Biochimica et Biophysica Acta 1828 (2013) 1989–2001

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

EpCAM: Structure and function in health and disease

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ARTICLE INFO

Article history:
Received 20 December 2012
Received in revised form 12 April 2013
Accepted 16 April 2013
Available online 23 April 2013

Keywords:
Epithelial Cell Adhesion Molecule (EpCAM)
Cancer
Cell–cell contact
Congenital tufting enteropathy
Regulated intramembrane proteolysis
Tetraspanin-enriched microdomains

ABSTRACT

Injection of tumor cells in mice more than 30 years ago resulted in the discovery of an epithelial antigen, later defined as a cell adhesion molecule (EpCAM). Although EpCAM has since evoked significant interest as a target in cancer therapy, mechanistic insights on the functions of this glycoprotein have been emerging only very recently. This may have been caused by the multitude of functions attributed to the glycoprotein, its localization at different subcellular sites and complex posttranslational modifications. Here, we review how EpCAM modifies cell–cell contact adhesion strength and tissue plasticity, and how it regulates cell proliferation and differentiation. Major knowledge derived from human diseases will be highlighted: Mutant EpCAM that is absent from the cell surface leads to fatal intestinal abnormalities (congenital tufting enteropathy). EpCAM-mediated cell proliferation in cancer may result from signaling (i) via regulated intramembrane proteolysis and/or (ii) the localization and association with binding partners in specialized membrane microdomains. New insight in EpCAM signaling will help to develop optimized cancer therapies and open new avenues in the field of regenerative medicine.

Contents

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URL: http://www.cellbiology.nl (B.N.G. Giepmans).

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http://dx.doi.org/10.1016/j.bbamem.2013.04.018

1. General introduction

In multicellular organisms, epithelium lining all external and internal surfaces provides a major barrier that prevents unrestricted exchange between the organism and the external world. The two main types of epithelium are (i) simple epithelium and (ii) stratified epithelium. Simple epithelium consists of a tightly packed and organized monolayer of polarized cells and can generally be found in organs in which absorption, secretion, or filtration occurs, such as in the intestine, the exocrine pancreas and the kidney. Stratified epithelium consists of multiple cell layers, and e.g. can be found in the skin, the cornea and the oral cavity. Characteristic features for simple epithelia are microvilli or cilia on the apical domain, high abundance of cell-cell junctions on the lateral domains, and cell attachment to basal membranes via focal adhesions and hemidesmosomes (Fig. 1). Dysfunction of epithelia due to cellular or molecular abnormalities can give rise to a number of diseases, including cancer.

The epithelial barrier function is primarily maintained by tight junctions (TJ; *Zonula occludens*; Fig. 1), which form an impermeable, or regulated semi-permeable, seal between the apical and basolateral domains of the cells. Cell adhesion is a critical regulator of cell polarity which in turn affects cellular functions as diverse as cell migration, proliferation and differentiation when epithelial cells adhere to neighboring cells, so-called contact inhibition [1]. Contact inhibition is crucial in organogenesis and wound healing [2,3]. In cancer, loss of contact inhibition may result in uncontrolled cell movement and proliferation, allowing cells to invade neighboring tissue and to metastasize [4]. Cell adhesion is mediated by transmembrane cell adhesion molecules (CAMs), some of which are subdivided into four families: cadherins, selectins, integrins, and immunoglobulin (Ig)-like CAMs (Fig. 1). Additionally, there are several CAMs that do not belong to any of these families [5]. In general, CAMs can promote cell–cell adhesion or cell–matrix adhesion by homophilic or heterophilic interaction (Fig. 1). One cell adhesion protein that does not fit into any of the "classical" families of adhesion molecules, which was discovered as one of the first cancer markers, is the epithelial cell adhesion molecule (EpCAM; CD326). In the 1970s, functional screens were performed for novel tumor-specific cell surface antigens [6,7], in which monoclonal antibodies were generated by immunization of mice with cancer cells and production of antibody-secreting hybridomas. Using the newly generated antibodies, EpCAM was identified as a tumor antigen [7]. EpCAM is a cell surface glycoprotein of approximately 40 kDa (detailed below) that is highly expressed in epithelial cancers and at lower levels in normal simple epithelia. Due to its simultaneous discovery by many research groups, EpCAM received a variety of names (Table 1). Several of these names are still in use, which complicates the linking of studies to the properties of EpCAM [8]. Since 2007, consensus has been reached to use EpCAM as the primary name [8].

Due to its frequent overexpression in tumors, EpCAM has been of significant interest for the diagnosis and therapy of various epithelial cancers, and several EpCAM-directed antibody- or vaccine-based clinical trials for a wide variety of carcinomas have been conducted [8,9]. Apart from being targeted as a molecular biomarker, EpCAM plays a morphoregulatory role in normal epithelia and stem/progenitor cells, as well as it may actively drive tumor progression in cancer cells. High expression of EpCAM is often associated with decreased patient survival [10–15]. Recently, mutations in the EpCAM gene have been discovered to be responsible for a disease that affects the intestinal epithelium, leading to intractable diarrhea in infants [16]. While the name EpCAM originates from studies demonstrating that the protein can promote homophilic adhesion [17], forced expression of EpCAM has also been shown to interfere with E-Cadherin-mediated cell adhesion, suggesting a role as a functional antagonist for classic cadherin-mediated cell–cell interactions [18]. In addition, EpCAM has been implicated to be involved

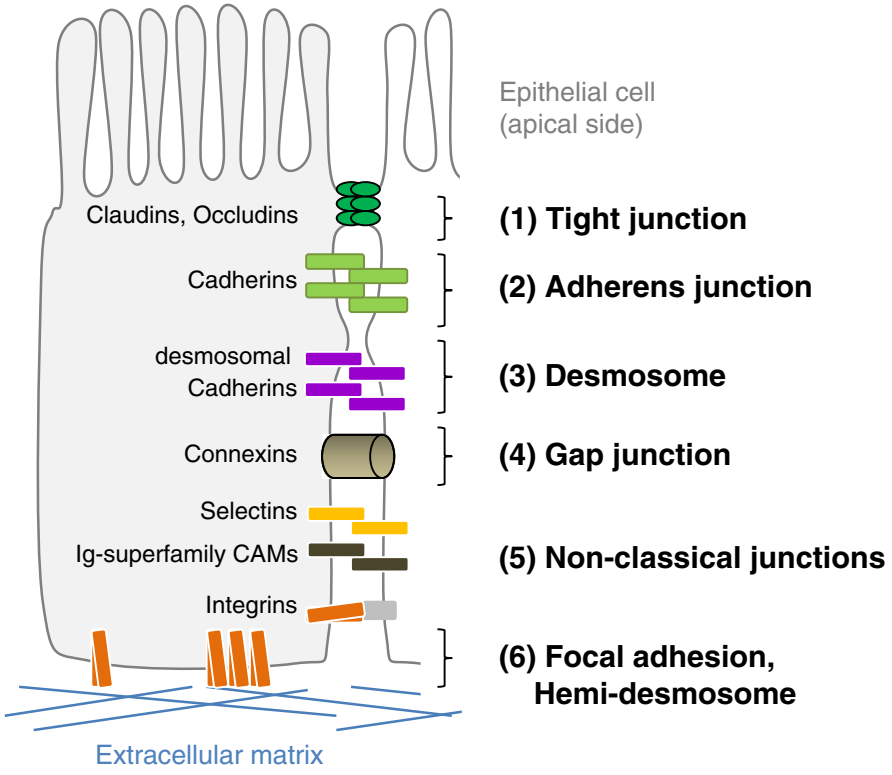

Fig. 1. Cell adhesion molecules (CAMs) and junctional complexes are abundant in epithelial tissues. (1) Tight junctions build a seal between adjacent cells and are connected to actin filaments. (2) Adherens junctions are plaques of classical cadherins linked to the actin cytoskeleton. (3) Desmosomes are formed by desmosomal cadherins, linked to intermediate filaments. (4) Gap junctions connect the cytoplasm of two adjacent cells and are linked to microfilaments. (5) Selectins, Ig-superfamily CAMs, but also other CAMs not belonging to the classical families can promote homophilic adhesion outside of junctions. Integrins bind in a heterophilic manner. (6) Focal adhesions (linked to actin) and hemi-desmosomes (linked to intermediate filaments) are cell–matrix junctions that are formed by integrins.

Table 1
EpCAM's various names based on monoclonal antibodies or cDNA clones. *murine EpCAM.

Abbreviation	Name	Ref.
(CO)17-1A	n.a., mAb from immunization with colon adenocarcinoma cells	[7]
TROP-1	Trophoblast cell-surface antigen	[21]
MH99	n.a. (mAb)	[22]
KS1/4	Carcinoma-associated glycoprotein	[23]
323/A3	n.a. (mAb)	[24]
HEA125	Human epithelium antigen	[25]
EGP34	Epithelial glycoprotein of M_r 34,000	[25]
ESA	Epithelial surface antigen	[26]
KSA	Adenocarcinoma-associated antigen	[27]
AUA1	n.a. (mAb)	[28]
gp 38	Glycoprotein of 38 kDa	[29]
Ber-EP4	n.a. (mAb)	[30]
MOC31	n.a. (mAb)	[31]
GA733-2	n.a., mAb from immunization with gastric adenocarcinoma cells	[32]
FU-MK-1	n.a. (mAb)	[33]
311-1K1	n.a. (from cDNA clone)	[34]
EGP-2	Epithelial glycoprotein-2	[35]
EGP40	Epithelial glycoprotein of Mr 40,000	[36]
Ep-CAM	Epithelial cell adhesion molecule	[17]
TACSTD-1	Tumor-associated calcium signal inducer 1	[37]
MK-1	n.a. (derived from mAb FU-MK-1)	[38]
CD326	Cluster of differentiation 326	[39]
G8.8*	n.a. (mAb recognizing murine EpCAM)	[40]

in a diversity of processes including cell proliferation, migration, adhesion, differentiation, and cell signaling [5,19]. Proliferation induced by EpCAM-overexpression has been shown to correlate with upregulation of cell cycle-related proteins [20]. Although several models of EpCAM signaling have been suggested, the molecular basis of EpCAM-mediated signaling in healthy tissue, cancer, and in conditions such as congenital tufting enteropathy is not yet fully understood. In this review, we provide a general overview of the current knowledge on EpCAM with a focus on functional and structural aspects of this molecule in health and disease.

2. EpCAM: gene, protein, and structure

Human EpCAM is a polypeptide of 314 amino acids (aa), consisting of a large extracellular domain (N-terminal) of 242 aa, a single-spanning transmembrane domain of 23 aa and a short cytoplasmic domain of 26 aa (C-terminal) [27] (Figs. 2 and 3). The gene encoding for human EpCAM is located on chromosome 2 (location 2p21) and has an estimated size of 14 kb [19,41,42]. Comparison of the genomic and cDNA sequences has shown that the EpCAM-encoding gene (*epcam*) consists of 9 coding exons: EpCAM's extracellular domain, including the signal peptide, is mainly encoded by exons 1 to 6, the transmembrane region by exon 7. The intracellular domain is encoded by exons 8 and 9 (last 13 amino acids) [19,42] (Fig. 2). The EpCAM gene is conserved in many species, including, mouse, rat and zebrafish. Moreover, the EpCAM protein seems to be highly conserved among higher vertebrates, showing up to 81% amino acid sequence homology between man and mouse, and up to 99% between man and gorilla.

2.1. The extracellular domain of human EpCAM

EpCAM´s extracellular domain starts with the signal peptide, which is cleaved off predominantly between alanine 23 and glutamine 24 [27,41,43] (Fig. 3). N-terminal sequencing revealed a minor alternative cleavage of the signal peptide after residue 21 (1%) [43].

Different models of the tertiary structure of EpCAM´s ectodomain have been developed, defining three motifs. Balzar and colleages have suggested that EpCAM´s extracellular domain contains a tandem of epidermal growth factor(EGF)-like repeats (amino acids 27–59 and 66–135), which closely resemble the fourth and fifth EGF-like motif in the rod domain of nidogen, a basement membrane glycoprotein involved in cell–matrix adhesion [44]. However, determination of the exact disulphide bond assignments of the protein by Chong and Speicher has revealed that the first motif in EpCAM's ectodomain shows a novel pattern of disulphide linkage that does not resemble an EGF-like domain [43]. The second motif does not represent an EGF-like repeat, but it rather resembles a thyroglobulin (TY) type 1A repeat, as proposed in earlier studies [41–43,45] (Fig. 3). Thyroglobulin type 1 domains are conserved in a number of proteins and capable of binding and thereby inhibiting certain cathepsins (cysteine proteases) which are involved in cancer progression [46,47]. Whether EpCAM plays a role as a substrate or inhibitor of cathepsins is not known. Following the TY-repeat, there is a third motif that is cysteine-free and unrelated to any known molecule.

EpCAM contains three N-glycosylation sites [26,48]. In insect cells, Asn198 is not glycosylated (Cysteine-poor region), whereas Asn111 is completely and Asn74 partially glycosylated [43] (TY-repeat, Fig. 3). However, in human and murine cell lines, point mutations of the potential N-glycosylation sites have shown that all three sites are glycosylated [49]. Glycosylation of Asn198 seems to be of high importance for EpCAM´s cell surface expression and protein stability [49]. Mutation of the Asn198 glycosylation site led to decreased expression of EpCAM. Furthermore, elimination of all three glycosylation sites reduced the half-life time of the protein from 21 to 7 h when compared to wt EpCAM. Since a number of cell surface molecules such as Notch, E-Cadherin, integrins, and CD44 are differentially glycosylated in carcinoma versus normal epithelia [50–53], differential glycosylation of EpCAM might be an important factor causing differences in the function of EpCAM in healthy versus malignant tissue. In head and neck carcinoma, EpCAM has been shown to be hyperglycosylated in comparison to healthy tissue [54].

2.2. The intracellular domain

Following the transmembrane domain, which is involved in the association with tight junction protein Claudin7 [55] (Fig. 3; see Section 3.3), EpCAM´s short cytoplasmic domain consists of 26 aa of which 14 are charged. Using EpCAM mutants with deletions in the

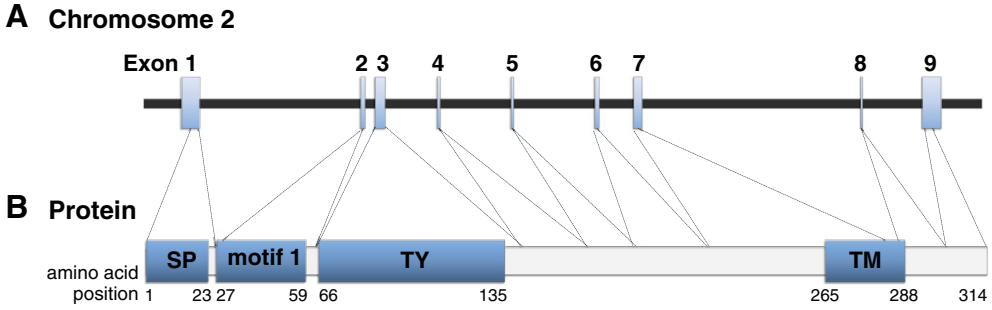

Fig. 2. Structure of the EpCAM encoding gene (A) and protein (B). The EpCAM encoding gene (*GA733-2*, *epcam*) consists of 9 exons located on chromosome 2. The protein is encoded as indicated. SP = signal peptide; TY = thyroglobulin-like domain; TM = transmembrane domain.

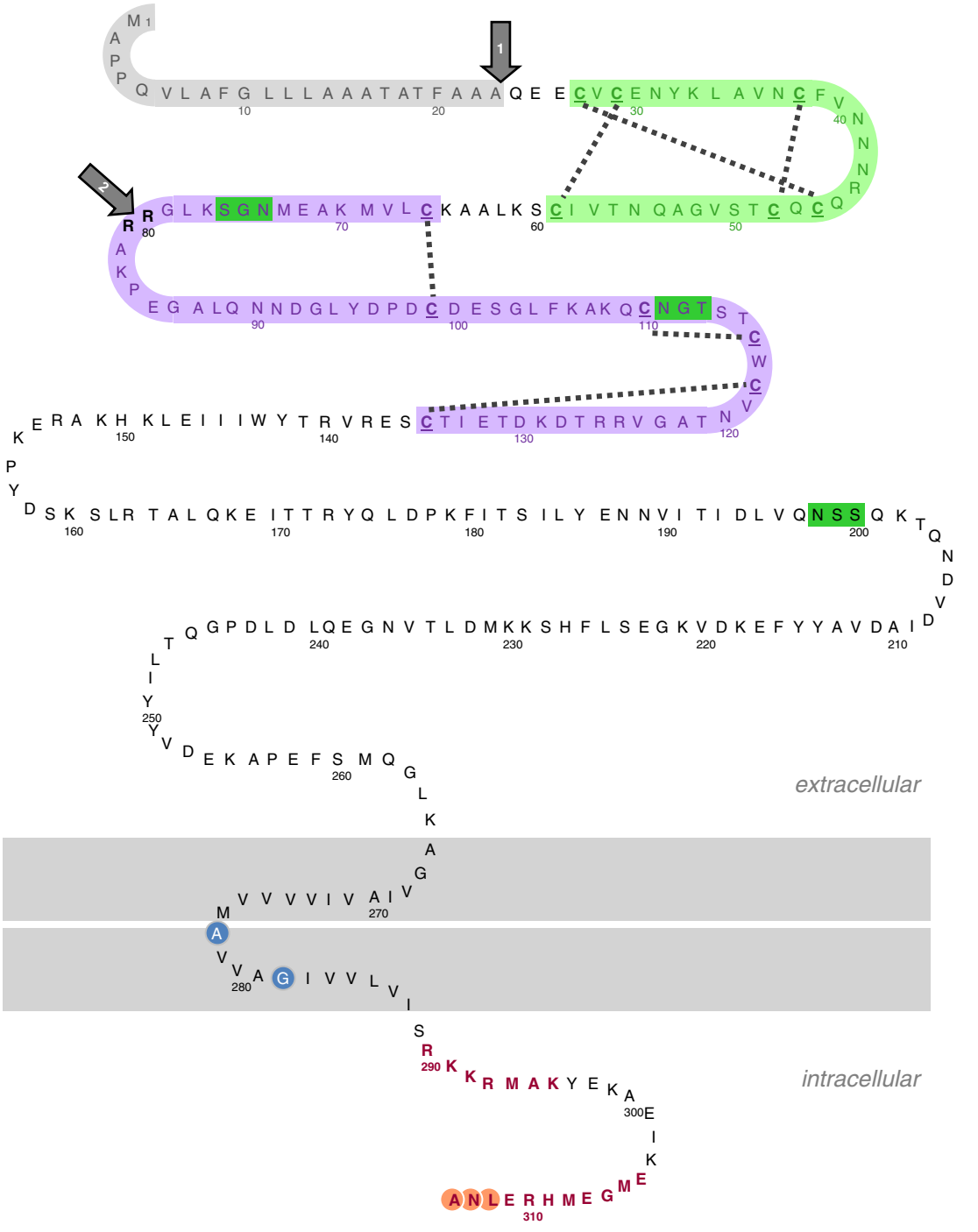

Fig. 3. Amino acid sequence of EpCAM. Reproduced and adapted from Schnell et al. [58].

intracellular domain, Balzar and colleagues [56] determined two potential α-actinin binding sites at positions 289 to 296 and 304 to 314 (Fig. 3), which are reportedly important for EpCAM´s localization at cell-cell contacts and therefore for the cell adhesion properties of the molecule [56] (see Section 3.1). At the C-terminus, amino acids Leu312, Asn313 and Ala314 display a putative PDZ binding site (Fig. 3). In most other cell-cell contact proteins, the hydrophobic C-terminal aa can interact with multi-PDZ domain proteins that are key in complex formation with signaling or structural proteins. Whether this is the case for EpCAM is currently not known.

2.3. EpCAM cleavage

Proteolytic cleavage of EpCAM at multiple sites has been implicated in its function. Like many other transmembrane proteins, EpCAM contains a signal peptide that is cleaved off by a signal peptidase (see before; Fig. 3, arrow 1). Furthermore, EpCAM can be cleaved between Arg80 and Arg81, located in the second motif (TY-repeat) of the ectodomain [27,42,49] (Fig. 3, arrow 2). The cleaved peptide has a molecular weight of 6 kDa and remains attached to the 32 kDa part via the disulphide bond between Cys6 and Cys7 (Fig. 4). Notably, the majority of mAbs that recognize EpCAM on cancer cells bind specifically to this 6 kDa part, underlining its high immunogenicity [48,57]. A number of proteases, including serine proteases trypsin and chymotrypsin, have been shown to be able to conduct this N-terminal cleavage of EpCAM [48], which occurs in a variety of epithelial cancer cell lines originating e.g. from colon, ovarian, and breast cancer [26]. Whether or not and to which extent N-terminal cleavage occurs depends on the presence and activity of the relevant proteases [26]. Although EpCAM's limited N-terminal proteolytic cleavage was discovered more than three decades ago and suggested to be of functional importance [48], the effects of this post-translational modification on EpCAM's structure and function are still unknown.

While the two cleavage sites described above are the only truly identified, EpCAM might be cleaved by additional proteases which will be discussed below. A comprehensive overview about the various proteolytic cleavage events in EpCAM is provided in Schnell et al. [58].

2.4. EpCAM's homologue TROP-2

The only molecule known to be homologous to EpCAM is the *GA733-1* gene product TROP-2 (*Trophoblast cell-surface antigen-2*), showing approximately 49% sequence identity and 67% similarity taking into account conserved substitutions [19,41]. The most prominent regions of homology are the transmembrane region, and a region in the extracellular domain that is homologous to the type 1 repeat of thyroglobulin. Like EpCAM, TROP-2 contains twelve cysteine residues. Remarkably, the positions of these cysteines as well as the overall distribution of hydrophilic and hydrophobic residues are conserved in both proteins [41]. Instead of three, TROP-2 (36 kDa) contains four N-glycosylation sites, resulting in a total molecular weight of 50 kDa. Two of these glycosylation sites are conserved in EpCAM [41]. Whereas the conserved tyrosine residue present in both proteins has never been reported to be phosphorylated, TROP-2 has been found phosphorylated within the cytoplasmic domain at Ser303, which is not conserved in EpCAM [59,60]. While phoshorylation of TROP-2 by protein kinase C may be essential for tumor growth promoting signaling of the protein [60], it does not seem to play a role in EpCAM-mediated signaling.

Like EpCAM, the TROP-2 protein is mainly expressed in epithelial tissue. However, expression levels do not seem to correlate: TROP-2 can be low in tissues with relatively high EpCAM expression, such as colon and lung tissue, whereas expression levels can be relatively high in EpCAM-negative epithelium [19]. Nevertheless, high levels of TROP-2 expression in a variety of late-stage epithelial carcinomas (pancreatic, colorectal, gastric and squamous cell-carcinoma of the oral cavity) are associated with decreased patient survival, as well as increased tumor aggressiveness and metastasis [60].

3. EpCAM function

3.1. EpCAM and adhesion?

While EpCAM was discovered in a tumor-marker assay, its function remained elusive. Sequence homology to nidogen, a basement membrane protein mediating cell-matrix adhesion, suggested that EpCAM might be involved in cell–matrix or cell–cell adhesion [42]. The name EpCAM -or epithelial cell adhesion molecule—was introduced by Litvinov and colleagues [17], who showed that EpCAM can mediate Ca^{2+}-independent homophilic cell–cell adhesion in cells that normally lack cell–cell interactions [17]. Expression of EpCAM in EpCAM-negative cells led to aggregation of cells and the formation of cell–cell contacts. In a mix of EpCAM-positive and EpCAM-negative L-cells, cell aggregates mainly consisted of EpCAM-positive cells (90%), pointing to homophilic adhesion. Using a range of EpCAM deletion mutants, Balzar et al. have demonstrated that all the motifs in EpCAM's extracellular domain are required for the formation of homophilic intermolecular binding and EpCAM accumulation at cell-cell adhesion sites [57]. However, also the short intracellular domain, which might connect EpCAM with the actin cytoskeleton via

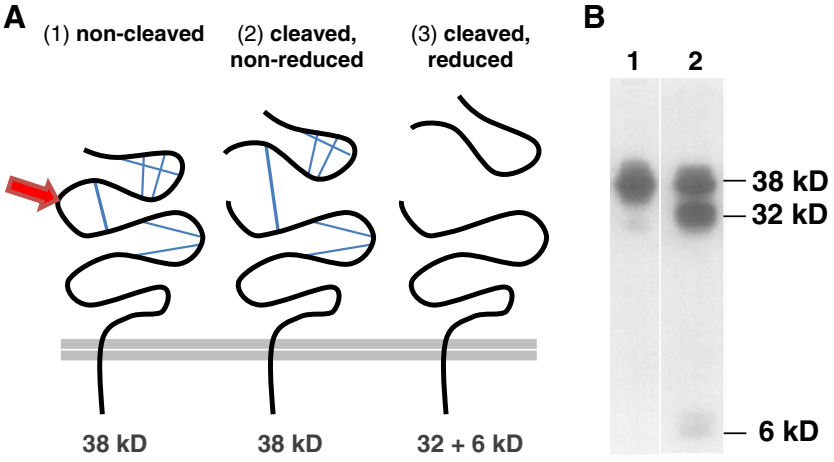

Fig. 4. N-terminal cleavage of EpCAM. (A) Schematic representation of EpCAM in its non-cleaved (1) and cleaved state (2). After reduction of disulfide bonds (blue lines), the cleaved part is no longer connected (3). Red arrow: Arg80/81 cleavage site. (B) Lysates of colon cancer cell line SW480 were immunoprecipitated with antibody MH99, recognizing EpCAM motif 1. [^{35}S]Methionine-labeled immunoprecipitates were analyzed under non-reducing (1) and reducing conditions (2) on 15% acrylamide gels (reproduced and adapted from Thampoe et al. [26]; Schnell et al. [58]).

α-actinin (see Section 2.2), is essential for EpCAM localization at cell-cell contacts and therefore for EpCAM's ability to mediate adhesion [56]. While it was concluded that EpCAM itself is an adhesion molecule, an alternative explanation of the effects in these expression studies is that EpCAM modulates classical cell–cell adhesion, which might be at the protein level, or by regulation of transcription (see below).

While being able to mediate homophilic adhesion when introduced in cells that lack their own means of cell-cell interaction, EpCAM is neither structurally related to any of the four major families of CAMs [18] nor associated with any classical junctional structure [61]. In human colon epithelium as well as in epithelial cell lines, EpCAM could not be detected at tight junctions, desmosomes or cell–matrix adhesions, whereas it partly co-localized with E-Cadherin at the lateral membrane [61,62].

When compared to E-Cadherin, EpCAM is a relatively weak cell–cell adhesion molecule [17,18]. Unlike E-Cadherin expressing cells, which are tightly connected due to adherens junctions, EpCAM expressing cells are only loosely interconnected [18]. Remarkably, when co-expressed in E-Cadherin expressing cells, EpCAM weakens E-cadherin-mediated intercellular adhesion [18]. This weakening is not due to lower E-Cadherin levels or lack of its cell-surface localization, but results from reduced stability of E-Cadherin-mediated adhesion due to disturbance of E-Cadherin association with the cytoskeleton via α-actinin [18]. Other typical epithelial features, such as contact inhibition and polarization, are less strong in EpCAM-expressing cells when compared to E-Cadherin-expressing cells [17]. Conditional knock-out of murine EpCAM in dendritic Langerhans cells, which also express E-Cadherin, attenuates the migration and motility of these cells due to increased adhesiveness [63]. These findings and the cancer cell invasion and metastasis promoting role of EpCAM in tumors suggest that EpCAM acts as a negative regulator of adhesion [63].

Being part of adherens junctions, E-Cadherin is fundamental for the maintenance of the normal architecture of epithelial tissues [64]. Loss of E-Cadherin expression can result in loss of cellular polarity and contact inhibition, unregulated growth and invasion of tumor cells in adjacent tissues [65–70]. In most epithelia, E-Cadherin and EpCAM are co-expressed during development and in post-natal life. However, an increased or *de novo* expression of EpCAM is often associated with tumoral transformation which can progress to malignancies and metaplastic behavior [17,18,71], whereas E-Cadherin exhibits tumor-suppressing and growth-inhibitory functions [72]. By weakening Cadherin-mediated cell–cell adhesion, EpCAM might foster higher cell plasticity within epithelial tissues, which in turn may help promote cell proliferation and motility both during morphogenesis, and during the development and progression of malignancies. In addition to affecting E-Cadherin mediated adhesion, EpCAM has recently been shown to be involved in the regulation of the epithelial integrity by affecting the composition and function of tight junctions via interaction with claudins [73,62].

In conclusion, although EpCAM is able to promote homophilic cell–cell interactions, its functional antagonism on E-Cadherin-mediated adhesions suggests that it operates as a modulator of the strength of cell adhesion rather than as a promoter of epithelial cell aggregation and junctional complex formation [63]. In this regard, compared to Cadherin-mediated adhesions, EpCAM´s anti-adhesive effect might well be tightly regulated and coordinated during morphogenesis and tissue regeneration [18] but disrupted during neoplasm development.

3.2. EpCAM and proliferation

Besides EpCAM's increased/*de novo* expression in metaplasia and neoplasia, its abundant expression on fast proliferating tumors points

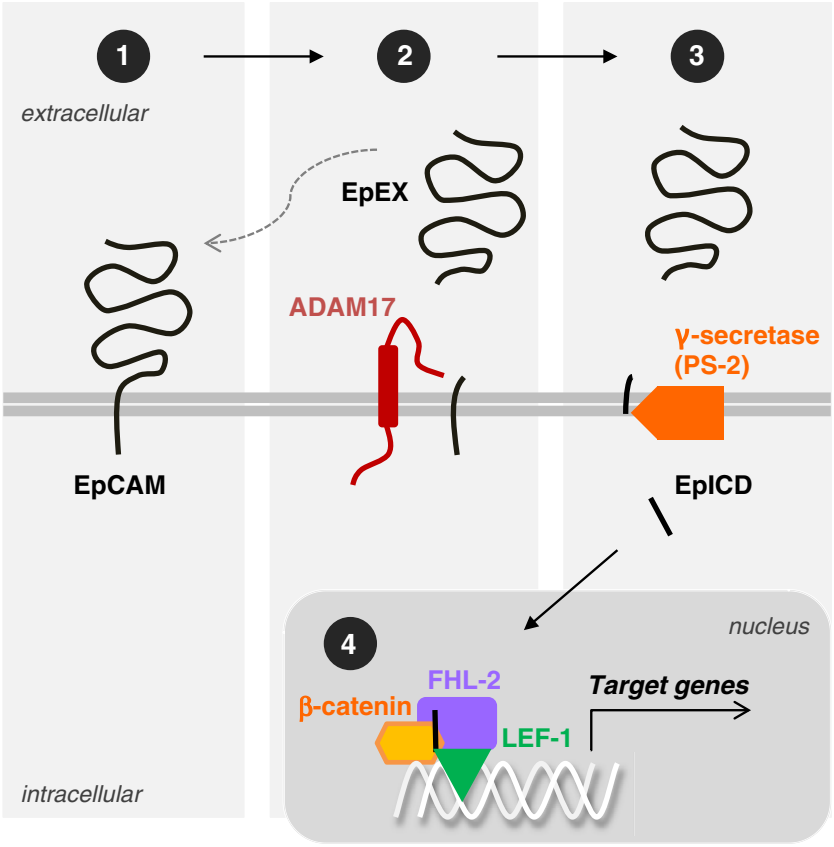

Fig. 5. EpCAM as a substrate for regulated intramembrane proteolysis (RIP). Full-length EpCAM (1) is cleaved by ADAM17, releasing EpCAM's ectodomain (EpEX) (2). EpEX might act as a homophilic ligand for non-cleaved EpCAM, inducing RIP (grey dotted arrow). Following the first cleavage step, EpCAM´s cytoplasmic tail (EpICD) is released due to cleavage by PS-2 (3). EpICD associates with FHL-2 and β-catenin and translocates to the nucleus (4), inducing transcription of EpCAM target genes via LEF-1 consensus sites. Adapted from Maetzel et al. [74]. See main text for details and abbreviations.

to a causal relationship and the involvement of EpCAM in growth control. Several *in vitro* and *in vivo* studies have shown that overexpression of EpCAM, or parts of it (see below), induces cell proliferation, whereas downregulation of EpCAM decreases cell proliferation [20,74–77]. Induction of EpCAM in some *in vitro* models results in rapid upregulation of the oncogenic transcription factor c-Myc, and consequently in upregulation of cell cycle-related proteins Cyclin A and E, as well as epidermal fatty acid binding protein [20,78]. Using EpCAM mutants, Münz and colleagues have shown that EpCAM´s intracellular domain is not only necessary but also sufficient to induce c-Myc upregulation [20]. While EpCAM-mediated induction of Cyclin A and E might be a secondary effect following upregulation of c-Myc, EpCAM has been shown to have a direct effect on Cyclin D1 at the transcriptional level [77]. A current model of EpCAM´s signaling mechanism suggests that EpCAM is subject to regulated intramembrane proteolysis (RIP; Fig. 5), and that the cleaved intracellular domain is responsible for the induction of EpCAM´s target genes [74,77] (see below; Fig. 5).

3.3. Models of EpCAM-mediated signaling

3.3.1. Regulated intramembrane proteolysis

The mechanism of EpCAM-induced proliferation in cancer cells has been shown to involve regulated intracellular membrane proteolysis (RIP) [74]. RIP describes an evolutionarily conserved mechanism combining regulated e.g. ligand-induced ectodomain shedding with the consecutive release of an intracellular domain (ICD) from transmembrane proteins [79] (Fig. 5). Both the shed ectodomain and/or the ICD may activate signaling events. Matrix metalloproteases (MMPs) and the disintegrin-type metalloproteases (ADAMs, *´A disintegrin and metalloprotease´*) are often involved in protein ectodomain shedding, whereas, in a second step, γ-secretases including presenilin-1 and 2 (PS-1, PS-2) as active subunits are generating the soluble ICD. Besides proteins such as Notch and amyloid precursor protein (APP), a number of CAMs have been shown to undergo proteolytic cleavage, including E-Cadherin, CD44 and L1-CAM [1,80–83]. While non-cleaved E-Cadherin, e.g., acts as a tumor suppressor, different proteolytic fragments of E-Cadherin have been shown to promote tumor progression [84].

The proteases implicated in RIP of EpCAM are metalloprotease ADAM17 (also known as TACE: *tumor necrosis factor-α-converting enzyme*) and a γ-secretase containing PS-2 [74]. The initial cleavage results in the release of a soluble fragment, called EpEX, which may act as a homophilic ligand for non-cleaved EpCAM. The cleaved intracellular peptide EpICD initiates signaling by association with β-catenin and four-and-a-half LIM domains protein 2 (FHL2). This complex may translocate to the nucleus, where it binds LEF-1 and DNA, resembling the canonical Wnt signaling pathway (Fig. 5). LEF-1 is known to be a major regulator of *c-myc* and Cyclin E, which represent wnt target genes and also EpCAM´s target genes [74]. Notably, since EpCAM itself has been shown to be induced by Wnt signaling [85], EpCAM cleavage may trigger a positive feedback loop on its expression [74]. Theoretically, EpCAM´s ability to weaken E-Cadherin mediated adhesion (discussed above) might lead to an increase in non-membrane bound β-catenin [18,86], which may be stabilized and protected from degradation due to association with EpICD and FHL-2 (Fig. 6). The fact that expression and regulation of ADAM17 and presenilins, as well as FHL2, differ between normal versus malignant tissue might explain EpCAM´s distinct functional properties in health and disease [74]. Recently, several additional cleavage sites in EpCAM´s ectodomain have been identified, suggesting that EpCAM signaling is regulated via different proteolytic pathways, possibly involved in controlling EpCAM's multiple functions [58]. Although EpCAM is detectable at low levels in sera of cancer patients [38,87,88], detection methods used did not distinguish between full-length EpCAM and its cleaved (partial) ectodomain. However, conclusions should be drawn with caution since EpCAM is also present on exosomes, microvesicles abundantly secreted by tumor cells [89].

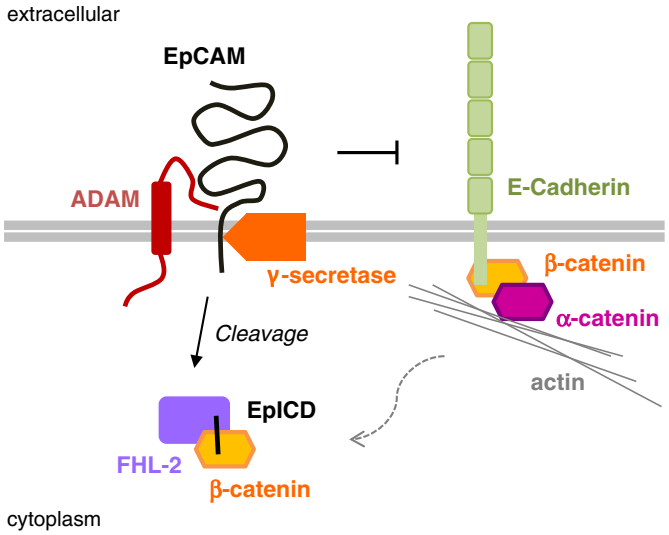

Fig. 6. EpCAM signaling in cross-talk with E-Cadherin. EpCAM may weaken E-Cadherin-mediated adhesion by interrupting the link between E-Cadherin and the actin cytoskeleton. This might in turn result in increased availability of non-bound β-catenin that may be stabilized by association with EpICD. Adapted from Maetzel et al. [74].

3.3.2. Tetraspanin-enriched microdomains

Proteolytic processing associated with EpCAM-mediated proliferation via RIP requires cell-to-cell contacts which represent the initial trigger events [90]. However, not only EpCAM's presence and homophilic interaction at the cell surface, but also localization in specific subdomains of the plasma membrane might be highly important for signaling. A number of cell signaling events are facilitated by specialized cell-cell contacts [2] (Fig. 1), and by other membrane microdomains such as lipid rafts, or so-called tetraspanin-enriched microdomains (TEMs), that can serve as signaling platforms. TEMs are organized macromolecular complexes formed by tetraspanins, integral transmembrane proteins that can interact with other tetraspanins, certain lipids, and a variety of transmembrane and cytosolic proteins [91–93]. Although lipid rafts and TEMs exhibit some similarities, they can be distinguished by their distinct protein composition [91]. EpCAM has been identified as a new molecule in TEMs, forming a primary complex with tetraspanin CD9 [94].

In metastasizing rat carcinoma cell lines, EpCAM has been found in a complex with tetraspanins CD9 and CO-029 (Tetraspanin8), as well as CD44 variant isoforms (CD44v4-7) (Fig. 7) [95], which are involved in tumor progression [96]. In contrast to CD9, which has a tumor-/metastasis-suppressing role, high levels of CO-029 correlate with metastasis and poor prognosis [92,94,97–99]. Another protein in the EpCAM-CD44v-tetraspanin complex has been identified as the tight junction protein Claudin7, which directly binds to EpCAM via an AxxxG motif within the transmembrane domain [55,100,101] (Fig. 3). Claudin7 recruits EpCAM into TEMs where it associates with CO-029 and CD44v6 [101] (Fig. 7). The direct association with Claudin7 seems to be crucial for EpCAM-specific functions: only in complex with Claudin7, EpCAM has been found to promote cell proliferation, apoptosis resistance, and tumorigenicity [55].

Interestingly, metalloproteases have also been shown to contribute to the tetraspanin web; ADAM10 for example associates with CD9 and other tetraspanins [94,102]. ADAM10 and ADAM17, which has been shown to be involved in EpCAM cleavage [74], are key players in ectodomain shedding or RIP [103–105]. Tetraspanins might play a role as regulators of ADAM-induced ectodomain shedding [102,106]: Antibodies against tetraspanins, including CD9, specifically induced ADAM10- but not ADAM17- dependent cleavage of TNF-α and EGF in certain cell lines [102]. Another study revealed that CD9 negatively regulates ADAM17 activity [107]. Furthermore, Tetraspanin12 has

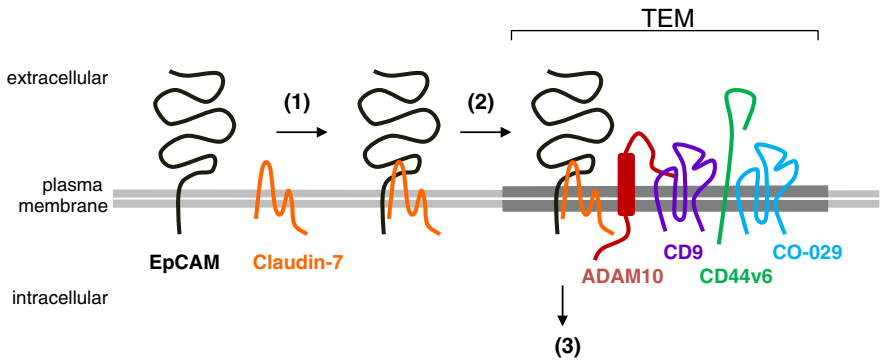

Fig. 7. EpCAM in tetraspanin-enriched microdomains (TEMs). EpCAM associates with Claudin-7 (1) and is recruited to TEMs (2), where it builds a complex with tetraspanins CD9 and CO-029, and CD44v6. ADAM10, also present in TEMs, might be involved in activating EpCAM signaling as presented in Fig. 6 (3).

been shown to promote ADAM10-mediated cleavage of APP and the maturation of ADAM10 in some cell lines [106]. Among the ADAM proteases, ADAM17 and ADAM10 are the most closely related [108] and often share substrates [103–105]. However, while a major fraction of ADAM10 is localized in TEMs, ADAM17 seems to be sequestered to lipid rafts [109] and has, except for one study [107], not been found to associate with tetraspanins [102,106]. The diverse localization of these two metalloproteases as well as their substrates might be an important factor in the regulation of ADAM-activity and substrate specificity. Notably, tetraspanins are not only involved in the regulation of ADAM activity, but were also shown to affect MMPs and to associate with γ-secretases (reviewed in Yáñez-Mó et al. [110]), implicating that TEMs might have an important role in the regulation of RIP processes. Since EpCAM associates with tetraspanins CD9 and CO-029 in TEMs, ADAM10 seems to be a likely candidate for the ectodomain cleavage of EpCAM (Fig. 7). Further investigation is needed to elucidate whether tetraspanins play a role in the regulation of EpCAM signaling.

4. EpCAM in health & disease

4.1. EpCAM in normal tissue and during development

4.1.1. EpCAM expression

In healthy adult tissue, EpCAM is expressed at the basolateral cell membrane of simple, pseudo-stratified, and transitional epithelia. No expression can be detected in the differentiated cells of normal squamous stratified epithelia. In adults, EpCAM is expressed in most organs and glands, with the highest expression in colon. Generally, the level of expression differs between tissues. Typically, in tissues where EpCAM is present it is high in proliferating cells, and low in differentiated cells. EpCAM is neither found in cells of lymphoid origin and bone-marrow-derived cells, nor in mesenchymal, muscular, and neuroendocrine tissues [25,111] (see Balzar et al. [19] and Went et al. [112]). In mice, the homologue of human EpCAM has been found in thymic epithelial cells, but also in thymocytes, T-cells and antigen-presenting dendritic cells [113,114]. In a number of epithelial tissues, active proliferation corresponds to increased EpCAM levels, whereas differentiation is associated with downregulation of the protein (reviewed in Balzar et al. [19]). In the intestinal epithelium for example, a gradient of EpCAM can be observed from crypts to villi [19]. This pattern corresponds to high EpCAM levels in the intestinal stem cells, located in the crypts [115], and decreasing levels when cells are differentiating, at the top of the villi. Besides in the gut, EpCAM can be found in stem/progenitor cells of other organs: e.g., hepatocytes are EpCAM-positive during embryonic liver development, whereas in adults, EpCAM is only expressed during liver regeneration processes in cells that morphologically resemble precursor stem cells [116]. EpCAM´s higher expression in fetal epithelia in comparison to adult mature tissues [23] points to a dynamic regulation of its expression during morphogenesis [117]. In the human fetal pancreas, the highest levels of EpCAM can be found in developing islets budding from pancreatic ducts. In the adult pancreas, levels are low in differentiated islet clusters but high in duct cells, a compartment that has been proposed to comprise progenitor cell populations for both the exocrine and the endocrine cell lineages [117].

4.1.2. Stem cell marker

EpCAM´s role in the switch between proliferation and differentiation is further sustained by the fact that EpCAM has been identified as a surface marker for pluripotent human embryonic stem cells (hESCs) [118,119] and murine embryonic stem cells (mESCs). In undifferentiated hESCs, EpCAM is co-expressed with pluripotency markers, including Octamer 4 (OCT4) and Sex-determining region Y-Box 2 (SOX2) [119,120]. Knock-down of EpCAM results in decreased cell proliferation and causes significant upregulation of endoderm- and mesoderm-associated genes. However, decreasing EpCAM levels by silencing did not significantly alter the expression of pluripotent markers. In mESCs, EpCAM expression has been found to be higher under self-renewal conditions than during differentiation [121]. Induction of differentiation caused downregulation of EpCAM along with decreased expression of c-Myc and pluripotent markers SOX2, Oct3/4, and Stat3. Downregulation of EpCAM induced similar effects [121]. Hence, EpCAM is strongly associated with the maintenance of the undifferentiated state of ESCs upstream of these pluripotency regulators. The natural mechanism of regulation of EpCAM levels in differentiating stem cells might be via epigenetic regulation of the *epcam* gene, not by direct methylation, but by activity of histones [120]. Although this regulation is indirect, modifying EpCAM levels in mouse ESCs has shown that EpCAM expression may be causally related to maintaining stem cells [122]: it may initiate the downstream markers mentioned above, and therefore will be a valuable target for future avenues to generate human induced pluripotent stem (iPS) cells.

4.1.3. Knock-down animal models

Due to the lack of genetic loss-of-function studies, the *in vivo* function of EpCAM remains poorly understood. Only recently, more and more EpCAM knock-out animal models are emerging. Loss of EpCAM in the developing zebrafish led to compromised epithelial plasticity and adhesiveness, with hyper-proliferation as a secondary consequence, possibly due to the loss of contact inhibition [123]. Compromised skin integrity also resulted in a higher susceptibility for bacterial infections and enhanced inflammation [123]. Homozygous deficient EpCAM$^{-/-}$ mice died *in utero*, revealing prominent placental abnormalities. EpCAM $^{-/+}$ mice were viable and did not show abnormalities [124]. Conditional knock-out of murine EpCAM in epidermal Langerhans cells, which represent skin-resident dendritic cells, resulted in a decrease in Langerhans cell motility and migration, and in compromised regulation of the skin inflammatory response [63]. Recently, a gene-trapped EpCAM knock-out mouse has

been obtained that is viable until postnatal day 4 [125], exhibiting the same histopathological features as seen in patients suffering from CTE (Section 4.3). Furthermore, two viable EpCAM knock-out mice have been developed showing intestinal defects [73] in concordance with what has been found in CTE and has been reported by Guerra et al. While cell surface EpCAM seems to be crucial for normal development [126], the molecular mechanism that lead to the intestinal abnormalities, and whether lower levels of tight junction proteins in mouse models for CTE are consequence or causative of the malformations in the absence of EpCAM remains to be identified.

4.2. EpCAM in cancer

4.2.1. Prognostic marker

EpCAM levels are increased in most epithelium-derived tumors. High expression levels of EpCAM usually correlate with poor prognosis, e.g. in breast cancer and ovarian cancer as well as in pancreatic, urothelial, and gallbladder carcinoma [10–15]. Exceptional cases are renal and thyroid carcinoma, where high levels of EpCAM have been shown to correlate with increased survival [127,128]. In several cancer types EpCAM seems to play a dual role, either promoting or reducing cancer progression [129]. Overviews of EpCAM regarding tumor prognosis are given in van der Gun et al. [129] and Patriarca et al. [130]. EpCAM's prognostic value has also been assessed in combination with E-Cadherin and tight junction protein Claudin7, which are both functionally related to EpCAM (Section 3.1). However, co-expression analysis did not improve EpCAM's prognostic value for the presence of nodal metastases in oral and oropharyngeal squamous cell carcinoma [131].

Since EpCAM might be subject to regulated proteolytic cleavage (Section 3.3), data regarding EpCAM expression based on immunohistochemistry should be interpreted with caution. Studies using an antibody against EpCAM's intracellular domain suggested that nuclear/cytoplasmic EpCAM staining correlates with the aggressiveness of thyroid cancer and overall patient survival, and that this nuclear and cytoplasmic staining can be specifically found in epithelial cancers in contrast to normal tissue [128,132,133]. Although we had similar staining results in oral and oropharyngeal squamous cell carcinoma material, we could not exclude artifacts due to non-specific binding of the primary antibody. Immunostaining artefacts that arise from routine procedures can easily be overlooked if the proper controls are lacking [134].

4.2.2. Cancer therapy

In addition to being of prognostic value, EpCAM is an attractive target for tumor diagnosis and therapy because of its tumor-specific overexpression. EpCAM has been used as a target for many immunotherapeutic approaches, including treatment with monoclonal antibodies [9] and the development cancer targeting antibodies [135]. Furthermore, vaccination strategies [136], an EpCAM-specific antibody fragment fused to TRAIL (*Tumor necrosis factor-related apoptosis-inducing ligand*) [137,138], and toxin-conjugated antibody fragments [130,139,140] have been developed. Since 2009, the anti-EpCAM trispecific antibody catumaxomab (Removab) has been authorized for treatment of malignant ascites in cancer patients; other EpCAM-directed antibodies and antibody-based constructs are currently in clinical development [8,9].

4.2.3. Lynch syndrome

In addition to its direct involvement in tumor biology (Section 3), EpCAM plays an indirect role in Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer; HNPCC), an autosomal dominant disorder that predisposes to colorectal adenocarcinoma (60–90%), endometrial carcinoma (20–60%), and various other cancers [141–143]. Tumors observed in Lynch syndrome families are diagnosed at an unusual early age and can be multiple. Lynch syndrome is caused by mutations in mismatch repair genes, including *MLH1* and *MHL2* (*mutL homolog 1* and *mutS homolog 2*). These mutations prevent the repair of DNA mistakes occurring during cell divisions that may eventually lead to the development of cancer. Mutations in *MLH1* and *MSH2* account for about 80% of Lynch syndrome cases [144].

Recently, heterozygous germline deletions in the EpCAM gene (*epcam*), located upstream of *MSH2*, have been shown to result in hypermethylation of the *MSH2* promoter region and subsequent loss of MSH2 expression [143]. Deletions of the last exons of *epcam* lead to extended transcription into the downstream gene, resulting in an *epcam-MSH2* fusion transcript and methylation induction of the *MSH2* promoter [143]. Nineteen different *epcam* deletions, all including the last two exons, have been identified in 45 Lynch syndrome families [141]. *Epcam* deletions account for approximately 20% of Lynch syndrome cases showing loss of mismatch protein expression but lacking the corresponding gene mutations [144]. In total, deletions in *epcam* inactivate *MSH2* in about 1% of individuals with Lynch syndrome. Due to the tissue-dependent levels of EpCAM expression, carriers of an *epcam* deletion have a high risk of colorectal carcinoma, whereas extra-colonic cancers are rarely found and the risk for endometrial cancer is reduced [142,145]. In the latter case, the cancer risk seems to correlate with the size and location of the deletion in the EpCAM gene. Thus, genetic screening for EpCAM might allow optimization of cancer surveillance programs for Lynch syndrome families [142].

Histological studies showed a lack of EpCAM expression in a number of tumors from patients with *epcam* germline deletions. In those cases, EpCAM was only absent in case of homozygous *epcam* deletion, resulting from the combination of a germline and a second somatic deletion [146,147]. Absence of EpCAM in these tumors seems to be in disagreement with the model of EpCAM being important in tumor development and progression. This raises the question of whether EpCAM is overexpressed as a result of tumoral transformation, rather than playing an active role as a proto-oncogene. In case of Lynch syndrome, the absence of mismatch repair proteins might be the dominant cause for the development of tumors, outrunning possible beneficial effects due to absence of EpCAM. Notably, only homozygous mutations in *epcam* result in loss of EpCAM expression combined with morphological abnormalities, as has been shown in knock-out mice (Section 4.1) and CTE (see below). Thus, while EpCAM is often associated with cancer progression, it might be secondary to tumor formation in some tumors. Absence of EpCAM might therefore not prevent tumor formation.

4.3. Mutations of EpCAM cause congenital tufting enteropathy

Apart from EpCAM´s indirect role in Lynch syndrome, homozygous mutations in the EpCAM gene have been identified in patients suffering from congenital tufting enteropathy (CTE) [16]. CTE is a rare autosomal recessive form of intractable diarrhea of infancy. Typically, patients develop chronic diarrhea within the first days after birth and show impaired growth. CTE is characterized by subtotal villous atrophy with crypt hyperplasia without evidence of inflammation. In the typical form, abnormalities are localized mainly in the intestinal epithelium and include disorganization of surface enterocytes with focal crowding, resembling tufts [148]. Most patients are dependent on parenteral nutrition to allow normal growth and development. The disease persists throughout life and imparts significant morbidity and mortality [16]. CTE was first described in 1994 [149] with an annual incidence of ~1/100,000 live births in Western Europe. As a result of its severity and rarity, the pathogenesis of CTE remains poorly understood.

In 2008, the genetic basis of CTE was uncovered: using single nucleotide polymorphism (SNP) genotyping in CTE patient material, Sivagnanam and colleagues [16,150] identified several *epcam* mutations, resulting in a single amino acid exchange, truncation or partial deletion of the EpCAM protein. Another case study revealed a single basepair insertion, leading to a frame shift and premature truncation of EpCAM [151]. Further studies revealed novel mutations resulting in premature stop codons and thereby truncation of the protein [152,153].

Generating all EpCAM mutants identified so far, we recently discovered that all the mutations in EpCAM result in absence of the protein from the plasma membrane [126]. This is due to either secretion or degradation of the truncated protein, or to retention of the aberrant protein in the endoplasmic reticulum. Considering EpCAM´s morphoregulatory role and its high expression in the intestinal crypts where the stem cells are located, absence of EpCAM might disturb normal intestinal development. Sivagnanam et al. speculate that EpCAM is involved in the development of the crypt villus axis, where epithelial cells originate from stem cells in the crypt and migrate to the tip of the villus [16]. EpCAM´s absence from the cell surface may not only preclude its adhesive function, but might also prevent EpCAM-mediated signaling, involving proteolytic cleavage and association with tetraspanins and other transmembrane proteins (Section 3.3). Recently, viable EpCAM knock-out mice have been generated that develop neonatal intestinal abnormalities [73,125]. In these models, loss of EpCAM has been shown to affect cell–cell junctions by influencing the expression and localization of either adherens junction proteins E-Cadherin and beta-catenin or tight junction-associated claudins, thereby influencing the epithelial integrity and barrier function. Since the knock-out mouse generated by Guerra and colleagues provides the typical histopathological features of CTE [125], it represents a promising model for further molecular studies and the development of CTE therapies.

Some cases of CTE have been reported as being associated with chronic arthritis [151] or malformations, including eye-associated abnormalities [154]. In a recent study, Roche and colleagues found that 10 out of 15 patients diagnosed with CTE exhibited ophthalmic functional disorders such as superficial punctate keratitis (SPK) and conjunctival erosive lesions, revealing that CTE might be a multifocal clinical disease [155]. Beside the characteristic CTE features in the intestinal epithelium, also conjunctival tissues showed disorganization of the surface epithelium with focal crowding, resembling tufts [155].

As a diagnostic feature for CTE, disorganization of surface enterocytes with tuft-like crowding has been considered unique until recently. However, since the genes for several congenital diarrheal disorders have been identified and tufts have been found unreliable as a distinctive feature for CTE, genetic testing is crucial for a correct diagnosis and optimal treatment [156].

5. Conclusions, remarks & future perspectives

Although EpCAM has been discovered as a tumor marker more than three decades ago and is since exploited as a tool for tumor diagnosis/prognosis and as a target for cancer therapy, it is still not fully understood how EpCAM is functionally involved in tumor biology. Only in recent years, models of EpCAM-mediated signaling on the molecular level emerged. The discovery of the causal correlation between EpCAM mutations and CTE highlights the importance of EpCAM in normal development. From these studies we learned that EpCAM needs to be present at the cell surface for proper function.

However, the signaling underlying balancing cell proliferation and differentiation remains pleiotropic. Post-translational modification of EpCAM such as glycosylation and proteolytic cleavage at various sites, as well as association with binding partners and localization in TEMs might be crucial for activation and regulation of EpCAM signaling and dictate the functional outcome.

The differences in EpCAM function in malignant versus healthy tissues might be explained by differential expression of the various proteins interacting with EpCAM. Although EpCAM-induced proliferation is normally triggered by increased, or *de novo*, expression in cancer as well as in developing or regenerating tissues, it remains to be determined if these events are regulated at the level of gene transcription, possibly involving the Wnt pathway [85]. While proteolytic cleavage of EpCAM may induce genes via Wnt, EpCAM itself may trigger a positive feedback loop on its expression [74]. Furthermore, ablation or downregulation of tumor suppressor protein p53 is associated with an increase in EpCAM expression [157], which might explain EpCAM´s frequent overexpression in tumors.

EpCAM's signaling mechanism and tissue-specificity need to be further clarified to utilize the new knowledge for therapeutic approaches in cancer and CTE. An important ingredient will be the three-dimensional structure of EpCAM. Progress toward the resolution of EpCAM's structural organization [158] may lead to an understanding of access to cleavage sites and mechanisms of EpCAM cleavage, as well as homophilic interactions. In addition, molecular modeling may aid in the design and evolution of small molecules to agonize and or antagonize EpCAM's function in patients.

We anticipate that further progress in uncovering EpCAM´s function in normal developing tissues and in the pathological conditions such as cancer and CTE will guide the use of this protein as a marker and/or a target to significantly improve the diagnosis, therapy and prognosis. Ultimately, studies focusing on the function of EpCAM will also open new avenues of research for tissue engineering and stem cell treatments.

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