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Review

The role of EpCAM in physiology and pathology of the epithelium

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Summary. Epithelial Cell Adhesion Molecule (EpCAM) has been discovered as one of the first tumor-specific antigens overexpressed in epithelial cancer. The present review focuses on the role of EpCAM in physiology and homeostasis of epithelia. Recent research pointed to a close interaction of EpCAM with other cell-cell contact molecules like E-cadherin and claudins and an intimate crosstalk with Wnt and TGF-beta signaling in the regulation of cell growth. Moreover, EpCAM has been shown to modulate trans-epithelial migration processes of white blood cells. Mutations of the EpCAM gene lead to disturbances of epithelial homeostasis and cellular differentiation from the stem cell compartment. In the intestinal tract EpCAM mutations contribute to congenital tufting enteropathy. Regarding tumorigenesis EpCAM can act as an oncogene still depending on additional driver mutations and epithelial phenotype of tumor cells. Tumor cells display increased EpCAM expression that often correlates with the loss of strict basolateral localization. Many tumors show enhanced regulated intramembrane proteolysis (RIP) of EpCAM and loose EpCAM expression under conditions of epithelial to mesenchymal transition. The resulting extracellular EpEX and intracellular EpICD fragments mediate proliferative signals to the cell. Resulting fragments can be validated either by sensitive enzyme-linked immune-sandwich assays (EpEX) or by immunohistochemistry (EpICD). The present review gives an overview on the detection of EpCAM fragments as predictive markers for disease progression and survival of cancer patients.

Key words: EpCAM, Wnt, TGF-β, EMT, EpEX, EpICD

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EpCAM in epithelial homeostasis

Epithelial Cell Adhesion Molecule (EpCAM, CD326) was described for the first time as cell surface cancer-associated antigen in 1979 (Herlyn et al., 1979; Koprowski et al., 1979; Baeuerle and Gires, 2007). EpCAM is a type-I single-span transmembrane glycoprotein consisting of an extracellular domain (EpEX) with an epidermal growth factor and thyroglobulin-like domain, followed by a trans-membrane domain and a short 26-amino acid intracellular domain, called EpICD. Elucidation of the 3D structure by crystallography revealed that EpEX domains form cis-dimers on the cell membrane and then intercellular trans tetramers, thereby mediating cell-cell contacts and inducing juxtracrine signaling (Pavsic et al., 2014). In addition, glycosylation of EpCAM, in particular at asparagine 198, seems to be very important for protein stability and trafficking to cell surface. Mutants of EpCAM at this site showed a decreased overall expression and half-life of the molecule (Munz et al., 2008).

In healthy tissues EpCAM is localized on the basolateral side of epithelial cells, in the intercellular spaces, where epithelial cells form tight junctions (Munz et al., 2008; Schmelzer and Reid, 2008). During

organogenesis EpCAM is highly expressed on a variety of human epithelia. Thus, EpCAM is present on most epithelia of the adult body, except squamous epithelium, and some specialized epithelia, like hepatocytes and keratinocytes (Moldenhauer et al., 1987). In skin tissue the expression of EpCAM is usually down-regulated as epithelial cells terminally differentiate (Klein et al., 1987). However, EpCAM expression levels frequently increase during inflammation and regeneration processes of epithelia (Trzpis et al., 2007, 2008; Gires, 2012; Dolle et al., 2015a).

Regarding the function of EpCAM in differentiation and maturation from progenitor cells it has been clearly shown that EpCAM has an important role in regulating formation of cell-cell contacts in tight junctions interacting with claudins (Lei et al., 2012) and E-cadherin (Litvinov et al., 1997; Martowicz et al., 2013). It has been shown that EpCAM is highly expressed in developing epithelium, including intestinal epithelium. At cell-cell junctions EpCAM colocalize with claudin-7. It has been suggested that EpCAM contributes to formation of intestinal barrier by recruiting claudins to cell-cell junctions, and intestinal epithelia of mice lacking EpCAM display epithelial barrier defects and die shortly after birth due to intestinal erosion (Lei et al., 2012). Moreover, loss of EpCAM in the developing skin of zebrafish leads to compromised plasticity and adhesiveness of epithelial cells (Slanchev et al., 2009).

On the other hand, in differentiated epithelial cells overexpression of EpCAM is able to down-regulate E-cadherin, and increasing expression of EpCAM in cadherin-positive cells leads to the gradual abrogation of adherens junctions (van der Gun et al., 2010). EpCAM rearranges the cytoskeleton of the cell by disrupting the link between α-catenin and actin filaments, thereby changing cell-cell adhesion from strong to weak, as a consequence leading to increased cell motility and migration (Winter et al., 2003, 2007; van der Gun et al., 2010).

EpCAM also has an important function to mediate migration of immune cells within epithelia. Next to epithelial cells EpCAM is strongly expressed on dendritic cells (Gaiser et al., 2012). Conditional deletion of EpCAM in Langerhans Cells (LC) of adult mice revealed inhibition of their migratory potential and suggested a role of EpCAM in promoting LC migration in epidermis by decreasing LC-keratinocyte adhesion. Loss of EpCAM in developing zebrafish skin resulted in a higher susceptibility for bacterial infections and stronger inflammatory responses due to compromised skin integrity (Slanchev et al., 2009). EpCAM overexpression in human mammary epithelial cells grown *in vivo* in chicken embryos resulted in enhanced formation of leukocyte clusters around xenografts in comparison to control-transfected cells (Martowicz et al., 2013).

Regulated Intramembrane proteolysis (RIP) of EpCAM after juxtacrine cell–cell contacts induces mitotic signaling by releasing the intracellular domain of EpCAM (EpICD) which acts as a transcription factor supporting *Wnt* signaling (Carpenter and Red, 2009; Maetzel et al., 2009). EpCAM is expressed in embryonic and somatic stem cells and is implicated in pluripotency (Gonzalez et al., 2009; Dolle et al., 2015b). In stem cells EpCAM is co-expressed with other pluripotency markers, such as *OCT4* and *SOX2*. The important role of EpCAM in stem cell biology was supported by experiments based on EpCAM knockdown by the use of siRNA in murine embryonic stem cells (Gonzalez et al., 2009; Huang et al., 2011). EpCAM downregulation resulted in reduced proliferation and diminished expression of stem cell markers. Also, induction of differentiation leads to downregulation of EpCAM together with decreased expression of *SOX2, c-MYC* and *OCT3/4*. Moreover, it has been suggested that EpCAM can act as a pro-survival factor counteracting terminal differentiation processes in normal mammary gland tissue. EpCAM overexpression in Human Mammary Epithelial cells could induce resistance to TGFβ1-mediated growth arrest and support longer proliferative capacity of the cells. Therefore EpCAM signaling contributes to renewal of epithelial cells by inhibiting TFG-β signaling (Martowicz et al., 2013).

EpCAM mutations and disease

Loss-of-function of EpCAM was studied in zebrafish mutants *in vivo*. Slanchev et al. found that during late development of the animals EpCAM is necessary to maintain epithelial integrity within the periderm of the skin, and loss of EpCAM leads to disrupted morphology of the underlying basal epidermis, as well as hyper-proliferation of skin cells (Slanchev et al., 2009). Moreover, EpCAM mutants display higher infection susceptibility and enhanced skin inflammation (Slanchev et al., 2009). EpCAM knockout mice displayed placental deficiencies (Nagao et al., 2009) and pups that are born failed to thrive and died soon after birth because of hemorrhagic diarrhea (Guerra et al., 2012). The intestinal tract of *EpCAM* knockout mice shows many tufts, villous atrophy and colon crypt hyperplasia (Guerra et al., 2012) similar to human congenital tufting enteropathy (CTE).

Moreover, EpCAM knock-out display a disturbed E-cadherin/β-catenin expression and subcellular localization (Guerra et al., 2012). Mutations in the human *EPCAM* gene on cytogenetic localization 2p21 (47,369,147 to 47,387,027) are strongly associated with congenital tufting enteropathy (CTE) (Sivagnanam et al., 2008; Sivagnanam et al., 2010). Several *EpCAM* homozygous or compound heterozygous mutations have been described in the literature, i.e. in the donor or acceptor splice sites of exon 4, in-frame exon skipping, nonsense mutations or insertions in exons 3, 5 and 6, leading to truncation of the protein in the extracellular domain (Sivagnanam et al., 2010). The prevalence of congenital tufting enteropathy has been estimated to be 1/50,000-100,000 per live births in Western Europe

(Goulet et al., 2007). Congenital tufting enteropathy is characterized by crypt hyperplasia and an increased number of mitotic figures in the crypts (Goulet et al., 2007). The altered villi are less able to absorb nutrients and fluids than normal tissue, which causes life-threatening diarrhea and poor growth (Reifen et al., 1994).

EpCAM in carcinogenesis

EpCAM is highly expressed on most carcinomas and therefore an attractive target for diagnostic and therapeutic intervention (Baeuerle and Gires, 2007; van der Gun et al., 2010). In comparison to normal epithelial tissue, where EpCAM primarily localizes to the basolateral membrane, carcinoma display an intense uniform membranous overexpression, frequently also associated with a cytoplasmic staining (Gastl et al., 2000; Gosens et al., 2007). Moreover, EpCAM is hyper-glycosylated in carcinoma tissue as compared with healthy autologous epithelia (Munz et al., 2008; Pauli et al., 2003). Hitherto, the "oncogenic" function of EpCAM is strongly dependent on tumor entity and differentiation status of the respective cancer cells (van

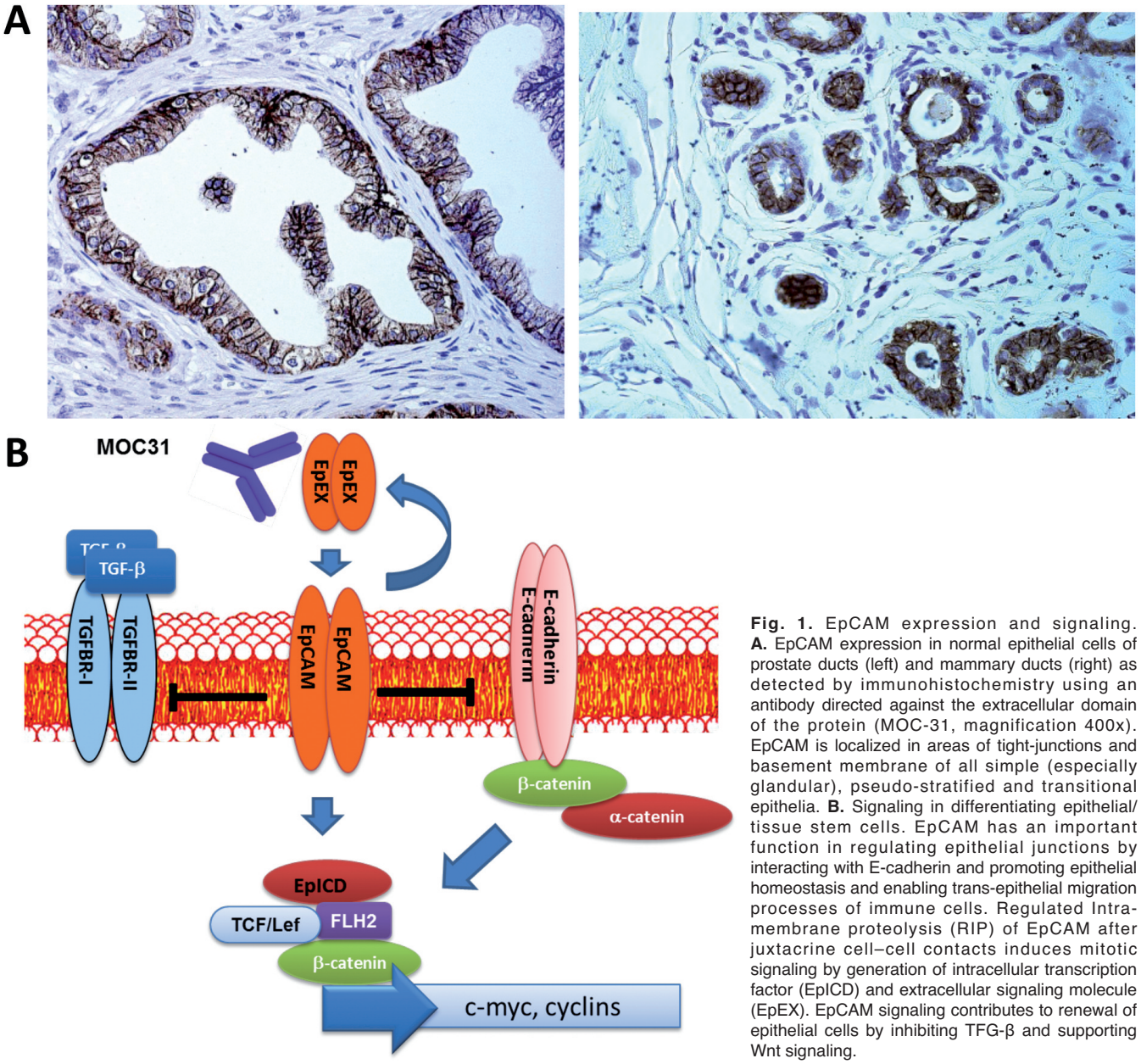

Fig. 1. EpCAM expression and signaling. **A.** EpCAM expression in normal epithelial cells of prostate ducts (left) and mammary ducts (right) as detected by immunohistochemistry using an antibody directed against the extracellular domain of the protein (MOC-31, magnification 400x). EpCAM is localized in areas of tight-junctions and basement membrane of all simple (especially glandular), pseudo-stratified and transitional epithelia. **B.** Signaling in differentiating epithelial/tissue stem cells. EpCAM has an important function in regulating epithelial junctions by interacting with E-cadherin and promoting epithelial homeostasis and enabling trans-epithelial migration processes of immune cells. Regulated Intra-membrane proteolysis (RIP) of EpCAM after juxtacrine cell–cell contacts induces mitotic signaling by generation of intracellular transcription factor (EpICD) and extracellular signaling molecule (EpEX). EpCAM signaling contributes to renewal of epithelial cells by inhibiting TFG-β and supporting Wnt signaling.

der Gun et al., 2010; Martowicz et al., 2012) and future research still has to clarify the function of EpCAM in processes such as invasion and metastasis of carcinoma cells.

Viral overexpression of EpCAM in human diploid epithelial cells displaying no mutations does not lead to immortalization and transformation of epithelial cells. These facts underlines that EpCAM is not a "classical" oncogene. But transfected epithelial cells show hyperplastic growth and resistance to inhibition by TGF-β when they grow *in vivo* (Martowicz et al., 2013). SV-40 large T- antigen transfected and immortalized human breast epithelial cells (MCF-10) additionally respond by increased *c-myc* expression (Martowicz et al., 2013). Advanced carcinoma cell lines displaying cancer driver mutations respond by an increased *Wnt* signaling and transcription of cell cycle progression genes, such as cyclin D1 (*CCND1*) and c-myc (*MYC*) (Maetzel et al., 2009; Chaves-Perez et al., 2013). Thus, knockdown of EpCAM in EpCAM^{high} breast carcinoma cell lines leads to reduced cell proliferation and less invasive growth *in vivo* (Martowicz et al., 2012). EpCAM is not so relevant in EpCAM^{low} breast carcinoma cells that already underwent epithelial to mesenchymal transition (EMT). Their growth is independent of EpCAM and EpCAM overexpression leads to inhibition of invasive growth into host tissue and recruitment of immune cells into the growth front of the tumor xenograft *in vivo* (Martowicz et al., 2012).

EpCAM overexpression supports EMT processes by down-regulating E-cadherin in E-cadherin^{high} carcinoma cells (Litvinov et al., 1997; Winter et al., 2007) and by

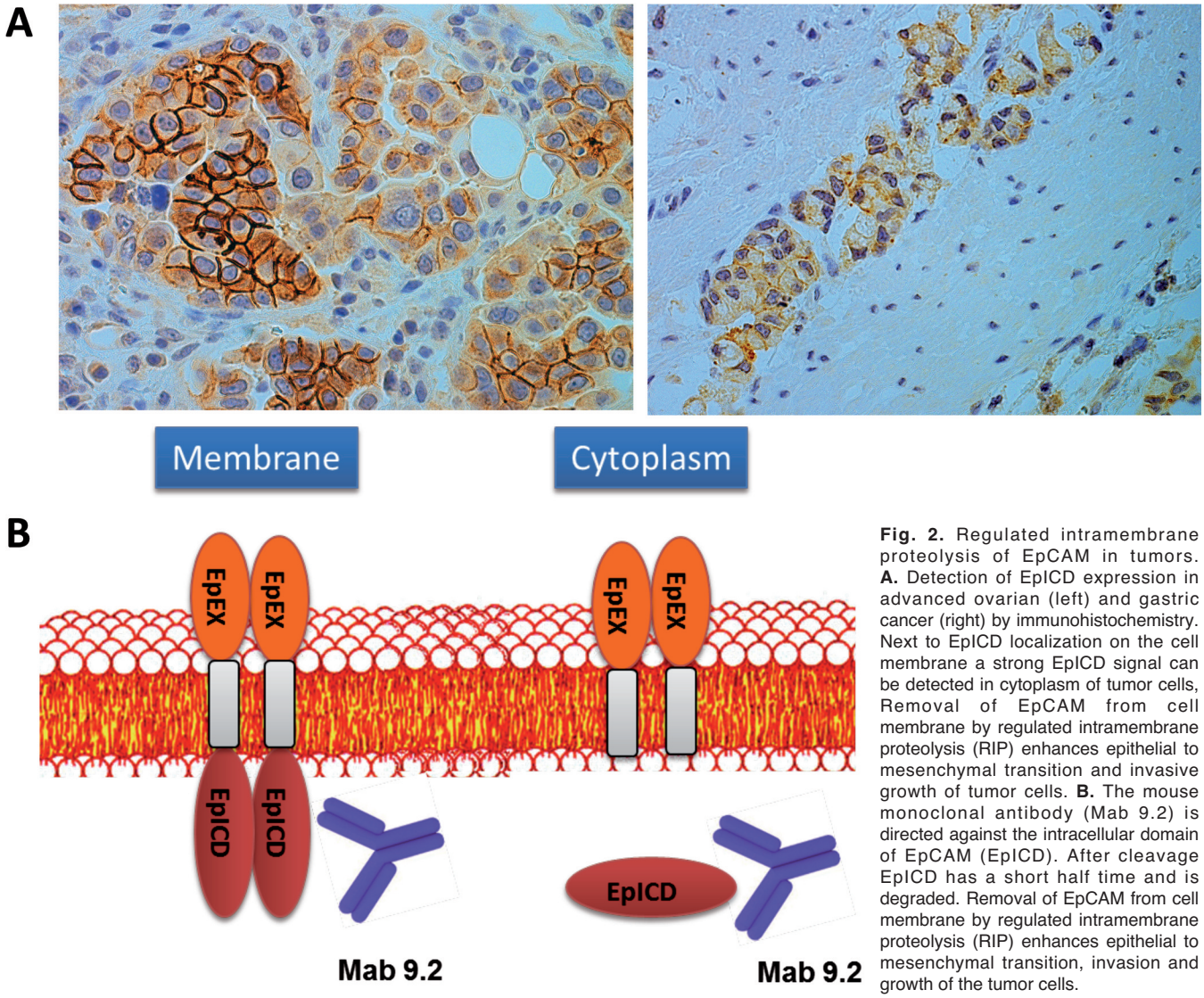

Fig. 2. Regulated intramembrane proteolysis of EpCAM in tumors. **A.** Detection of EpICD expression in advanced ovarian (left) and gastric cancer (right) by immunohistochemistry. Next to EpICD localization on the cell membrane a strong EpICD signal can be detected in cytoplasm of tumor cells, Removal of EpCAM from cell membrane by regulated intramembrane proteolysis (RIP) enhances epithelial to mesenchymal transition and invasive growth of tumor cells. **B.** The mouse monoclonal antibody (Mab 9.2) is directed against the intracellular domain of EpCAM (EpICD). After cleavage EpICD has a short half time and is degraded. Removal of EpCAM from cell membrane by regulated intramembrane proteolysis (RIP) enhances epithelial to mesenchymal transition, invasion and growth of the tumor cells.

diminishing claudin-7 mediated tight junctions, making cancer cells more prone to metastasis (Philip et al., 2015). EMT has been shown to be a requisite for metastasis by enhancing motility, invasion and dissemination in epithelial cancer cells (Kalluri, 2009) The reverse process, termed mesenchymal-to-epithelial transition (MET), then allows cancer cells to regain their epithelial features, including adhesion and proliferation (Ruscetti et al., 2015), thereby promoting the development of macro-metastases from single disseminated tumor cells. These regulatory mechanisms have also been shown for EpCAM in prostate cancer cells. Induction of resistance to chemotherapy was associated with EMT and loss of EpCAM, whereas treatment of chemo-resistant cells with miRNA 200 or 205 inhibited EMT, induced MET and restored EpCAM expression (Massoner et al., 2014).

A novel mechanism of EpCAM signaling by regulated membrane proteolysis (RIP) was recently described in cancer cells (Carpenter and Red, 2009; Maetzel et al., 2009). Signaling and proteolytical cleavage of EpCAM occurs by juxtacine cell-cell interactions (Denzel et al., 2009). In one step, the extracellular domain of EpCAM (EpEX) is cleaved off from the remaining molecule by the tumor necrosis factor alpha converting enzyme (*TACE, ADAM17*). It was found that the soluble EpEX provides a positive feedback loop and enhances RIP of EpCAM in a paracrine way (Denzel et al., 2009). In addition, the c-terminus of EpCAM- is cleaved by the γ-secretase complex, which contains presenilin-2 (*PS-2*), thereby generating an intracellular domain of EPCAM (EpICD) that can translocate to the nucleus and act as transcription factor. It associates with adaptor protein four and half LIM domain protein 2 (*FHL2*) in the cytosol. After the shuttle to the nucleus, EpICD associates with β-catenin and lymphoid enhancer binding factor 1 (*LEF-1*) and enhances transcription of Wnt signaling target genes, such as c-myc (*MYC*), cyclin-A (*CCNA*) and cyclin E (*CCNE*) (Munz et al., 2004).

RIP of EpCAM seems to be a bad prognostic marker for tumors and reflects their aggressive growth behavior in different epithelial tumor entities (Fong et al., 2014b). A detailed immunohistochemical analysis of full length EpCAM (EpEX epitope) and C-terminus (EpICD epitope) on serial sections of primary tumor samples revealed that loss of membranous EpICD expression was a frequent event and predicted a poor prognosis in patients with pancreatic cancer (Fong et al., 2014a). Similar results were obtained in colorectal carcinoma patients indicating that increased RIP and generation of EpICD promotes invasion and metastasis (Seeber et al., unpublished data).

Regarding the cleaved extracellular domain it has been demonstrated that soluble EpEX induces RIP and supports invasion of breast cancer cells (Sankpal et al., 2011). EpCAM containing exosomes released by tumor cells can also be found in biological fluids (Runz et al., 2007). Exosomes are able to bind to EpCAM expressing target cells to in auto/paracrine way to induce RIP and pro-survival signaling. Interestingly, EpCAM containing exosomes have been observed to increase in a variety of cancer patients but not in healthy individuals (Madhavan et al., 2015; Rupp et al., 2011). Measurement of cleaved (EpEX) or soluble EpCAM in exosomes was done in serum of cancer patients (Petsch et al., 2011; Gebauer et al., 2014; Tas et al., 2014). In comparison to healthy age-matched probands not all tumor entities displayed a clear positive result for EpCAM, i. e. patients with pancreatic cancer have elevated EpCAM levels in serum, but not lung or breast cancer patients (Petsch et al., 2011; Gebauer et al., 2014; Tas et al., 2014). A diagnostic and predictive measurement of EpCAM in serum is further hampered due to the fact that nearly 30% of probands with no cancer have equal concentrations like cancer patients in serum (Untergasser and coworker, unpublished data). The origin for soluble EpCAM in a subgroup of healthy persons is still unclear. Interestingly, EpCAM is more predictive for the measurement of urine in patients with bladder cancer (Bryan et al., 2014). Similar to EGFR EpCAM strongly correlates with bad prognosis and survival of patients (Bryan et al., 2015). Recently, our group found by a validated sandwich ELISA that soluble EpCAM is present in high amounts in ascites and correlates with positive cytology, i.e. the presence of tumor cells in the peritoneum (Seeber et al., 2015b).

Due to the high expression of EpCAM on tumor cells in EpCAM based immunotherapies have been developed (Sebastian, 2010). In 2000 the first bispecific and trifunctional anti-EpCAM mouse/rat hybrid antibody, also known as catumaxomab, was presented to treat patients (Zeidler et al., 2000). The two Fab-domains bind to EpCAM and to the CD3 T cell receptor and the Fc-region activates accessory cells such as NK cells. A multicenter phase I/II trial investigated the tolerability and effectiveness of catumaxomab in patients with ovarian cancer with the result that catumaxomab eliminates ovarian cancer cells in the peritoneum with an acceptable safety profile (Burges et al., 2007). Although only a subgroup of patients benefited from treatment, catumaxomab obtained approval from the European Medical Agency in 2009 for intraperitoneal application in patients with malignant ascites. Recently, our group could demonstrate that soluble EpCAM inhibits catumaxomab efficacy *in vitro* and *in vivo*. When soluble EpCAM was detected in ascites, patients treated with catumaxomab had a shorter survival compared to those where no EpCAM was found (Seeber et al., 2015a,b). Thus, high levels of soluble EpCAM/EpEX in the microenvironment of the tumor and EpICD cleavage on the cell membrane of tumor cells are a clear sign of tumor progression in epithelial-like cancers, leading to a more invasive phenotype, enhanced epithelial-to mesenchymal transition and formation of metastasis.

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