic protein 7), precludes the action of TGF-β by promoting the conversion of mesenchymal to epithelial cells [80]. Addition of BMP-7 to CCA cells led to an inhibition of TGF-β1-induced EMT by decreasing nuclear expression of TWIST and cell migratory ability [41].

Tumor necrosis factor-α (TNF-α) reduced the expression of epithelial makers (E-cadherin and CK19) and enhanced the expression of mesenchymal markers (S100A4, SNAIL and ZEB2) favoring CCA cell migration *in vitro* [56, 60]. In addition, TNF-α was able to induce the expression of MMP9, largely related with tumor invasiveness in CCA [81, 82].

Interleukin-6 (IL-6). Patients with CCA display high levels of IL-6 in serum and in malignant cholangiocytes [83, 84]. Interestingly, IL-6 is not only expressed by malignant cholangiocytes, but also by surrounding cells (*e.g.* fibroblasts, hematopoietic cells and macrophages) in human CCA tumors [71, 72]. IL-6 triggers EMT in CCA cells by promoting membrane E-cadherin down-regulation, cell scattering and up-regulation of mesenchymal markers N-cadherin and vimentin [71, 85]. The suppressor of cytokine signaling 3 (SOCS3), which regulates IL-6/STAT3 pathway by antagonizing STAT3 tyrosine phosphorylation, inhibits IL-6-induced EMT [85]. Furthermore, a crosstalk between IL-6 and TGF-β1 in EMT has been emphasized in CCA [71]. Both factors induce endogenous expression of IL-6 and TGF-β1 in CCA cells through Smad4. Thus, inhibition of Smad4 halted the IL-6/TGF-β1 crosstalk loop, and reversed IL-6/TGF-β1-induced EMT. In human samples, all protagonists, IL-6, TGF-β1, Smad4 along with the mesenchymal marker N-cadherin are expressed at the invasion front of tumor cells, suggesting that Smad4 may represent a therapeutic target not only to halt CCA progression, but also to control pro-inflammatory environment maintenance [71].

Receptor tyrosine kinases (Figure 2B)

Epidermal Growth Factor Receptor (EGFR) expression and signaling are strongly associated with CCA development and progression [70, 86-88]. We recently showed that ectopic cytoplasmic localization of E-cadherin is correlated with EGFR overexpression in human iCCA and pCCA [29]. Interestingly, E-cadherin also displayed a cytoplasmic pattern in xenografted tumors, whereas the mice treatment with gefitinib restored the membranous expression of E-cadherin. *In vitro*, EGF and HB-EGF, two EGFR ligands, induced scattering of CCA cells that resulted from the disruption of adherens junctions [29, 68]. In EGF-stimulated CCA cells, EMT-TFs (SLUG and ZEB1) and mesenchymal markers (N-cadherin and α-SMA) were induced, favoring cell invasiveness through cytoskeleton remodeling [29, 89]. We obtained similar results after down-regulating the PDZ scaffold protein EBP50 which led to the implementation of an EMT program through EGFR activation with the subsequent

acquisition of invasive and migratory properties by CCA cells [86]. Besides EBP50, SOX4 transcription factor has been described as a potent inducer of EMT in CCA cells possibly through modulation of EGFR expression [90].

Eph Receptors and their ligands are expressed at very low levels in normal cholangiocytes [91, 92]. Among Eph receptors, EphA2 and EphB2 were increased in CCA tumors and correlated with the metastatic status of patients and poorer tumor differentiation [91, 92]. Overexpression of EphA2 in CCA cells induced a down-regulation of cell-cell junction proteins and an up-regulation of mesenchymal markers, leading to the acquisition of fibroblastic appearance and invasive properties.

G protein-coupled receptors (Figure 2C)

H4 Histamine receptor (H4HR). While H1HR and H2HR stimulate biliary hyperplasia and CCA growth, H3HR and H4HR decrease CCA progression [93]. H4HR is up-regulated in human CCA cells compared to non-malignant tissue [94]. CCA cells treated with the specific H4HR agonist clobenpropit induced a down-regulation of fibronectin, vimentin and S100A4, while expression of epithelial markers CK7, CK8 and CK19 was maintained. Consequently, clobenpropit treatment reduced invasive and metastatic potential of CCA cells [94].

C-X-C-motif Chemokine Receptor-4 (*CXCR4)* binds the stromal-derived-factor-1 (SDF-1; also called CXCL12). CXCR4 is expressed by tumor cells in human CCA but not in the adjacent non-tumor tissue [95, 96]. High expression of CXCR4 is associated with metastasis and poor clinical outcome of iCCA. *In vitro*, CXCR4 acts as a potent activator of EMT by increasing expression of SLUG, vimentin and MMP-9, promoting cell migration and invasion through a β-catenin-dependent mechanism [96].

Development-related pathways (Figure 2D)

Notch signaling. Notch-1 and Notch-4 expression is up-regulated in tumor cells from iCCA compared with adjacent non-tumor liver tissues [97-100]. In eCCA, the four Notch receptors were overexpressed [101] and high expression of Notch-1 and Notch-3 was related to advanced TNM stage and advanced T stage, respectively, suggesting a contribution of both

receptors to eCCA progression. After stimulation, Notch is cleaved by a γ-secretase and the Notch intracellular domain is released and translocated into the nucleus to activate target gene transcription. As in many other cancers, Notch signaling activation led to EMT induction in CCA cells. Notch-1 overexpression causes an increase in α-SMA and vimentin expression and a decrease in E-cadherin protein levels. All these events were accompanied by a cellular morphological change and cytoskeleton reorganization characteristic of EMT activation [98]. Consistently, Notch-1 inhibition by a γ-secretase inhibitor decreased vimentin and SNAIL expression, as well as impaired invasion and migration in CCA cells [102]. Among Notch signaling target genes, Sox9 plays a fundamental role in biliary pathophysiology [103, 104]. Sox9 has been recently linked to the activation of EMT in CCA [105]. Since Sox9 has been described as a Notch1 mediator in EMT activation in lung adenocarcinoma [106], it could play a similar role in CCA.

Hedgehog signaling. Upon Hedgehog ligand binding, GPCR-like protein Smoothened is released by Patched receptor and allows generating activated GLI1-3. Hedgehog signaling components are overexpressed in iCCA [107-109], and it has been shown that GLI1 and GLI2 overexpression was associated with intrahepatic metastasis and poorer OS and DFS [108]. A role of Hedgehog signaling in EMT has been described in human CCA cells and CCA xenografted tumors by modulating E-cadherin expression [107] Consistently, inhibition of Hedgehog signaling by cyclopamine [107] or capsaicin [110] up-regulated E-cadherin expression [107] and impaired EMT in CCA [110].

microRNAs (miRs) (Figure 2E)

miRs are deeply involved in EMT regulation [14]. So far, 6 miRs including miR-221 [111], mir-200c [112], miR-204 [113] miR-214 [114], miR-34a [76] and miR-21 [115] have been described to regulate EMT in CCA. With the exception of miR-221 and miR-21, which are up-regulated in CCA tissue and associated with poor survival, all the others are down-regulated in human CCA.

Transcriptomic profiling of iCCA tissues revealed that a signalling pathway linking miR-200c to EMT is preferentially activated in iCCA that display stem cell gene expression traits [112]. Ingenuity Pathway Analysis showed that miR-200c was negatively correlated with genes from the TGF-β signalling pathway, and NCAM1 (Neural Cell Adhesion Molecule 1) was experimentally demonstrated to be a direct target of miR-200c. Forced expression of miR-200c in HuH28, a CCA cell line that displays a fibroblastic-like cell morphology and low levels of miR-200c, led to an inhibition of EMT with suppression of mesenchymal gene expression (ZEB1/2, vimentin and N-cadherin) and increment of E-cadherin. Conversely, miR-200c down-regulation in HuCC-T1, a CCA cell line with epithelial appearances and high expression of miR-200c, led to an activation of EMT with an induction of mesenchymal markers and a repression of E-cadherin [112].

miR-204, miR-214 and miR-34a inhibit EMT in CCA by targeting SLUG, TWIST and Smad4, respectively [76, 113, 114], while miR-221 and miR-21 positively regulate EMT by directly repressing PTEN and by unidentified mechanisms, respectively [111, 115].

Additional regulatory factors

Unconjugated primary bile acid, chenodeoxycholic acid, and the secondary bile acid, lithocholic acid, induce SNAIL expression and E-cadherin down-regulation in CCA cells, at least in part, through two transcription factors, Nuclear factor-Y and Stimulating protein 1 [116]. Whether these effects involve the bile acid receptors, Farnesoid X Receptor and G protein-coupled bile acid receptor 1 (TGR5), remains to be elucidated. However, since bile acids can activate EGFR [117], they may induce indirectly EMT through EGFR signaling pathway.

Hepatitis C virus core (HCVc) in CCA tissues was associated with decreased E-cadherin expression and increased N-cadherin, vimentin and fibronectin expression [118, 119]. A correlation between HCVc and metastasis in lymph nodes and other organs was evidenced. Consistently, expression of HCVc in CCA cells induced a fibroblastic and scattered appearance, along with an augmentation of vimentin and fibronectin, and a down-regulation

of E-cadherin, that may be mediated by lysyl oxidase homolog 2 [118, 119]. These effects may be of special relevance in areas of East-Asia with high HCV prevalence or countries where the diagnosis of primary tumors in livers with a cirrhotic background has been recently increasing [2].

Small proline rich protein 2a (SPRR2a) overexpression in CCA cells induced EMT, which in turn promotes invasiveness [59, 120]. Further experiments showed that SPRR2A acts as a transcriptional corepressor with ZEB1 to repress miR-200c/141 transcription in CCA cells in order to maintain a mesenchymal phenotype [59].

In addition, 14-3-3zeta and aPKC-ı [121, 122], adrenomedullin [123], Forkhead box protein C2 [124],–WAVE3 [125] and Fibroblast growth factor (FGF)19/FGFR4 axis [126] promote EMT in CCA. Conversely, FBXW7 [127, 128], thymosin β10 [129] and MAP3K4 [130] act as negative regulators of EMT through inhibition of mTOR, ERK1/2 and NFκB pathways, respectively.

EMT regulation by the microenvironment in cholangiocarcinoma

For a long time perceived as a tissue composed only of cancer cells, the tumor tissue is in fact, like all tissues, composed of different cellular and acellular components. It is now well established that all these components constitute a favorable environment for tumor development. CCA is characterized by a prominent desmoplastic stroma [3], which is composed primarily by cancer-associated fibroblasts (CAFs) and to a lesser proportion of tumor-associated macrophages (TAM) and vascular cells. As discussed below, stromal cells play a key role in CCA progression through reciprocal interactions with malignant cells that lead to EMT activation.

Cancer-associated fibroblasts (CAFs) and hepatic stellate cells (HSCs) (Figure 3A)

CAFs are probably derived from activated HSCs and/or portal (or periductal) fibroblasts in the liver [131] although a circulating bone-marrow-derived precursor cell origin has also been suggested. The possibility that cholangiocytes could also feed the pool of stroma myofibroblasts has been refuted in different models of fibrosis [132] and more recently in a

murine model of xenografted tumors [133]. CAFs have a crucial role in favoring CCA progression, and more particularly in promoting EMT, through interactive autocrine and paracrine signaling pathways [68, 131]. We have recently shown that CAFs from human iCCA synthesize HB-EGF, which activates EGFR in CCA cells leading to the disruption of cell-cell junctions and an increase of invasiveness [68]. Furthermore, we described a paracrine reciprocal loop in which CCA cells produced TGF-β1 that stimulates HB-EGF expression by CAF. Thus, this cyclic interplay between tumor and stroma cells, contributes to EMT of CCA cells through a constant activation of EGFR pathway.

Regarding the intercellular dialogue between HSCs and CCA cells, the SDF-1/CXCR4 axis has been described as a major interaction pathway, which could contribute to stromal fibrosis in CCA [75, 95]. It has been shown that SDF-1 and CXCR4 are expressed by HSCs in human iCCA. *In vitro*, angiotensin II and TGF-β enhanced the release of SDF-1 by LI-90 cells (a human HSC line), which in turn, promoted activation of LI-90 cells. Furthermore, SDF-1 reduced E-cadherin expression and enhanced nuclear β-catenin and vimentin expression in CXCR4-expressing CCA cells [75]. In this study, angiotensin II was shown to act not only on HSCs, but also on CCA tumor cells by inducing EMT.

Tumor-associated macrophages (TAMs) and mast cells (Figure 3B)

TAMs represent the major class of immune cells within the tumor microenvironment and derive from circulating monocytes that infiltrate tumor tissues and differentiate into macrophages. Many infiltrating CD68-positive macrophages and TNF-α-positive macrophages exist at the iCCA interface [134, 135]. TAMs secrete several factors that influence EMT in CCA tumor cells. CCA cells cultured in presence of conditioned media from activated TAMs experienced an accumulation of β-catenin in cell cytoplasm [46] down-regulation of E-cadherin and CK19, and an increment in the expression of mesenchymal markers (*i.e.* S100A4, N-cadherin and MMP9) [72, 136], as well as an increment in cell migration. This augmented migration could explain the association between the extrahepatic metastases and the high density of TAMs described in patients with CCA [136]. The

paracrine stimulation of EMT in CCA tumor cells by TAMs may be related to the production and secretion of interleukins, TNF-α and TGF-β1 by activated macrophages [72]. More recently, TAMs have been described as potent providers of Wnt ligands [137], which are known to induce EMT in other cancers [138, 139].

Mast cells are master regulators of the immune system, which are involved in liver pathogenesis [140]. Inhibition of mast cell-derived histamine prevents EMT switch and ECM breakdown in CCA cells through H1HR and H2HR [141]. Moreover, if the SCF/Kit pathway involved in tumor mast cell recruitment is inhibited, the paracrine influence of mast cells on CCA is vanished [141], suggesting a role of mast cells/tumor cells interaction in the promotion of EMT and progression of CCA.

The information summarized above points out the role of the different cell types within the tumor, and not only cancer cells, in CCA progression, and more specifically in the activation of EMT that may lead to metastasis. Although not related to EMT, a recent study [142] showed the possibility of targeting CAFs in a syngeneic rat model with a pro-apoptotic drug called Navitoclax, which suppressed tumor growth and improved host survival. Therefore, efforts should be directed to the development of targeted therapies against both tumor cells and their interactions with the surrounding stroma, in order to reduce metastasis and improve patient conditions.

EMT and therapeutic considerations

Chemoresistance

Drug resistance is a challenge constantly faced by clinicians regarding the use of antitumor agents in patients. In CCA, EMT has been identified *in vitro* as a mechanism of resistance against gemcitabine and cisplatin [71, 143], both used in combination as the standard of care for patients with CCA [9]. Yamada *et al.*, recently showed that CCA cell lines exhibiting mesenchymal traits were more resistant to gemcitabine than CCA cell lines having a prominent epithelial phenotype [71]. IL-6 and TGF-β1, through an autocrine and crosstalk loop involving Smad4, were incriminated in the resistance to gemcitabine by inducing EMT.

Indeed, establishment of gemcitabine-resistant CCA cell lines showed that these cells displayed a reduced expression of E-cadherin and an increased expression of N-cadherin and vimentin [71]. In addition, inhibition of SLUG sensitized CCA cells to cisplatin through up-regulation of the proapoptotic protein PUMA [143]. It is noteworthy that the activity of EMT-TFs is not restricted to the repression of E-cadherin promoter, but they also trigger cell survival by promoting apoptosis escape of tumor cells through inhibition of PUMA-mediated cell death in CCA [15, 144].

Something that is worth to be mentioned is the increasing evidence suggesting that EMT could be associated with other features such as the acquisition of cancer stem cell (CSC) properties [145]. The acquisition of CSC properties by tumor mesenchymal-like cells in response to EMT could have major consequences, in particular by fostering tumor heterogeneity and by contributing to resistance to anticancer drugs in CCA, a tumor containing a strong contingent of CSC [55]. In fact, Shuang *et al.* have recently found, both in iCCA and eCCA, a significant correlation between TGF-β1 and ALDH1 (aldehyde dehydrogenase 1), a functional CSC marker. In addition, TGF-β1-induced EMT in CCA cells resulted in an acquisition of mesenchymal traits, as well as ALDH expression, which were accompanied by a decreased sensitivity to 5-fluorouracil [69].

By helping cells to counteract endogenous safeguard mechanisms, EMT may confer resistance to cell death triggered by chemotherapeutic agents. However, it still remains unclear whether chemotherapeutic agents directly induce EMT or lead to the selection of tumor cells that already display EMT traits.

Targeting EMT pathways

The first thought when it comes to target EMT would be playing with E-cadherin repressors. However, the master EMT regulators are transcription factors, what make them quite difficult to target. Thus, the most useful alternative strategy would be the use of drugs that negatively regulate EMT, though only few studies testing chemical agents against EMT in CCA have been performed. Paclitaxel is a chemotherapeutic agent that stabilizes microtubules and

arrests cell cycle. At low doses, paclitaxel has been shown to inhibit TGF-β-induced EMT in CCA cells [74]. FTY720, a synthetic sphingosine immunosuppressant, inhibits EMT and favors reversion of EMT that is called MET (mesenchymal-epithelial transition) in CCA cells and, prevents metastasis *in vivo* after implantation of CCA cells into the peritoneal cavity of immunocompromised mice [146]. Recently, taking advantage of the presence of CXCR4 at the surface of CCA cells, a polymeric CXCR4 antagonist capable of delivering miR-200c has been developed to inhibit EMT inducer ZEB1 in CCA cells [147]. miR-200c was chosen as a potential treatment for its ability to target ZEB1. Thus the combination CXCR4 antagonist/miR200c offers a promising antimetastatic strategy for the CCA treament, which must be tested in preclinical models to move forward. In addition, as displayed above, EMT is highly regulated by a large number of signaling pathways. These pathways are targetable by drugs currently tested in preclinical studies or clinical trials in CCA, such as the EGFR inhibitor erlotinib (see reviews [13, 148]).

However, in many cases the prolonged use of these drugs results in the opposite undesired effect, the induction of an EMT program as a chemoresistance mechanism through the activation of compensatory alternative pathways. This issue has already been described in non-small cell lung cancer, where src/FAK signaling pathway was up-regulated in a resistant cell line to erlotinib leading to EMT [149]. Furthermore, our preliminary results suggest that EMT is induced as a mechanism involved in acquired resistance to erlotinib in human CCA cells, probably through an up-regulation of IGF signaling [150].

Final remarks and future perspectives

Given the large body of evidence displayed in this review, there is no doubt of the prominent role of EMT in CCA progression. It becomes imperative to consider EMT as another player in the high metastatic and chemoresistant features characteristic of this tumor. However, some points should be taken in consideration before jumping to patient treatment. Definition of proper combinations of epithelial and mesenchymal markers is eagerly needed in order to select the appropriate patients for the available therapies and the prediction of possible

outcomes. Among these markers, proper characterization of EMT-TFs is of special importance, since they are deeply involved in the induction of the highly chemoresistant CSC phenotype that has not been profoundly investigated in CCA. Furthermore, since CCA is a very complex and heterogeneous tumor with a prominent stromal component that interacts closely with cancer cells, strong effort should be directed to the development of combinational therapies directed towards both cancer and stromal cells. Finally, the possibility to inhibit signaling pathways involved in this complex process with single molecules against receptors or intracellular kinases should be taken with extreme care, given that the inhibition of these pathways could also trigger compensatory pathways leading to EMT induction as a mechanism of resistance.

Altogether, EMT has become an attractive therapeutic target for CCA. However, due to the redundancies and bypasses among the different signaling pathways and cell types involved, further studies focused on the development of combination therapeutics targeting EMT in CCA are eagerly needed.

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Author names in bold designate shared co-first authorship.

Figure legends

Figure 1.

Cancer cell EMT events. The diagram shows a group of cells progressively engaged in EMT and the differential changes in epithelial and mesenchymal markers during this process. A typical epithelial sheet (A) contains polarized epithelial cells on top of a basement membrane. Cells are joined together by several cell-cell junctions, tight junctions, GAP junctions and adherens junctions containing E-cadherin/β-catenin. (B) Upon stimuli, increment in the expression of EMT-inducing transcription factors (EMT-TFs) leads to the down-regulation and disassembly of cell-cell junctions with the consequent loss of epithelia integrity. (C) EMT-TFs stimulate a mesenchymal phenotype, which includes reorganization of the actin cytoskeleton and secretion of matrix metalloproteinases allowing the dissolution of the basement membrane and the mobility of the resulting mesenchymal-like cells. The different proteins expressed through EMT are listed in the box.

Figure 2.

Regulatory networks involved in EMT regulation in CCA. Cytokines (A), tyrosine kinase receptors (B), G protein coupled receptors (C) and receptors involved in developmental processes (D) play a role in the induction of EMT program by activating intracellular signaling pathways in CCA tumor cells. miRNAs target several EMT regulatory proteins (E). Abbreviations: CXCR4, chemokine receptor type 4; EBP50, ezrin-radixin-moesin-binding phosphoprotein 50; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EphA2, ephrin type-A receptor 2; GLI, glioma-associated oncogene; H4HR, H4 histamine receptor; HB-EGF, heparin-binding EGF-like growth factor; IL, interleukin; ILR, interleukin receptor; miR, microRNA; NICD, notch intracellular domain; PTCH, patched receptor; SDF-1, stromal cell-derived factor 1; SMO, smoothened; SOX4, sex determining region Y box 4; SOX9, sex determining region Y box 9; Sp1, specificity protein 1; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming-growth factor-β; TGFβR, transforming-growth factor-β receptor; TNF-α, tumor

necrosis factor-α; TNFR, tumor necrosis factor receptor; ZEB, zinc finger E-box binding homeobox.

Figure 3.

Model depicting the reciprocal paracrine loop between CCA cells and cells from the tumor microenvironment involved in EMT regulation. (A) Hepatic myofibroblasts produce the EGFR ligand, HB-EGF, which activates EGFR at the CCA cell surface. EGFR activation leads to the stimulation of its downstream pathways and eventually to the activation of an EMT program. In addition, EGFR signaling also triggers TGF-β production by CCA cells, which results in myofibroblast activation and in increased HB-EGF synthesis by myofibroblasts. Hepatic stellate cells produce SDF-1, which, together with ANGII induce β-catenin nuclear translocation through their receptors. Furthermore, both SDF-1 and ANGII are able to promote hepatic stellate cell activation. (B) LPS activated macrophages produce different factors, including several ILs, TGF-β, TNF-α and WNT ligands that promote EMT by signaling through their receptors. Recruited mast cells release histamine that induce EMT in CCA cells through HRs signaling. In turn, CCA cells produce SCF that promote mast cell migration and recruitment. Each color indicates a group of signaling pathways described in the text. Abbreviations: ANGII, angiotensin II; AT1, angiotensin II receptor type 1; CXCR4, chemokine receptor type 4; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; FDZ, frizzled receptor, HB-EGF, heparin-binding EGF-like growth factor; HRs, histamine receptors; IL, interleukin; ILR, interleukin receptor; LPS, lipopolysaccharide; SCF, stem cell factor; SDF-1, stromal cell-derived factor 1; TGF-β, transforming-growth factor-β; TGFβR, Transforming-growth factor-β receptor; TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor.

Table 1 | **Clinical relevance of EMT in human cholangiocarcinoma**

Epithelial markers	CCA subtype	Number of samples	Membrane expression/ localization	% of cases	Association with clinical parameters	Ref.
E-cadherin	CCA	35	Reduced	62.9	Tumor differentiation Infiltration status Lymph node metastasis Clinical TNM staging Median survival Independent prognostic factor in univariate and multivariate analyses	[19]
	CCA	47	Reduced	45	Tumor Grade	[20]
	CCA	83	Reduced	51.8	Poor tumor differentiation	[26]
	CCA	140	Reduced	52	Positive metastasis status	[28]
	iCCA	31	Reduced	61.3	Not correlated	[21]
	iCCA	119	Reduced	41.2	Not Correlated	[24]
	iCCA	96	Reduced	43.8	Lymph node metastasis Advanced pTNM stage Poor differentiation Poorer overall survival Independent prognostic factor in multivariate analysis	[25]
	iCCA	83	Reduced	51.8	Poor histological differentiation	[26]
	iCCA	100	Cytoplasmic	50	Tumor size Presence of satellite nodules	[29]
	iCCA	85	Reduced	16.5	Poor tumor differentiation	[31]
	iCCA	140	Reduced	55	Lymphatic metastasis Poorer overall survival Poorer disease free survival	[32]
	iCCA	102	Reduced	44.1	Not correlated	[35]
	iCCA	42	Reduced Absent	64.3 19	Tumor grade pTNM stage Intrahepatic metastasis Poorer survival	[36]
	eCCA	38	Reduced	42.1	Lymph node metastasis Tumour stage Lymphatic invasion Blood vessel invasion Overal lower survival	[18]
	eCCA (pCCA)	52	Reduced	36.5	Not correlated	[37]
	eCCA (pCCA)	47	Reduced	40.4	Not correlated	[39]
	eCCA	117	Reduced	82.1	Poorer overall survival Independent prognostic factor	[40]
β-catenin	CCA	47	Reduced	58	Tumor differentiation grade	[20]
	iCCA	31	Nuclear	16.1	Not correlated	[21]
	iCCA	119	Loss or delocalization	57.1	Not correlated	[24]
	iCCA	85	Reduced / nuclear	14.1	Poor tumor differentiation Tumour size Lymph node metastasis	[31]
	iCCA	140	Cytoplasmic or nuclear	45.7	Poorer overall survival	[32]
	iCCA	71	Reduced Nuclear	82 15	Poor tumor differentiation	[44]
	iCCA	24	Cytoplasmic or nuclear	58.3	Tumor size	[45]
	iCCA	38	Reduced / cytoplasmic	73.7	Not correlated	[46]
	eCCA	79	Nuclear	12.6	Not correlated	[47]

CK19	iCCA	119	Reduced	42	Neural invasion Intrahepatic metastasis Undifferentiated tumor Poor overall survival and disease free survival	[24]
Mesenchymal markers	**CCA subtype**	**Number of samples**	**Expression**	**%**	**Association with clinical parameters**	**Ref.**
N-cadherin	CCA	30	Increased	53.3	Not correlated	[41]
	iCCA	96	Increased	57.3	Higher recurrence rate of vascular invasion Poorer overall survival	[25]
	iCCA	29	Increased	79	Not correlated	[34]
	eCCA	38	Increased	23.7	Not correlated	[18]
	eCCA (pCCA)	23	Increased	30.4	Not correlated	[34]
	eCCA	117	Increased	18.8	Poorer overall survival Independent prognostic factor in multivariate analysis	[40]
Vimentin	iCCA	119	Increased	20.2	Not correlated	[24]
	iCCA	96	Increased	37.5	Lymph node metastasis Advanced pTNM stage Poorly differentiated type Poorer overall survival	[25]
	iCCA	85	Increased	21.2	Poor differentiation Higher stage tumor	[31]
	iCCA	140	Increased	55.7	Lymphatic metastasis Poorer overall survival Poorer disease free survival	[32]
	iCCA	102	Increased	43.1	Portal vein invasion Tumor size	[35]
	iCCA	23	Increased	69.6	Not correlated	[49]
	iCCA	21	Increased	23.8	Tumor grade differentiation Poorer overall survival Independent prognostic factor in multivariate analysis	[50]
	eCCA	117	Increased	13.7	Poorer overall survival	[40]
	eCCA (pCCA)	17	Increased	11.8	Not correlated with clinico-pathological features	[49]
S100A4	CCA	86	Absent Low nuclear High nuclear	57 22.1 20.9	Overall survival after surgical resection Increased metastasis after surgical resection	[54]
	CCA	50	mRNA overexpression	52	Lymph node metastasis	[60]
	iCCA	119	Increased	30.3	Angiolymphatic invasion Neural invasion Intrahepatic metastasis Undifferentiated tumor	[24]
	iCCA	65	Increased	49.2	Vascular invasion Lymph node metastasis TNM stage Poorer overall survival rate Independent prognostic factor in multivariate analysis	[53]
	eCCA	117	Increased	11.1	Poorer overall survival Independent prognostic factor in multivariate analysis	[40]
SNAIL	CCA	50	mRNA overexpression	66	Metastasis stage	[56]
	iCCA	119	Increased	28.6	Angiolymphatic invasion Neural invasion Intrahepatic metastasis	[24]

	iCCA	140	High/Low	48.6/51.4	Undifferentiated tumor Lymphatic metastasis Poorer overall survival Poorer disease free survival	[32]
	eCCA (pCCA)	52	mRNA overexpression	23	Not correlated	[37]
	eCCA (pCCA)	47	High/Low/Negative	38/36/12	Poorer overall survival	[39]
	eCCA (pCCA)	37	Increased and nuclear	54	Lymph node metastasis Poorer overall survival	[48]
SLUG	iCCA	36	Increased	72.2	Lymph node metastasis Lymphovascular invasion Distant metastasis Hematogenous recurrence Lymph node recurrence Poorer overall survival	[57]
	eCCA (pCCA)	52	mRNA overexpression	34.6	Nodal metastasis Distant metastasis Poorer overall survival	[37]
Twist	CCA	30	Increased and nuclear	26.7	Poorer overall survival	[41]
ZEB1	iCCA	102	Nuclear	43.1	Nodal metastasis Tumor stage Undifferentiated-type histology Lymph node metastasis Portal vein invasion Poorer overall survival	[35]
ZEB2	CCA	165	Cytoplasmic/Nuclear	51/12.8	Lymph node metastasis	[60]

Abbreviations: CCA, cholangiocarcinoma; eCCA, extrahepatic CCA; EMT, Epithelial-mesenchymal transition; iCCA, intrahepatic CCA; pCCA, perhilar CCA; Ref, references; S100A4, S100 Calcium Binding Protein A4; ZEB, zinc finger E-box binding homeobox. In most cases, expression of the markers was analyzed at the protein level. When mRNA has been analyzed, the information is mentioned.

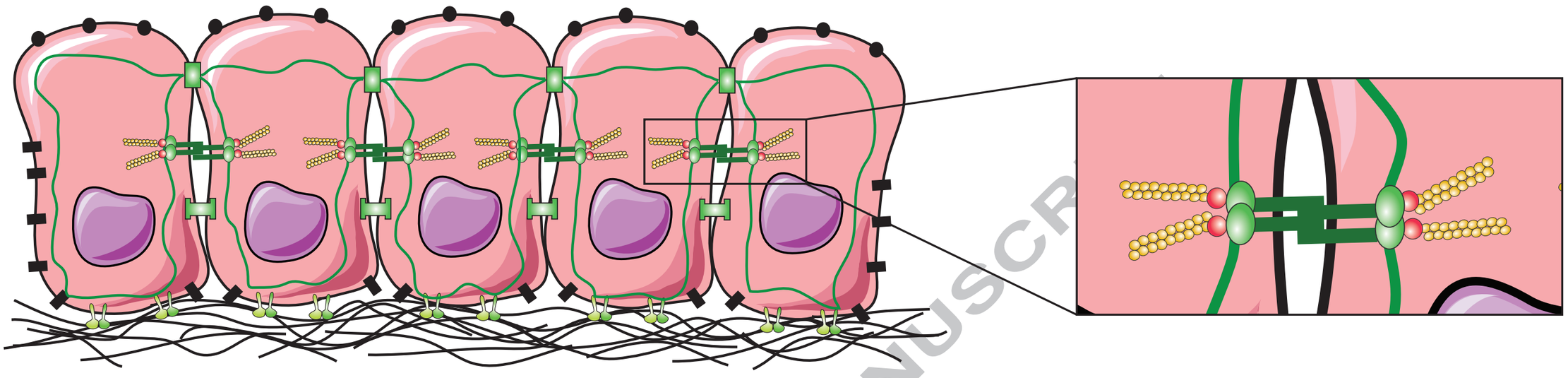
Table 2 | **Regulation of EMT in human cholangiocarcinoma cells**

EMT Inducer	EMT Inhibitor	Receptor /Target	Epithelial Marker	Mesenchymal Marker	Biological Effect	Cell lines	Ref.
ADM		Unknown	E-cadherin ZO-1	N-cadherin Vimentin ZEB1		HuCC-T1 HuH-28	[123]
ANGII		AT1	β-catenin E-cadherin	Vimentin	Migration	CCKS-1 HuCC-T1	[75]
Bile acids		Unknown	E-cadherin	Snail		HuCC-T1	[116]
	Capsaicin	Hh signaling	E-cadherin	N-cadherin Vimentin	Invasion Migration	SZ1 TFK-1	[110]
	Clobenpropit	H4HR	CK7 CK8 CK19	Fibronectin MMP-1/-2/ -3/-9/-11 S100A4 Vimentin		Mz-ChA-1	[94]
	Cyclopamine	Hh signaling	E-cadherin		Invasion Migration	SZ1 TFK-1	[107]
EGF	Gefitinib	EGFR	β-catenin DSP 1/2 E-cadherin ZO-1	α-SMA Fibronectin MMP-1/-9 N-cadherin SLUG Vimentin ZEB-1	Invasion Migration	Choi-CK Mz-ChA-1 SK-ChA-1	[29] [89]
	FTY720	STAT3 signaling	E-cadherin	N-cadherin TWIST1 Vimentin	Invasion	HuCC-T1 QBC939 TFK-1	[146]
	γ-secretase inhibitor	Notch signaling	β-catenin E-cadherin	SNAIL Vimentin	Invasion Migration	SZ1 TFK-1	[102]
FGF19	AP24354	FGFR4	E-cadherin	N-cadherin SNAIL Vimentin	Invasion	RBE	[126]
HB-EGF	Gefitinib	EGFR	β-catenin E-cadherin		Invasion Migration	EGI-I Mz-ChA-1 SK-ChA-1	[68]
Histamine	Cromolyn sodium	HR	E-cadherin	Paxilin S100A4 Vimentin MMP-2/-3/-9		Mz-ChA-1	[141]
IL-6		IL6R	E-cadherin	N-cadherin SNAIL Vimentin	Invasion Migration	CCLP1 HCCC9810 HuCC-T1 KMCH Mz-ChA-1 RBE	[71] [85]
	PCX/miR200c	CXCR4/ ZEB1		ZEB1	Migration	HuCC-T1	[147]
SDF1	AMD3100	CXCR4	β-catenin E-cadherin	MMP-9 SLUG Vimentin	Invasion Migration	CCKS-1 HuCC-T1	[95] [96] [75]
TGF-β	BMP-7 Paclitaxel TGF-βsRII	TGFβR	β-catenin CK19 E-cadherin	α-SMA Col1A1 Fibronectin MMP-2 N-cadherin S100A4 SNAIL TWIST Vimentin	Invasion Migration	BECs CCKS-1 CCLP1 GBC-SD HuCC-T1 KMCH M139 M213 Mz-ChA-1	[18] [41] [48] [69] [74] [76] [75]

						QBC939 TFK-1	
TNF-α		TNFR	CK19 E-cadherin	MMP-9 S100A4 SNAIL Vimentin ZEB2	Invasion Migration	CCKS-1 HuCC-T1 M139 M213 M214	[56] [60] [81] [82]
WNT3		β-catenin signaling	β-catenin			M214	[46]

Abbreviations: ADM, adrenomedullin; ANGII, angiotensin II; AT1, angiotensin II receptor type 1; BMP-7, bone morphogenetic protein-7; CK, cytokeratin; CXCR4, chemokine receptor type 4; DSP, desmoplakin; EGF, epidermal growth factor; FGF19, fibroblast growth factor 19; FTY720, fingolimod; HB-EGF, heparin-binding EGF-like growth factor; Hh, hedgehog; HR, histamine receptor; IL, interleukin; MMP, matrix metalloproteinase; Ref, references; S100A4, S100 Calcium Binding Protein A4; SDF-1, stromal cell-derived factor 1; TGF-β, transforming-growth factor-β; TGF-βsRII, soluble TGF-β type II receptor; TNF-α, tumor necrosis factor-α; WNT3, wingless-Type MMTV Integration Site Family, Member 3; ZO-1, zonula occludens 1.

A



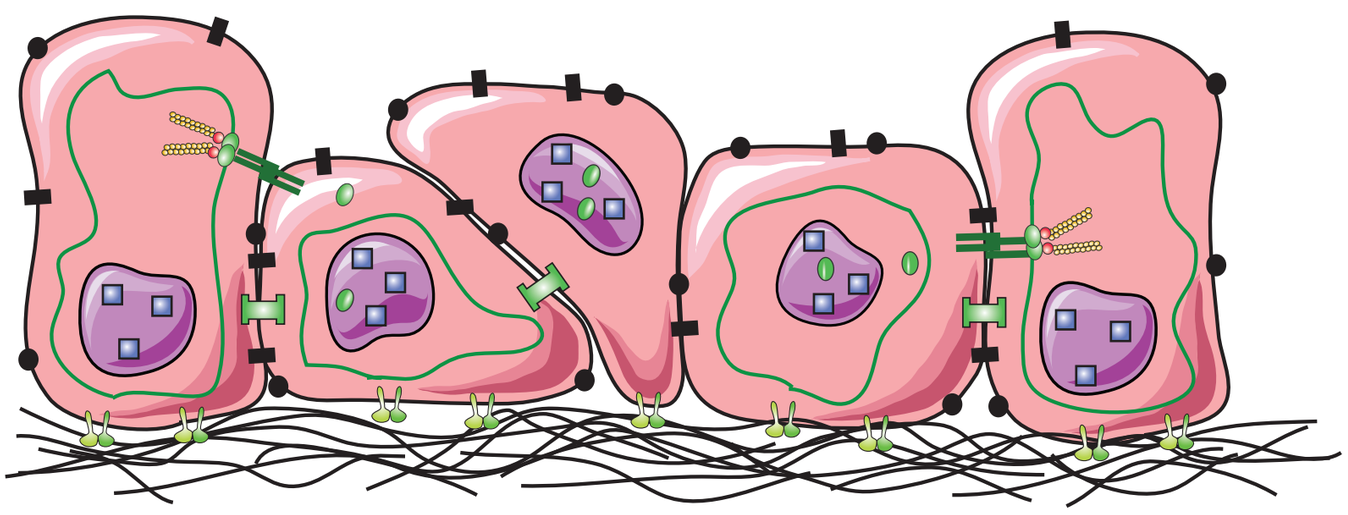
EMT stimulus

EMT-TFs increment

E-cadherin deregulation

Loss of Apico-Basal polarity

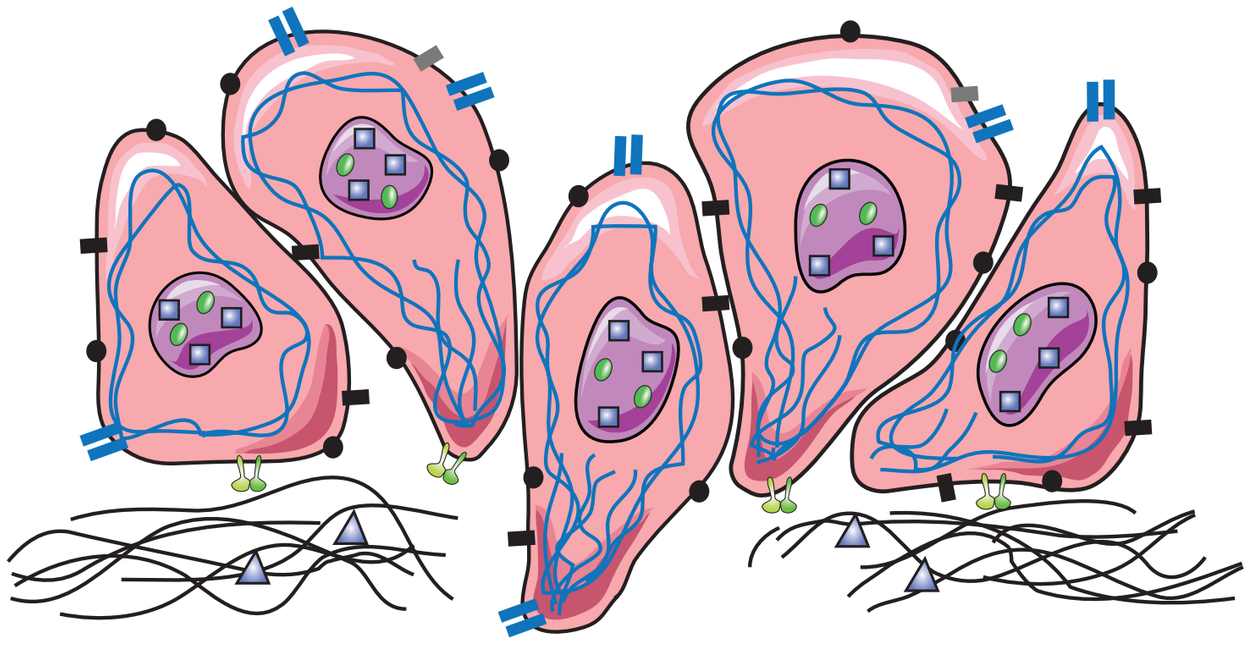
B



Gain of mesenchymal proteins

Migration and invasion

C



- Basement membrane
- Apical membrane components
- Basolateral membrane components
- Integrins
- Cytokeratin intermediate filaments
- GAP junctions
- Tight junctions
- E-cadherin
- β-catenin
- α-catenin
- Actin cytoskeleton
- Vimentin
- EMT-inducing transcriptional factors
- Matrix metalloproteinases
- N-cadherin

FIGURE 1

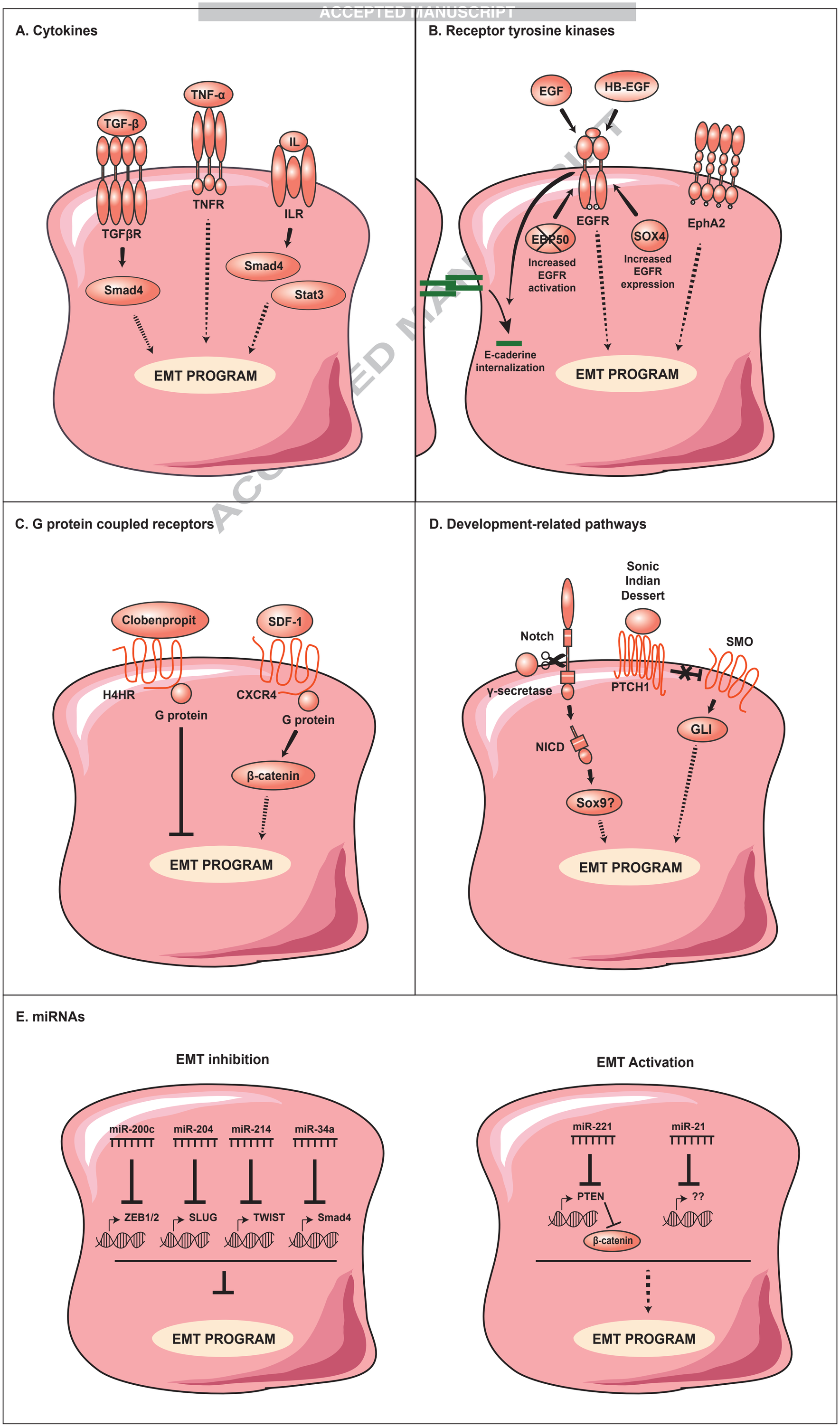


FIGURE 2

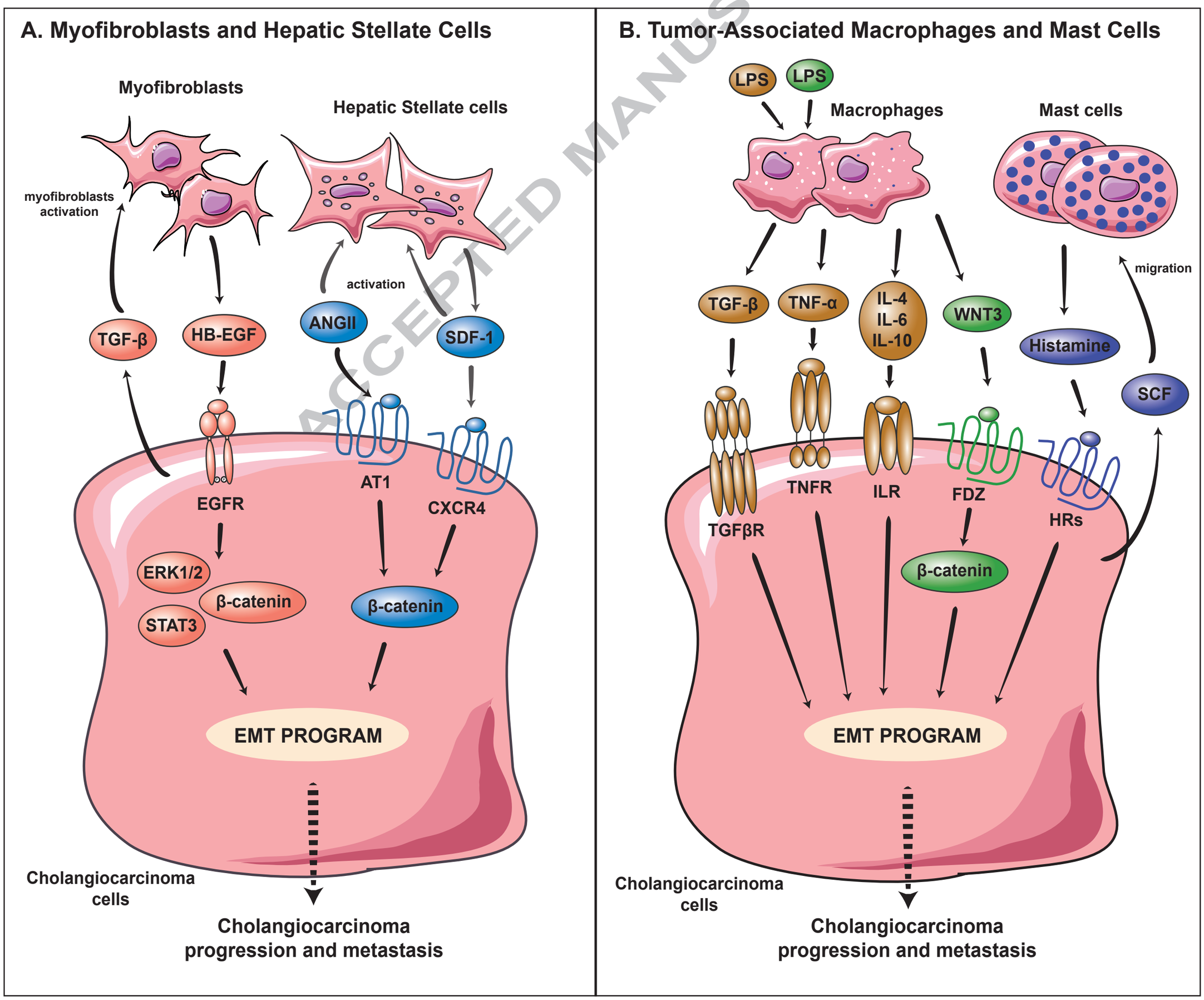

A. Myofibroblasts and Hepatic Stellate Cells
Myofibroblasts
Hepatic Stellate cells
myofibroblasts activation
TGF-β
HB-EGF
ANGII
SDF-1
activation
EGFR
AT1
CXCR4
ERK1/2
STAT3
β-catenin
β-catenin
EMT PROGRAM
Cholangiocarcinoma cells
Cholangiocarcinoma progression and metastasis
B. Tumor-Associated Macrophages and Mast Cells
LPS
LPS
Macrophages
Mast cells
migration
TGF-β
TNF-α
IL-4
IL-6
IL-10
WNT3
Histamine
SCF
TGFβR
TNFR
ILR
FDZ
HRs
β-catenin
EMT PROGRAM
Cholangiocarcinoma cells
Cholangiocarcinoma progression and metastasis

FIGURE 3