# Co-ordination of epithelial junctional systems, and its disturbance during the development of a malignant phenotype

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# CO-ORDINATION OF epithelial junctional SYSTEMS, AND ITS disturbance DURING THE DEVELOPMENT OF A MALIGNANT phenotype

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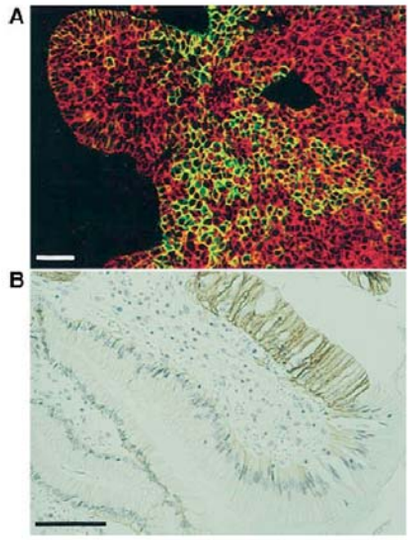


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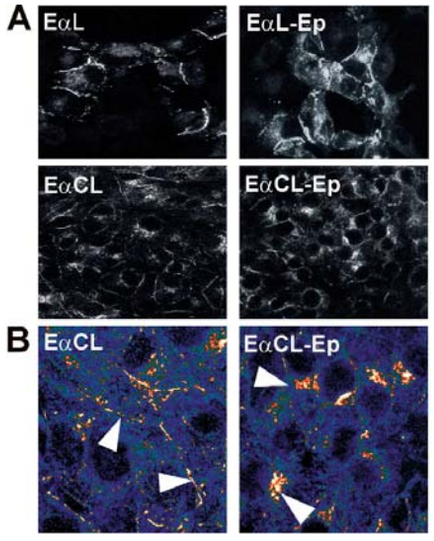


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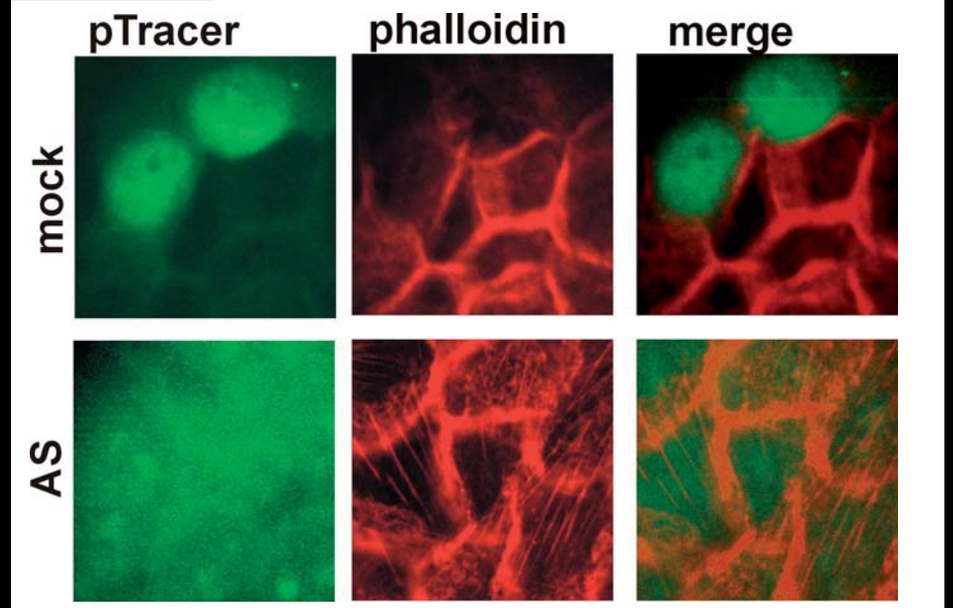


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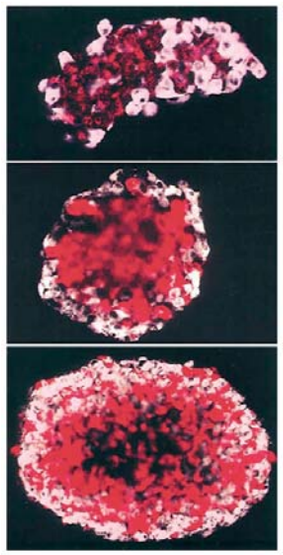

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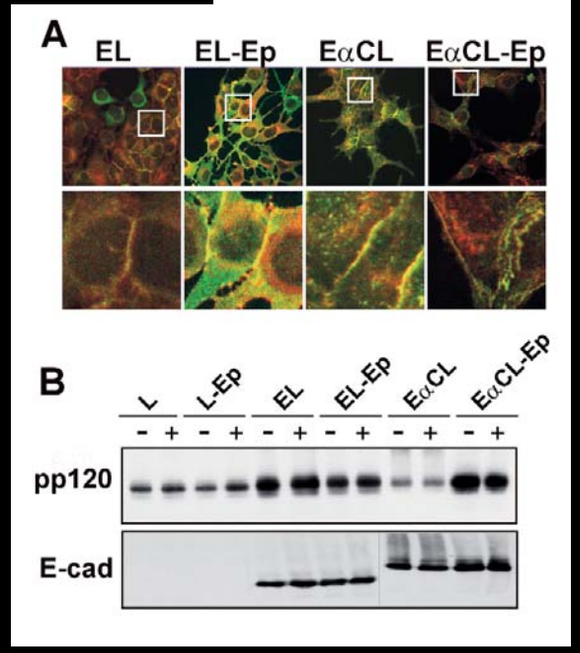


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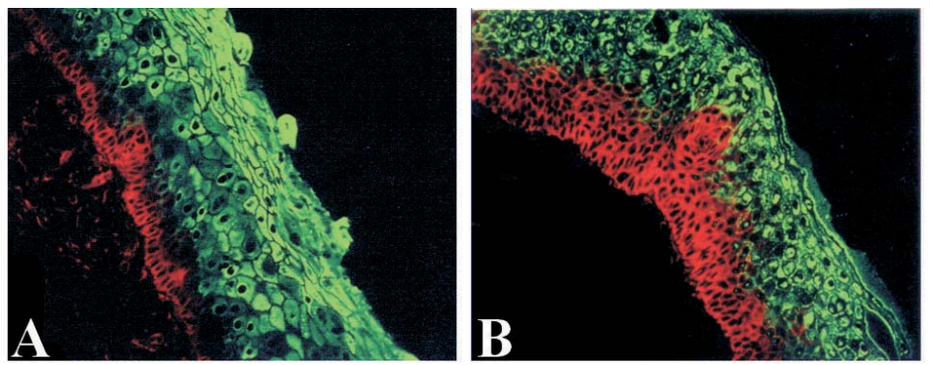


Chapter 1

# General introduction

Function defines form and form defines function. Multicellular organisms have developed organs, tissues and compartments with cells specifically differentiated to perform a certain function efficiently. For instance, an adequate barrier protects the internal organs from the outside, the epithelium. In more complex animals, the epithelium is a dynamic tissue layer with highly differentiated, tightly interconnected cells. Depending on its microenvironment, the epithelial pluripotent stem cell may mature in different directions. For example, the intestinal epithelium has divergent biological tasks: being the barrier between the body and the outside world, digesting food, absorption of the resulting mix while keeping the indigestible bulk and associated microflora inside the lumen. The intestinal stem cell can therefore diffentiate into different celltype that performs specific tasks [Radte and Clevers, 2005].

In order to grow, repair or adapt, differentiated cells and pluripotent proliferative cells have to be in perfect balance. A well-organized intercellular communication system is required to balance growth, repair and differentiation for the development and maintenance of adult tissue.

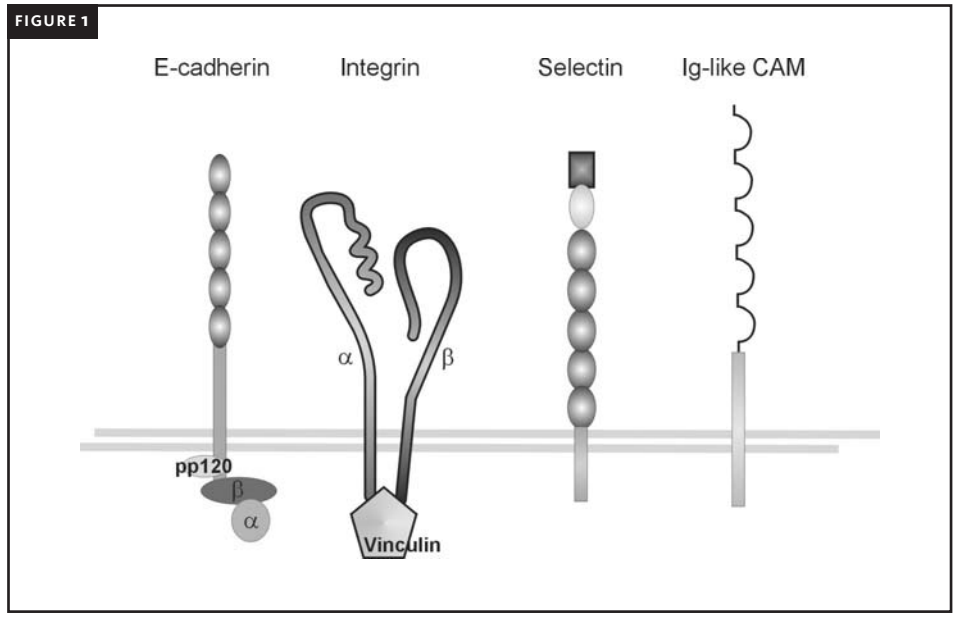
## Cell adhesion molecules (CAMs)

Both local and systemic communication are a prerequisite for well-organized and functioning tissues. Systemic communication is e.g. mediated by hormones, enzymes? and cytokines. Local intercellular communication in a tissue, also involves many different types of molecules. Since the last decade, it has become clear that cell adhesion molecules (CAMs) also serve as communication and regulatory modalities. CAMs are known to maintain tissue integrity, but accumulating data suggests that the distinction between adhesion and signaling can no longer easily be made. Since CAMs participate in tissue development, migration, differentiation and cell death they would be better defined as morphoregulating molecules. A clear example is the formation of a double neural tube as a result of β-catenin overexpression [Funayama et al, 1995].

Cellular adhesion molecules (CAMs) are classified into four major groups: integrins, selectins, immunoglobulin-like (Ig-like) super family and cadherins, (see figure 1). However, other (families of) adhesion molecules have been described, for example, mucins, syndecans, CD44, protein zero and Ep-CAM.

Integrins mediate cell adhesion to extracellular matrix (ECM) components as well as intercellular adhesion. These cell surface receptors are present in neural, endothelial and epithelial tissue. Each integrin is a heterodimer, where ligand and function specificity is determined by combinations of different α and β sub-units. The affinity of integrins for their specific ligands may vary, resulting in dynamic cell-ECM or cell-cell interactions. Integrins also participate in outside-in signaling that can lead to

FIGURE 1




changes in gene expression [Wei et al., 1997; Gimond et al., 1999; Kim and Yamada, 1997].

Selectins participate in the homing of lymphocytes to lymph nodes, as well as in leukocyte extravasation in inflamed tissue. They are expressed on leukocytes and endothelium and are transmembrane or GDI-linked molecules. The molecule consists of a $Ca^{2+}$-dependent C-type lectin domain, a single EGF-like repeat and several repeats that can bind molecules of the complement cascade, and mediates heterotypic adhesion between leucocytes and endothelium.

The members of the Ig-like superfamily of CAMs (IgSF) are required in different stages of brain development, including neuronal migration, axon pathfinding, target recognition and synapse formation. In the adult, functions of neuronal networks are maintained by a diversity of transmembrane - and secreted members of IgSF proteins in the nervous system. The complexity of IgSF protein function results from different levels of regulation including gene expression, protein localization, and protein interactions. IgSF proteins all consist of immunoglobulin (Ig) domains, which are double, 100 amino acid β-pleated sheets, stabilized by disulphide bonds. Often, fibronectin III repeats are present in the molecule. Both heterophilic and homophilic adhesions can be mediated by Ig-like molecules, as was demonstrated for N-CAM and ICAM-1/2/3 (binding to integrins).

The members of the cadherin superfamily (classical, non-classical, protocadherins and desmosomes) mediate neural and epithelial cell-cell adhesion and thereby maintain cell polarity and regulate differentiation. [Birchmeier 1995]. Classical cadherins are expressed in a wide variety of epithelial and neural tissues. The classical cadherins, i.e. E-, P-, and N-cadherin, are type I molecules that mediate epithelial or neuronal homotypic cell-cell adhesions. These transmembrane molecules are composed of five repeating, $Ca^{2+}$-binding domains in the extracellular domain that mediate the homotypic cell-cell adhesion. These molecules are involved in embryonic development and in maturing, differentiating tissues. In the following paragraph, classical cadherins will be discussed more extensively.

## The cadherin/catenin adhesion complex

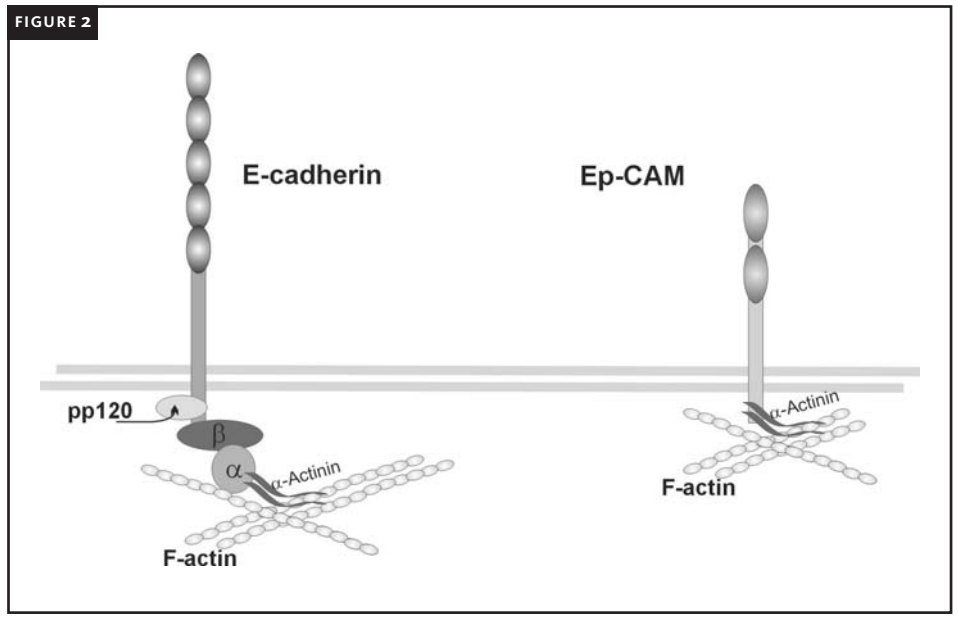
Classical cadherins play a critical role in epithelia, where they establish and maintain cell polarity [McNeil et al., 1990; Natkhe et al., 1993], morphogenisis [Wheelock et al., 1992; Larue et al., 1996] and regulation of proliferation and programmed cell death [Hermiston et al., 1995; Hermiston et al., 1996; Takahashi et al., 1996; Zhu et al., 1996]. A dynamic regulation of cadherins is a prerequisite for directing these cellular processes.

Several cadherins, classical and non-classical, can be expressed in one type of tissue. Although different classical cadherins bind to the same cytosolic proteins via their cytoplasmic tails, downstream signals originating from cadherin-mediated contacts are both cadherin-specific and cell-context-specific [for review see Wheelock and Johnson, 2004]. During the gradual transformation of squamous epithelium towards low- and high grade squamous intraepithelial lesions (SILs), both qualitative as well as quantitative changes in expression patterns were observed [De Boer et al., 1999]. E-cadherin expression virtually disappears from the intercellular connections, while P-cadherin becomes the predominant cadherin in high-grade SILs and modulates the expression of differentiation markers (involucrin, cytokeratin-13). While P-cadherin emerges, γ-catenin replaces β-catenin, and becomes the predominant catenin linking cadherins to the cytoskeleton. Also, the assembly of desmosomes is affected during progression of SILs and is accompanied by a dramatically decreased expression of desmogleins and desmoplakins (I, II). The authors concluded that, during development of cervical lesions, substantial changes occur in cell-cell junctions [De Boer et al., 1999]. These result in cellular junctions dissimilar from those of reserve cells, basal cells, or cells of immature squamous metaplasia, despite morphological similarity between these cell types and cells of high-grade lesions [De Boer et al., 1999]. In some cases of primary squamous cell carcinoma of the oral cavity, the loss of immunohistochemical staining of the desmosomal components desmoglein and desmoplakin correlated well with the loss of differentiation, degree of invasion and presence of lymph node metastases [Hiraki et al.,1996].

The intercellular domain of E-cadherin can bind several intracellular accessory molecules, together forming a complex that is able to bind to the cytoskeleton or to start signal transduction (see figure 2): β-catenin links E-cadherin to α-catenin that can bind to the F-actin cytoskeleton. The binding to the cytoskeleton strongly anchores the cell-cell adhesion complex

Cadherin-independent pools of α-catenin and β-catenin have been localized at the lateral membrane. It was suggested that these pools are nothing more than reservoirs

FIGURE 2




that provide catenins for the assembly of the cadherin adhesion complex. However, other cytosolic proteins may bind the catenins, thereby generating transduction of various signals. First, β-Catenin was found to be a key player in *Wnt*1- signaling. In vertebrates, binding of Wnt (growth factor/ proto-oncogene) to its receptor, ultimately results in β-catenin or plakoglobin inducing embryonic axis formation in *Xenopus laevis*. Second, the tumor suppressor gene product Adenomatous Polyposis Coli (APC) protein is known to bind γ- and β-catenin.Wild type APC is responsible for cytoplasmic degradation of β-catenin. In colorectal cancer cells expressing a mutant APC protein, the mutant APC cannot bind to cytosolic β-catenin, and elimination of cytosolic β-catenin does not occur; Third, an EGF-receptor-induced tyrosine phosphorylation of β-catenin and plakoglobin suppresses E-cadherin function.

Destabilization of the cadherin mediated adhesion complexes is achieved, for example by GTPase effector IQGAP1, that binds β-catenin or α-catenin. Also, pp120 can shuttle between cadherin mediated adhesion complexes and a cytoplasmic pool. A selective uncoupling of pp120 from E-cadherin destroys the strong adhesion mediated by E-cadherin [Aono et al., 1999, Thoreson et al., 2000].

Obviously, cadherin expression and cadherin signaling are modulated by a complex of pathways, involving many other molecules [for review see Wheelock and Johnson, 2004].

## Ep-CAM

Besides cadherins, most epithelial cells also express the epithelial cell adhesion molecule named Ep-CAM. In 1979 Ep-CAM was first identified as a human tumour-associated antigen expressed by colon, gastric and pancreatic carcinomas and at the time named mAb17-1A [Herlyn et al., 1979]. Later investigations have demonstrated Ep-CAM expression in breast, prostate, ovarian, lung and renal cancer [Spizzo et al., 2004, 2006; Went et al., 2005, 2006]. There are several synonyms for Ep-CAM since Ep-CAM has been independently identified in different studies. In each the antigen was named after the antibody recognising it. Different names are still being used today. Therefore, the use of "EpCAM" was unanimously proposed by the scientific community to be used, to facilitate comprehension [Baeurle and Gires, 2007]. Moreover, Ep-CAM was included in the human cluster of differentiation as CD326.

A *de novo* expression in squamous carcinomas and an overexpression of Ep-CAM in other carcinomas explains why Ep-CAM was recognized as a pan-carcinoma antigen. Later publications demonstrated that Ep-CAM is also expressed in most simple, columnar epithelia, but is absent in mature squamous epithelium [Litvinov et al., 1994a,b]. Ep-CAM expression is associated with proliferation *in vitro* [Litvinov et al., 1996].

Ep-CAM is the product of the GA733-2 gene on chromosome 4q [Linnenbach et al., 1993]. The GA733-2 gene consists of 9 exons, all of which (partly) contain protein-coding sequences. No TATA or CAAT boxes have been identified but the putative promotor sequence contains consensus binding sites for Sp1 and AP-1 transcription factors. Exon 1, the 5'-UTR, encodes a signal peptide sequence. Exons 2 to 6 encode the extracellular domain of the Ep-CAM molecule, exon 7 for the transmembrane region, and exon 8 and 9 for the cytoplasmic domain. The 3'-UTR may encode a signal for specific degradation of mRNA encoding cytokines, lymphokines or some proto-oncogenes. Another sequence has been identified as a consensus sequence in the 3' region of inflammatory mediators. These sequences suggest that Ep-CAM expression may be regulated at the mRNA level.

The encoded molecule is composed of 314 amino acids, comprising a 42 kDa type I glycoprotein that mediates $Ca^{2+}$ -independent homotypic cellular adhesions. Ep-CAM is capable of mediating cell aggregation, preventing cell scattering and directing cell segregation [Litvinov et al., 1994a]. The cytoplasmic domain is necessary for localization at cell-cell boundaries and adhesion regulation through the cytoskeleton which is most likely performed via α-Actinin [Balzar et al., 1998]. (see figure 2). Ep-CAM shows adhesive properties when introduced into murine fibroblasts that lack the capacity to form intercellular interactions [Litvinov et al., 1994b; Balzar et al., 1999]. Further studies revealed that Ep-CAM mediated adhesions can not be stucturally distinguished from E-cadherin mediated adhesions but co-localize . However, Ep-CAM mediated adhesions could not be detected in tight junctions and desmosomes [Balzar et al., 1999]. A model for the development of Ep-CAM mediated adhesion has been proposed by Balzar et al., [Balzar et al., 1999]. This model is based on the observation that when introduced in fibroblasts both E-cadherin and Ep-CAM compete for the same type of anchor sites on F-actin; in initial cell-cell contacts. In mature cell-cell contacts, the underlying cortical

actin contains both adhesion molecules in close proximity but in distinct clusters. In polarized cells, primary cell-cell contacts also contain Ep-CAM and E-cadherin clusters. The release of ZO-1 (carried by the cadherins to the apical part of the lateral membrane), leads to the formation of the apical junctional complex including the tight junction and adherens junction. Ep-CAM molecules are 'squeezed out' and directed to more basal regions of the lateral membrane [Balzar et al., 1999].

## Ep-CAM as signaling adhesion molecule

Until recently, distinct roles for cell adhesion and signaling pathways were assigned to adhesion molecules and signal receptors, respectively. However, quantitavive changes, in for example E-cadherin, affect crucial parameters of epithelial morphogenesis [Gumbiner 1996]. The suggestion that adhesion systems within a cell may act as regulators of other adhesion systems, opens new perspectives on signaling that is relevant for understanding of rearrangements in cellular or tissue organization [Edelman 1993; Rosales et al., 1995; Gumbiner 1996]. Many studies since have been focused on the signaling properties of adhesion molecules.

Several studies suggest that Ep-CAM expression affects intercellular processes. Mammary gland development is affected at all stages by introduced Ep-CAM overexpression in transgenic mice [Balzar et al., 2001]. Ep-CAM expression is detected at low levels in the primary ducts of mammary gland epithelia of virgin mice. During pregnancy, the expression levels gradually increase, parallel to the proliferative status. During the differentiating stage of lactation, Ep-CAM expression in the ducts returns to instrinsic levels and almost disappears from the alveolar cells. E-cadherin expression remains virtually unchanged during the stages of active proliferation or differentiation. Ep-CAM not only is associated, but also is directly involved in mammary gland morphogenesis. In Ep-CAM transgenic virgin mice, elevated Ep-CAM expression increases the active growth of primary ducts and induces secondary branching and budding of mammary gland epithelium [Balzar et al., 2001]. A complete cycle of pregnancy, delivery and lactation results in extreme ductal hyperplasia and lobular hypoplasia, while involution is partially disturbed because Ep-CAM expression prevents apoptosis and sustains cell proliferation preventing involution of the mammary gland to pre-pregnancy morphology.

Tubular branching of the mammary gland can be blocked by activation of phosphoinositide 3-kinase (Pi3K), as was observed in WAP/TGFα-transgenic mice or in a transgenic mice model mimicking insuline-like growth factor signaling that was treated with Pi3K-inhibitor [Sandgren et al., 1995; Neuenschwander et al., 1996]. The results of the mammary gland model in Ep-CAM transgenic mice suggest a morphoregulating role for Ep-CAM, and it is plausible that this signaling pathway involves Pi3K.

## Ep-CAM as an oncogenic signaling molecule

Recently, EpCAM was shown to interact directly with CD44v4-v7 [Schmidt et al., 2004], a tumor metastasis-promoting CAM, and with claudin-7, a tight junction protein [Ladwein et al., 2005].





This complex of CD44v4-v7-tetraspanin-EpCAM-claudin-7 is formed by pancreatic tumor cell lines [Ladwein et al., 2005]. These carcinoma-specific complexes can influence cell-cell adhesion, cell-matrix adhesion, and apoptosis resistance, and they appear to be involved in processes that promote carcinogenesis, i.e., metastasis [Schmidt et al., 2004]. The EpCAM-claudin-7 complex is frequently observed in highly metastatic tumours, such as pancreatic and colorectal carcinomas, supporting the hypothesis that the tetraspanin complexes support tumor progression by promoting metastasis.

## Pi3K

Phosphoinositide 3-kinase (Pi3K) phosphorylates the 3' position of the inositol ring on phosphaditylinositol. Many cellular functions are affected by the activity of Pi3K, since it is essential in signal transduction and membrane traffic. The enzyme is ubiquitously expressed, and is activated upon binding of the regulatory subunit p85 to receptors associated with growth factors. The p85 subunit subsequently binds the catalytic subunit p110α, which then catalyzes the phosphorylation of phosphoinositide 3,4-biphosphate (PIP2). A critical downstream target in this signaling transduction pathway is the activation of protein Ser-Thr kinase AKT1, also known as PKB, that effects multiple cellular processes, including cellular proliferation, apoptosis and cell scattering [Royal et al., 1997]. It also contributes to tumorigenesis [Klippel et al., 1998, Kennedy et al., 1997]. The gene PIK3CA, which encodes the p110α subunit, is localized on 3q26. The 3q amplification is also the most consistent chromosomal aberration found in cervical cancer, and is implicated in the progression of dysplastic cervical epithelial cells into invasive cancer [for review see Rooney 1999; Ried et al., 1999].

An *in vitro* model of epithelial MDCK cells demonstrated that the engagement of E-cadherins in homotypic cellular adhesions promotes an increase of Pi3K-dependent activation of Akt and the translocation of Akt to the nucleus [Pece et al., 1999]. The activation of Pi3K in response to adherens junction assembly involves the concomitant recruitment of Pi3K to E-cadherin-containing protein complexes [Pece et al.,1999]. Pi3K activation modulates the E-cadherin mediated adhesions and both Pi3K and E-cadherin can bind α-Actinin [Shibasaki 1994]. A signaling pathway involving both Ep-CAM and Pi3K is plausible and this pathway may affect the regulation of cadherin-mediated adhesion complexes.

Expression of exogenous p85 in mouse keratinocytes increases the metabolic stability of β-catenin and increases the signaling of β-catenin leading to transcription of a reporter gene [Espada et al., 2005]. Both effects are results of the formation of β-catenin/p85 and inhibition of β-catenin/APC complexes and these are independent of PI3K or GSK activity. These findings suggest that p85 can act as a direct metabolic regulator of β-catenin activity [Espada et al., 2005].

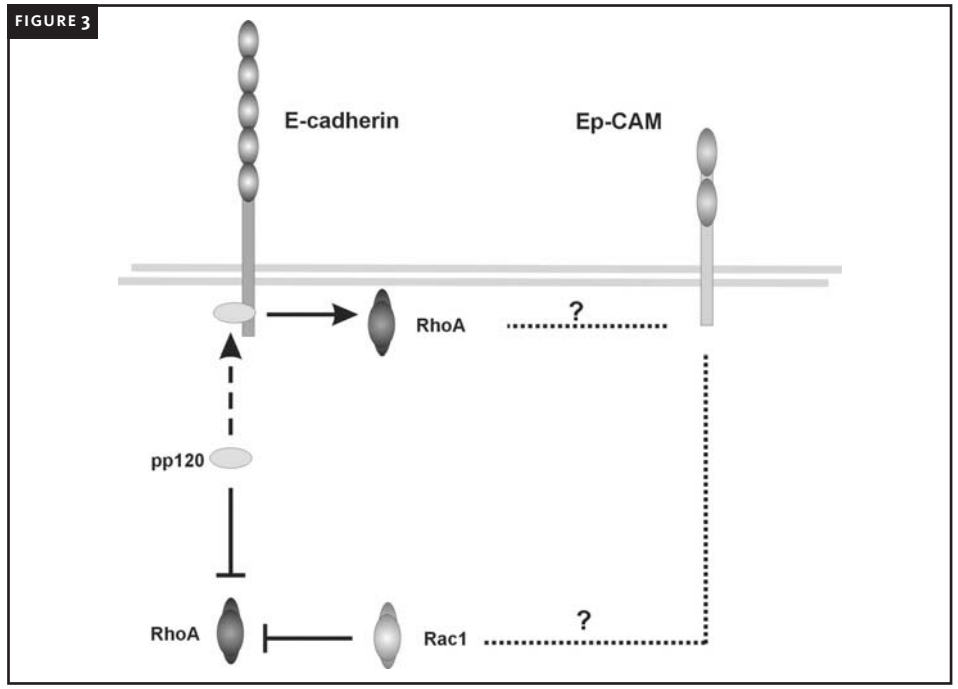
## GTPases

Over the years, it has been demonstrated that cadherin- mediated adhesion requires the activation of cytosolic proteins of the Rho family of small GTPases [Braga et

al., 1997; Hordijk et al., 1997]. The Rho small GTPases belong to the Ras superfamily of GTPases and are regulated by guanide nucleotides. GTPases bound to guanide triphosphate (GTP) are in an active state and ready for signaling. Upon hydrolysis of GTP and liberation of the phosphate (resulting in guanide diphosphate, GDP), the associated GTPase is inactivated. Members of the Rho family are Rho, Rac and Cdc42, participate in several cellular processes including actin cytoskeleton reorganization involving cell adhesion, cytokinesis and motility [Van-Aelst and D'Souza 1997]. In epithelial cells, the activity of small Rho GTPases is required in both the formation as well as the disassembly of cadherin- mediated junctions [Braga et al., 1999; Hordijk et al., 1997; Takaishi et al., 1997; Zhong et al., 1997]. RhoGTPase Cdc42 and Rac1 regulate the basolateral trafficking for the post-Golgi transport of E-cadherin before p120 interacts with E-cadherin, resulting in polarized cells [Wang et al., 2005]. These actions of the GTPases are also involved in the transformation to a malignant phenotype.

A specific target for Rho is pp120 [Anastasiades et al, 2000; Noren et al 2000]. Not only can pp120 regulate cadherins, expression of pp120 in fibroblasts also induces enhanced motility and marked changes in cell shape [Reynolds et al., 1996]. Expression of pp120 leads to inhibition of Rho (see figure 3). Furthermore, it has been suggested that Rac activation in these cells may strengthen the cadherin – dependent adhesion [Hordijk et al., 1997; Takaishi et al., 1997]. On the other hand, Rac activation in keratinocytes can specifically remove cadherins from newly formed and stable cell-cell contacts [Braga et al., 2000].

FIGURE 3










It is clear that Rho activity is essential for cadherin -dependent adhesion [Braga et al., 1997; Takaishi et al., 1997]. The bacterial toxin C3 transferase inhibits the endogenous Rho, which results in a very fast removal of cadherins from newly formed or mature junctions.

Experimental data demonstrated that Cdc42 inhibition resulted in a reduction of aggregation capacity observed in E-cadherin transfected fibroblasts [Fukata et al., 1999], but Cdc42 inhibition does not prevent cadherin localization at junctions.

A specific target of both Rac and Cdc42 is IQGAP1 [Fukata et al., 1999]. It interacts with β-catenin in the same region as α-catenin does, and competition between _-catenin and IQGAP1 can regulate the binding of E-cadherin/ β-catenin to the cytoskeleton. The postulated hypothesis of initial and mature cell-cell contacts mediated by either Ep-CAM or E-cadherin implies the transformation of both stress fibers and cortical actin.

Embryonic development, cell proliferation, maturation, differentiation, epithelial to mesenchymal transformation, carcinogenesis are just a few examples of processes that involve coherent signaling pathways between adhesion molecules, growth factors, intracellular transporters, and many other molecules in order to maintain tissue integrity in high eukaryotes.

## Aim and outline of the investigations

This thesis aims to explore the signaling pathways activated by Ep-CAM, and their putative morphoregulatory function. In **chapter 2**, the biological characteristics and expression patterns of Ep-CAM that are known from the literature are summarized. In general, ectopic Ep-CAM abrogates the adherens junctions that are mediated by E-cadherin. **Chapter 3** is focused on the effects of Ep-CAM on E-cadherin- and N-cadherin- mediated adhesions in both double transfected murine fibroblasts as well as Ep-CAM transfected human epithelial cells. Subsequently, the site of interaction in the E-cadherin adhesion complex that [could be affected]. by Ep-CAM is studied in two cell models (**chapter 4**). Ep-CAM is introduced into fibroblasts that express either one of two different E-cadherin/α-catenin fusion proteins, and the effect of Ep-CAM expression on the chimeric molecules is studied. The results indicate that Ep-CAM most likely shifts the adhesion strength from strong to weak by affecting E-cadherin at the juxtamembrane domain, or the COOH- terminal region of α-catenin. **Chapter 5** describes the reversebility of this mechanism; decreasing Ep-CAM expression in tumor cells leads to the restoration of E-cadherin- mediated adhesions and the epithelial phenotype. The results of many studies of the effect of on Pi3K manipulation in epithelial cells are similar to those observed for Ep-CAM. In **chapter 6**, the role of Ep-CAM in signal transduction pathways involving Pi3-kinase will be discussed. Finally, the potential diagnostic value of Ep-CAM expression in epithelial tumors is reviewed (**chapter 7**). The last chapter (**chapter 8**) summarizes the results presented in preceding chapters and discusses future prospectives.

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Chapter 2

# The biology of 17-1A antigen (Ep-CAM)

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## Abstract

The glycoprotein recognized by the monoclonal antibody 17-1A is present on most carcinomas, which makes it an attractive target for immunotherapy. Indeed, adjuvant treatment with mAb 17-1A did successfully reduce the 5 years mortality among colorectal cancer patients with minimal residual disease. Currently the antibody is approved for clinical use in Germany, and is on its way for approval in a number of other countries. New immunotherapeutic strategies targeting the 17-1A antigen are in development or even in early phase clinical trials. Therefore, a better understanding of the biology of the 17-1A antigen may result in improved strategies for the treatment and diagnosis of human carcinomas. In this review the properties of the 17-1A antigen are discussed concerning tumor biology and the function of the molecule. This 40 kDa glycoprotein functions as an **E**pithelial **C**ell **A**dhesion **M**olecule, therefore the name Ep-CAM was suggested. Ep-CAM mediates $Ca^{2+}$ independent homotypic cell-cell adhesions. Formation of Ep-CAM mediated adhesions has a negative regulatory effect on adhesions mediated by classic cadherins, which may have strong effects on the differentiation and growth of epithelial cells. Indeed, in vivo expression of Ep-CAM is related to increased epithelial proliferation and negatively correlates with cell differentiation. A regulatory function of Ep-CAM in the morphogenesis of epithelial tissue has been demonstrated for a number of tissues, in particular pancreas and mammary gland. The function of Ep-CAM should be taken into consideration when developing new therapeutic approaches targeting this molecule.

## Introduction

The monoclonal antibody (CO)17-1A was one of the first mAbs generated against an antigen that is often present at the cell surface of human carcinoma cells (Herlyn et al., 1979; Koprowski et al., 1979). Since mAb 17-1A recognizes a tumor-associated antigen expressed by a majority of human epithelial neoplasias, the 17-1A antigen had attracted major attention as a target for immunotherapy to combat human carcinomas (Ragnhammar et al., 1993; Mellstedt et al., 1991; Riethmuller et al., 1994/1998). Already by the early '90s, hundreds of patients were treated with the 17-1A

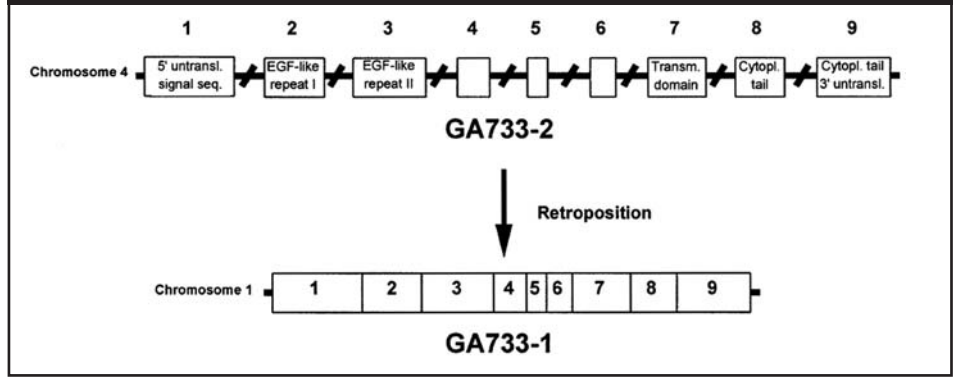
mAb, but the treatment applied as various forms was not effective against solid tumors. However, the post-operative treatment of colorectal cancer patients gave promising results, since adjuvant treatment with 17-1A successfully reduced the 7 years mortality among Duke's C colorectal cancer patients with minimal residual disease (Riethmuller et al., 1994/1998). In line with these results, the antibody was approved in 1995 for routine clinical use in Germany. Moreover, other immunotherapy strategies targeting the 17-1A antigen are in the process of development, suggesting that in the near future this molecule will be (widely) targeted in new clinical trials to combat carcinomas.

The 40 kDa glycoprotein recognized by 17-1A has been described under various names originating from the name of the respective mAb (e.g. MH99, AUA1, MOC31, 323/A3, KS1/4, GA733, HEA125 etc.) that was raised against the molecule (Herlyn et al., 1979; Mattes et al., 1983; Durbin et al., 1990; De Ley et al., 1988; Edwards et al., 1986; Varki et al., 1984; Momburg et al., 1987). The corresponding cDNA had been independently cloned by a number of groups using different names (e.g. KSA, EGP, EGP40, GA733-2) for the encoded molecule (Perez and Walker, 1989; Strnad et al., 1989; Simon et al., 1990; Szala et al., 1990). A few years ago, we have demonstrated that the 17-1A antigen can function as a homophilic cell-cell adhesion molecule, and suggested the name Ep-CAM (**E**pithelial **C**ell **A**dhesion **M**olecule) that reflects its tissue specificity and function (Litvinov et al., 1994a/b). This review discusses the properties of the 17-1A antigen, alias Ep-CAM. Recent findings point towards a major morphoregulatory function for this molecule, not only relevant for epithelial tissue development, but also for carcinogenesis and tumor progression. A better understanding of the biology of the 17-1A antigen may prove useful in developing improved strategies for the treatment and diagnosis of human carcinomas. The name Ep-CAM will be used further in this review irrespective of the designation chosen for this molecule by the original group whose results are discussed.

## The gene

Ep-CAM is encoded by the GA733-2 gene (Linnenbach et al., 1993). The human gene with a minimal estimated size of approximately 14 kb is located on chromosome 4. The GA733-2 locus maps to chromosomal region 4q. The putative GA733-2 promoter contains consensus binding sites for the Sp1 and AP-1 transcription factors, but no TATA or CAAT boxes were found (Linnenbach et al., 1993). Comparison between genomic and cDNA sequences reveals that the GA733-2 gene consists of nine coding exons as depicted in figure 1 (Linnenbach et al., 1993). Amino acid sequences encoded by exon 1 correspond to a signal peptide sequence. Exons 2 to 6 encode for sequences forming the extracellular domain. The transmembrane region is encoded by exon 7. Exon 8 encodes a 15 amino acid portion of the cytoplasmic domain, including a cluster of six positively charged amino acids. Exon 9 encodes the remaining 13 amino acids of the cytoplasmic domain, the stop codon, and the 3'-untranslated region.

**FIGURE 1** Structure of the GA733-2 and GA733-1 genes (after Linnenbach et al [19]). The GA733-2 gene, encoding the human transmembrane glycoprotein Ep-CAM, consists of a total of nine exons located on chromosome 4. The retroposition of GA733-2 mRNA into chromosome 1 resulted in the intron-less GA733-1 gene.




The transcribed mRNA is approximately 1.5 kB. The 5'-untranslated regions from independently isolated Ep-CAM mRNAs revealed a variation in the size of this region, containing either 16 or 160 bases (Perez and Walker, 1989; Simon et al., 1990). All reported sequences for Ep-CAM mRNAs were identical in their coding part, namely an open reading frame of 942 bases. The 3'-untranslated region contains two possibly important motifs (Perez and Walker, 1989). The first sequence (ATTTA) has been proposed to be a signal for specific degradation of mRNA for cytokines, lymphokines, and some proto-oncogenes (Shaw and Kamen, 1986). The second sequence (TTATTTAT) has been identified as a consensus sequence in the 3' region of the inflammatory mediators (Caput et al., 1986). This suggests that the expression of Ep-CAM may be highly regulated at the mRNA level. We have analyzed Ep-CAM mRNAs from a large number of carcinoma cell lines, but found no variations in the splicing of exons encoding the intracellular domain of the molecule (unpublished results). Moreover, no mRNA splice variants that encode different isoforms of Ep-CAM have been reported so far.

## The GA733 gene family

The only molecule known to be homologous to Ep-CAM is the GA733-1 gene product (or EGP-1/Trop-2) that shares 49% homology with the Ep-CAM amino acid sequence. Taking into account conserved substitutions, the two antigens have a similarity of 67% (Szala et al., 1990). Ep-CAM and GA733-1 share two highly homologous regions (Szala et al., 1990). The first region spans 39 residues of the extracellular domain, and is characterized by 79% identical amino acids and a 97% similarity. The second region represents the transmembrane domain. The combined results of molecular cloning, DNA sequencing, amino acid sequence comparison, and chromosome mapping studies indicate that both exon shuffling and retroposition have been factors in the evolution of the GA733 gene family (Linnenbach et al., 1993). The intronless

GA733-1 gene probably originates from the retroposition of Ep-CAM mRNA into a region on chromosome 1 as depicted in figure 1. The retroposition event, which resulted in the GA733-1 gene, most likely preceded the divergence of avian and mammalian species approximately 300 million years ago (Linnenbach et al., 1993). After retroposition, the GA733-1 evolved independently of Ep-CAM, and may be viewed as a molecule that belongs to the GA733 family rather than a protein closely related to Ep-CAM.

The molecule encoded by the GA733-1 gene is slightly larger than Ep-CAM (Linnenbach et al., 1993). The polypeptide backbone contains four N-linked glycosylation sites that result in a 50 kD glycoprotein. The molecule can be phosphorylated within its cytoplasmic tail at serine 303 that is absent in the Ep-CAM cytoplasmic domain (Basu et al., 1995). The conserved tyrosine residue that is present in both molecules has never been reported to be phosporylated. Phosphorylation of the cytoplasmic serine residue suggests a possible role for GA733-1 as cell surface receptor involved in signal transduction. Indeed, stimulation of human carcinoma cells with anti-GA733-1 mAbs induced an intracellular calcium signal (Ripani et al., 1998).

At least five mAbs are known that specifically recognize GA733-1, namely RS7, MR23, 162-46.2, MOv 16, T16 (Ripani et al., 1998; Stein et al., 1994; Fornaro et al., 1995). The five mAbs do not reveal cross-reactivity with Ep-CAM epitopes (our unpublished result; Ripani et al., 1998). Moreover, no mAb specific for Ep-CAM has been shown to react with the GA733-1 protein.

The GA733-1 molecule is also mainly expressed in epithelial tissue. GA733-1 expression levels are low in tissues with a relatively high Ep-CAM expression, such as colon and lung tissue (Stein et al., 1994). In contrast, GA733-1 is expressed at relatively high levels in Ep-CAM negative epithelium. In skin, the GA733-1 molecule is present in supra-basal and spinous layers of the epidermis with increased expression levels in the more differentiated cells (Klein et al., 1987). It is also interesting that a substantial decrease in GA733-1 expression was detected in breast carcinoma cell lines as compared to immortalized normal epithelium (our unpublished results), but the significance of this is unclear. Recently, the GA733-1 gene was genetically linked to the autosomal recessive disorder Gelatinous Drop-Like corneal Dystrophy (GDLD), since 33 out of 40 disease alleles (83%) in a panel of Japanese families were reported to have deleterious mutations causing truncation of the GA733-1 protein (Tsujikawa et al., 1999). The function of GA733-1, possibly overlapping with that of Ep-CAM, is unknown and requires further investigation.

### Evolutional conservation of Ep-CAM

Using Southern blot analysis, sequences homologous to the human GA733-1 and GA733-2 genes were detected in monkey, mouse, hamster, and chicken (Linnenbach et al., 1993). Polyclonal antibodies directed against human Ep-CAM detected homologous proteins in mice, rats, and non-human primates (Zaloudik et al., 1997). MAbs 17-1A and 323/A3 cross-react with hamster and rhesus monkey Ep-CAM respectively (our unpublished results). Thus, Ep-CAM seems to be highly conserved among high vertebra, which is even better illustrated by the homology between mouse and human Ep-CAM. The nucleotide sequence of mouse Ep-CAM is 80% identical to human Ep-CAM. The homology of the amino acid sequence is even higher, namely 82% (Bergsagel et al., 1992).

## Tissue Pattern Expression

### Expression during embryonic development

Relatively little information is available concerning the expression of Ep-CAM during human embryonic development. Ep-CAM expression is detected in fetal lung, kidney (proximal tubules), liver, pancreas, skin, and germ cells. At different stages of lung development, epithelial cells express Ep-CAM (Kasper et al., 1995). As early as the embryonic stage (week 7-8 embryo), relatively high levels of Ep-CAM are expressed by the epithelial cells of the primordial lung. In fact, during the remaining period of development (pseudoglandular, canalicular, and alveolar stage), and throughout adult life, epithelial cells of the lung reveal immuno-reactivity for Ep-CAM (Kasper et al., 1995). The majority of hepatocyte precursors are Ep-CAM positive in the liver of week 8 embryos, but negative in adult liver (De Boer et al., 1999). During pancreatic development Ep-CAM is co-expressed with N-CAM and E-cadherin in week 18 embryos (Cirulli et al., 1995). Moreover, it was reported that in fetal pancreas the highest Ep-CAM expression was detected in developing islet-like cell clusters budding from the ductal epithelium (Cirulli et al., 1998). These cell clusters with an up-regulated Ep-CAM expression (during development) are thought to become the early endocrine cells. In fetal skin of 8-12 week embryos, keratinocytes are Ep-CAM positive, and during development of the hair follicle high levels of Ep-CAM expression were detected (our unpublished results). For the formation of the primitive sex cords in humans, Ep-CAM is expressed at the embryonic stages by germ cells during migration at day 10.5 and early gonad assembly at day 12.5 (Anderson et al., 1999). In human embryos, no Ep-CAM expression was observed in thymus, but in mice Ep-CAM is expressed in thymus epithelium and thymocytes in adult tissue, and even higher in fetal thymus (Farr et al., 1991; Nelson et al., 1996).

In studies using the mAb's GZ1, GZ2, and GZ20 that recognize rat Ep-CAM, the molecule was reported to be expressed during the very early stages of embryogenesis (Schiechl et al., 1986/1987; Tarmann et al., 1990). Immunocytochemistry revealed the expression of Ep-CAM in fertilized oocytes, the two-cell stage, and some cells of the morula. At the blastocyst stage, the zona pellucida showed no Ep-CAM staining, whereas the trophoblast and inner-cell mass were positive. Rat embryos of 8.5 days (at the stage of the three germ layers) revealed positive staining for both the ectoderm and entoderm, while mesoderm was negative. At day 11.5, rat embryos expressed Ep-CAM in all epithelia including those of mesodermal origin, such as the Wolffian duct and coelomic epithelium. In contrast, neural and connective tissue revealed no Ep-CAM expression. Embryos of 14.5 days showed expression in all epithelia, except the

Müllerian duct. Finally, day 17.5 embryos expressed Ep-CAM in all epithelia including glandular tissue such as pancreas anlage and thyroid, whereas neural tissue, lymphatic organs, muscular tissue, liver parenchyma, and the gonads remained negative (Tarmann et al., 1990). These studies suggest an important role for Ep-CAM in embryonic development. However, to evaluate the importance of Ep-CAM during embryogenesis or morphogenesis of individual tissue, further studies (especially with tissue-targeted gene disruption) are required.

**TABLE 1** Distribution of Ep-CAM in normal human adult tissues. The level of Ep-CAM expression by specific cell types within epithelial tissues is indicated by - for no expression, + for low expression levels, ++ for intermediate expression levels, and +++ for high expression levels. Data on distribution and expression levels was summarised from multiple studies using different Ep-CAM specific monoclonal antibodies including unpublished data from own lab.

| Tissue | +/– | Distribution |
|---|---|---|
| Oral cavity | + | Stratified squamous epithelial mucosa –, secretory cells + |
| Oesophagus | + | Stratified squamous epithelium –, columnar epithelium +/– |
| Stomach | + | Mucosa + |
| Duodenum, jejunum, ilium | + | Mucosa ++ |
| Colon, rectum | + | Mucosa +++ |
| Salivary gland | + | Acinar cells +, duct cells +, basal and suprabasal cells +, squamous epithelium – |
| Pancreas | + | Ductal epithelium ++, acini ++, islets + |
| Liver | + | Bile ducts ++, Bile canaliculi +/–, Hepatocytes – |
| Gallbladder | + | Glandular epithelium ++ |
| Trachea | + | Mucosa ++ |
| Bronchi | + | Mucosa ++, |
| Lung acini | + | Ciliated bronchioli epithelium +, alveolar duct +, alveoli (pneumocytes) + |
| Skin | +/– | Keratinocytes –, melanocytes –, hair follicle +, sweat gland +, dermis – |
| Kidney | + | Distal tubules ++, proximal tubules +, collecting ducts ++, Henle's loop +, Bowman's capsule +, Capillary tufts – |
| Ureter | + | Transitional epithelium + |
| Bladder | + | Transitional epithelium (urothelium)+ |
| Urethra | + | Transitional epithelium + |
| Thyroid gland | + | Follicular epithelium ++, C cells + |
| Parathyroid gland | + | Chief and oxyphil cells ++ |
| Adrenal gland | + | Cortical epithelium +, medullary chromaffin cells – |
| Pituitary gland | + | Adenohypophyseal cells ++, pituicytes – |
| Testis | – | Some spermatogonia + |
| Epididymis | + | Ciliated, basal, and cuboidal cells ++ |
| Prostate, seminal vesicles | + | Secretory, basal, and ductal cells ++ |
| Ovary | + | Oocytes ++, follicular epithelial cells – |
| Oviduct | + | (Non-)ciliated cells + |
| Uterus/cervix | + | Endometrium ++, myometrium –, endocervical glands ++, ectocervical squamous epithelium – |
| Mammary gland | + | Ductal epithelium ++, alveoli + |
| Thymus | + | Medullary epithelium +, Hassal bodies +, cortical epithelium – |
| Tonsil | + | Crypt epithelium + |
| Spleen | – | |
| Lymph node | – | |
| Bone-marrow-derived cells | – | |
| Skeletal muscle | – | |
| Brain | – | |
| Vessels | – | |
| Connective tissue | – | |

## Expression in normal adult tissues

Based on immunohistochemical data, Ep-CAM is a strictly epithelial molecule in adult humans (Table 1). Ep-CAM is detected at the baso-lateral cell membrane of all simple (especially glandular), pseudo-stratified, and transitional epithelia. In contrast, normal squamous stratified epithelia are negative for Ep-CAM. In adult human tissues no expression was found in mesenchymal, muscular, and neuro-endocrine tissues (Momburg et al., 1987; Bumol et al., 1988). Further, no Ep-CAM expression was detected in cells of lymphoid origin. The level of expression may differ significantly between the individual tissue types. In the gastrointestinal tract, gastric epithelium expresses very low levels of Ep-CAM. Expression levels are substantially higher in small intestine, and in colon Ep-CAM is probably expressed at the highest levels among all epithelial cell types. Glandular epithelium of the gall-bladder shows Ep-CAM expression (Momburg et al., 1987), but the transitional epithelium (urothelium) of the bladder is only slightly positive (Zorzos et al., 1995). In the lower respiratory tract, the trachea, bronchi, bronchioles, and alveoli are Ep-CAM positive (Momburg et al., 1987). In adult liver the bile ducts are Ep-CAM positive, whereas hepatocytes are negative (De Boer et al., 1999). Most epithelial cells of the kidney, such as cells of the proximal tubules, distal tubules, and ducts, reveal Ep-CAM expression (Momburg et al., 1987). In pancreas Ep-CAM expression has been detected in the ductal epithelium, acini, and some expression in the islets (Momburg et al., 1987; Cirulli et al., 1998). In skin, the sweat ducts and the proliferative zone of the hair follicle reveal Ep-CAM staining, whereas keratinocytes and melanocytes are mainly negative (Momburg et al., 1987; Tsubura et al., 1992). Within the basal layers of the epidermis some Ep-CAM reactivity can be observed in the reserve cells, since mAb MH99 was reported to be reactive with some cells within the basal layer of skin keratinocytes (Klein et al., 1987). The glands of the endocrine system (thyroid, parathyroid, pituitary and adrenal glands) contain Ep-CAM positive epithelium (Momburg et al., 1987). In mammary glands, the ductal epithelium reveals relatively high Ep-CAM expression as compared to the lower levels in alveolar epithelium (Edwards et al., 1986; Momburg et al., 1987). Ep-CAM expression is detected in most epithelial tissues of the female genital tract (ovaries, oviducts, cervix, and uterus). Normal endocervical glandular epithelium (both columnar and reserve cells) reveals high expression levels for Ep-CAM, whereas ectocervical squamous epithelial cells do not express the molecule (Litvinov et al., 1996). Some Ep-CAM expression may be detected in the basal cells of morphologically normal ectocervical tissue, but only in areas bordering lesions of cervical intraepithelial neoplasia (Litvinov et al., 1996). In tissues of the male genital tract, some of the epithelial cells in testis, epididymis, seminal vesicle, and prostate reveal Ep-CAM expression (Momburg et al., 1987).

## Correlation with benign and malignant tumor development

Active proliferation in a number of epithelial tissues is associated with increased or *de novo* Ep-CAM expression (Zorzos et al., 1995; Litvinov et al., 1996; High et al., 1996). This is especially evident in tissues that normally reveal no or low levels of Ep-CAM expression, such as squamous epithelium. At the early stages of neoplasias of the uterine cervix, *de novo* expression of Ep-CAM is often observed in areas with atypical, undifferentiated cells of the squamous epithelium (Litvinov et al., 1996). Thus, in cervical intraepithelial neoplasia (CIN) grades I and II, the basal and suprabasal cells are Ep-CAM positive, while grade III lesions reveal up to 100% positive cells in all layers of the squamous epithelium. Moreover, a clear increase in both the number of positive cells

and the level of Ep-CAM expression is observed during the progression from CIN I to CIN III. Expression of Ep-CAM in atypical cells of CIN lesions correlated with the disappearance of markers for squamous differentiation and enhanced proliferation. In weak, mild, and severe oral mucosal dysplasias high levels of Ep-CAM expression were detected in basal and suprabasal cells with a clear border between Ep-CAM positive dysplastic cells and Ep-CAM negative normal epithelial cells (High et al., 1996).

In glandular epithelium of the gastrointestinal tract, one can observe a clear gradient of decreasing expression of Ep-CAM from crypts to villae (Schiechl et al., 1986/1987). The level of Ep-CAM expression correlates with the proliferative activity of intestinal cells, and inversely correlates with their differentiation (Schiechl et al., 1986). Dysplastic or metaplastic proliferation corresponds to an increase (sometimes to very high levels) in Ep-CAM expression. In gastric epithelium that normally expresses low levels of Ep-CAM, a strong expression of Ep-CAM is observed in proliferative metaplastic lesions, such as intestinal metaplasia (unpublished results). Even in colon, where the epithelium expresses the highest levels of Ep-CAM, the development of polyps is reported to be associated with an increased expression of the molecule. Hepatocytes are Ep-CAM positive during embryonic development (week 8 embryos), but negative in adult liver (De Boer et al., 1999). However, during liver regeneration processes cells that morphologically resemble precursor stem cells are Ep-CAM positive, but as they mature into hepatocytes, they become again Ep-CAM-negative (De Boer et al., 1999). Dysplastic lesions of the bladder epithelium (urothelium) reveal increased Ep-CAM expression as compared to normal urothelium (Zorzos et al., 1995).

**TABLE 2** Ep-CAM expression in human malignant neoplasias. Most carcinomas express Ep-CAM, whereas tumors derived from non-epithelial tissues are Ep-CAM negative. The level of Ep-CAM expression by tumor cells is indicated by - for no expression, + for low expression levels, ++ for intermediate expression levels, and +++ for high expression levels. Data on distribution and expression levels was obtained from multiple studies using different Ep-CAM specific monoclonal antibodies and reflects the pattern of Ep-CAM expression rather than a reactivity of a particular antibody.

| Type of tumor | Ep-CAM expression |
|---|---|
| Oral mucosal carcinoma: | |
| Basal cell carcinoma | ++ |
| Squamous cell carcinoma | + |
| Laryngeal (squamous cell) carcinoma | + |
| Esophageal (squamous cell) carcinoma | + |
| Gastric adenocarcinoma | ++ |
| Carcinoma of small intestine | +++ |
| Colorectal adenocarcinoma | +++ |
| Pancreatic carcinoma | ++ |
| Liver carcinoma: | |
| Hepatocellular carcinoma | – |
| Cholangiocarcinoma | ++ |
| Biliary duct carcinoma | ++ |
| Lung carcinoma | +++ |
| Skin carcinoma: | |
| Basal cell carcinoma of skin | ++ |
| Squamous cell carcinoma of skin | – |
| Renal cell carcinoma | ++ |
| Transitional cell carcinoma of bladder | ++ |
| Thyroid carcinoma | ++ |
| Prostate carcinoma | ++ |
| Ovarian carcinoma | ++ |
| Endometrium carcinoma | ++ |
| Cervical carcinoma: | |
| Squamous cell carcinoma of cervix | ++ |
| Adenocarcinoma of cervix | +++ |
| Mammary carcinoma | ++ |
| Mesotheliomas: | |
| Non-epithelioid | – |
| Epithelioid | ++ |
| Germ cell tumour | +/– |
| Wilms' tumor (epithelial component) | +/– |
| Melanoma | – |
| Sarcoma | – |
| Lymphoma | – |
| Meningioma | – |

Malignant proliferation is nearly always associated with Ep-CAM expression at some stage of tumor development. Most carcinomas, but no other tumor types express high levels of Ep-CAM (table 2). However, Ep-CAM expression in carcinomas can be heterogeneous, and is probably affected by a shift of tumor cell differentiation to either mesenchymal or squamous (in squamous carcinomas) cell phenotypes. It has been reported for dysplastic oral mucosa that well-differentiated squamous cell carcinomas are negative for Ep-CAM, whereas poorly differentiated squamous cell carcinomas are Ep-CAM positive (High et al., 1996). Most squamous carcinomas are Ep-CAM positive, except for (Ep-CAM negative) squamous carcinoma of the skin. The expression of Ep-CAM distinguishes squamous cell carcinoma of the skin from the Ep-CAM positive basal cell carcinoma (Tellechea et al., 1993). In the majority of squamous and adenocarcinomas of the uterine cervix varying levels of Ep-CAM expression were detected (Litvinov et al., 1996). However, as compared to CIN III, some decrease in the expression (both intensity and number of positive cells) is observed in areas of squamous differentiation.

## Ep-CAM as a marker protein for differential diagnosis

In human tissue Ep-CAM is expressed only in epithelium and neoplasias derived from epithelia. Therefore, the molecule may be used as a marker to distinguish epithelial neoplasias from neoplasias derived from non-epithelial tissues. Ep-CAM positive tumors are derived from epithelial cells, whereas Ep-CAM negative tumors may be originating from non-epithelial as well as epithelial tissues. Further, Ep-CAM may be used as a marker to histologically differentiate between epithelial neoplasias. Occasionally, difficulties in the histological differential diagnosis between basal-cell carcinoma (BCC) and squamous-cell carcinoma (SCC) of the skin may arise. Basal squamous cell epithelioma, a tumor combining morphological properties of BCC and SCC, is one common example of these difficulties, but also other histological types of BCC may be erroneously interpreted as SCC (Tellechea et al., 1993). Staining for Ep-CAM demonstrated that all BCC's were diffusely and intensely labeled, whereas none of the SCC's expressed Ep-CAM, irrespective of histological type or grade of differentiation (Tellechea et al., 1993). In liver neoplasias, Ep-CAM was expressed in almost all cholangiocarcinomas, whereas the majority of hepatocellular carcinomas were Ep-CAM negative, suggesting that hepatocellular carcinoma originates from a highly differentiated precursor (De Boer et al., 1999). The results also indicate that Ep-CAM can be used as an additional immunohistochemical marker to distinguish cholangiocarcinoma from hepatocellular carcinoma due to the differential expression in these epithelial tumors. Finally, it was demonstrated that Ep-CAM could be used as a marker to discriminate carcinomas from Ep-CAM negative mesotheliomas, except for the epithelioid type (Ryan et al., 1997).

It has been demonstrated for squamous cell carcinomas of the head and neck that expression of the classic cell adhesion molecule, E-cadherin, correlates inversely with lymph node metastasis (Schipper et al., 1991). It is also reported that E-cadherin

functions as an invasion suppressor molecule (Behrens et al., 1989). Since Ep-CAM functions as an intercellular adhesion molecule, like E-cadherin, it has been claimed that the expression might reduce the metastasizing capability of tumor cells, and correlate with a better prognosis of carcinoma patients (Basak et al., 1998). Thus, Ep-CAM may also be useful as a marker for increased malignant potential in the diagnosis of cancer patients. Indeed, a lack of Ep-CAM expression in the primary tumor of laryngeal carcinomas significantly correlated with nodal metastasis (Takes et al., 1997). Further, the expression of Ep-CAM increases from baseline levels in normal oral epithelium to high levels in mild, moderate, and severe dysplasias, but both invasive well-differentiated and invasive poorly-differentiated squamous cell carcinoma revealed reduced or no Ep-CAM expression (High et al., 1996). In contrast, Ep-CAM was reported to function as a marker for increased malignant potential in transitional cell carcinoma of the bladder, since the percentage of cells expressing Ep-CAM increases from grade I to grade III (Zorzos et al., 1995). Further, in a large cohort of primary breast tumors, high levels of Ep-CAM expression correlated with larger tumor size and infiltrated lymph nodes (Tandon et al., 1990). Thus, it seems that Ep-CAM plays a dual role in tumorigenesis, which requires further investigation.

## Immunohistochemical detection

The results obtained by immunohistochemical analysis of human tissues with a variety of mAbs against Ep-CAM were approximately identical. All published antibodies react with cryostat tissue sections, and most of them do not loose their reactivity to the antigen after paraformaldehyde (4%) fixation of tissues, but are sensitive to glutaraldehyde or formalin fixation and tissue embedding in parrafin. A simple antigen retrieval procedure (treatment with trypsin/pronase) allows immunohistochemical staining with anti-Ep-CAM antibodies of formalin fixed, and parrafin embedded tissues. Some of the mAbs were reported to have reactivity with Ep-CAM on routinely fixed histological material without pre-treatment, but the reactivity was substantially reduced. The areas of relatively low Ep-CAM expression that are well detected on frozen sections may be negative in trypsine pre-treated formalin sections, whereas the areas of high Ep-CAM expression are well detected in pre-treated formalin sections (unpublished results). Moreover, histological data obtained with low affinity mAbs (e.g. 17-1A or AUA-1) may differ from the results obtained with high affinity mAbs (e.g. 323A3 or GA733), as mainly the areas of high expression levels are detected with low-affinity mAbs in immuno-histochemistry.

## The Ep-CAM molecule

The largest open reading frame of Ep-CAM encodes for a 314 amino acid polypeptide (figure 2A), which contains a 23 amino acid leader sequence, a 242 amino acid extracellular domain with two EGF-like repeats within the cysteine-rich N-terminal part, a 23 amino acid transmembrane domain, and a 26 amino acid cytoplasmic domain.





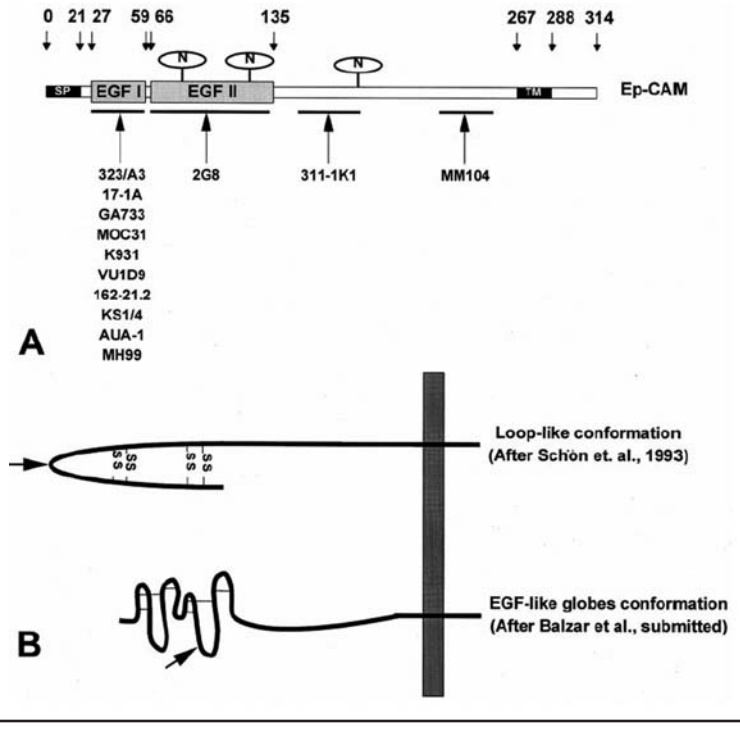
## The extracellular domain

When mice are immunized with cells derived from human carcinomas, the extracellular domain of the Ep-CAM molecule is one of the most immuno-dominant epitopes at the cell surface. This has resulted in a large number of monoclonal antibodies developed to tumor associated antigens which are specifically reactive with the Ep-CAM extracellular domain. Figure 2A presents a list of monoclonal antibodies directed against epitopes on the Ep-CAM molecule. Studies with mutant Ep-CAM molecules showed that all known mAbs have their epitopes located within one of the structural domains of the Ep-CAM extracellular domain (Balzar et al., submitted). Thus, mAbs 17-1A, 323/A3, KS1/4, GA733, MOC31, VU1D9, K931 all react with one of the partially overlapping epitopes in the first EGF-like repeat. Previous cross-inhibition studies showed that these mAbs can be subdivided into three major groups according to epitopes (Schon et al., 1993). MAb 2G8 recognises the second EGF-like repeat, while mAbs MM104, and 311-1K1 react with the cystein-poor region.

The 265 amino acid containing extracellular domain starts at the N-terminus with a signal sequence containing 11 hydrophobic residues. Signal peptidase cleavage probably occurs seven residues after the core sequence, between alanine 23 and glutamine 24 (Szala et al., 1990). Sequence analysis of the Ep-CAM extracellular domain shows that the N-terminal cysteine-rich half contains two EGF-like repeats with the motifs $CX_1CX_8CX_7CX_1CX_{10}C$ (position 27-59) and $CX_{32}CX_{10}CX_5CX_1CX_{16}C$ (position 66-135), followed by a cysteine-poor domain (Figure 2A). The first and second EGF-like repeats of Ep-CAM are closely related to respectively the fourth and fifth EGF-like repeats within the rod-like domain of nidogen (positions 776-809 and 819-889), a laminin-binding extracellular matrix protein (Simon et al., 1990; Mann et al., 1989). Within the second EGF repeat there is a high homology region (SD>7) between Ep-CAM and nidogen molecules that is not limited by cystein residues only. The second EGF-like repeat of Ep-CAM also shows homologies to the thyroglobulin type I repeat (96-160; $CX_{23}CX_{10}CX_6CX_1CX_{19}C$) which can also be found in placental protein 12 (150-225; $CX_{29}CX_{10}CX_{10}CX_1CX_{20}C$), and an inhibitor of cysteine proteinases, Equistatin, isolated from the sea anemone Actinia equina (Lenarcic et al., 1997).

In contrast to the fourth repeat of nidogen, the first repeat of Ep-CAM does not contain a potential β-hydrohylation site, which makes $Ca^{2+}$ binding by Ep-CAM highly unlikely. According to their structure and some consensus motifs, the EGF-like repeats can be subdivided into three major types I, II, and III (Apella et al., 1988). The EGF-like repeats of Ep-CAM reveal the closest homology to repeats of types II and III, which are also present in molecules like nidogen, EGF precursors, the LDL receptor, L-selectin, and PECAM (Apella et al., 1988; Davis et al., 1990). The high homology between the EGF-like repeats in Ep-CAM and the rod-like domain of nidogen would predict an Ep-CAM extracellular domain structure as depicted in figure 2B. This structure differs from a loop-like structure as was previously suggested (Schon et al., 1993; Thampoe et al., 1988; Helfrich et al., 1994). However, when the EGF-like repeats of Ep-CAM are expressed individually, they

**FIGURE 2** Structure of Ep-CAM. (A) The Ep-CAM polypeptide consists of 314 amino acids. The numbers above the short black arrows indicate the amino acid residues. The encircled N indicates sites for N-glycosylation. Below: the epitopes recognized by the indicated Ep-CAM-specific mAbs. (B) Conformation of the Ep-CAM protein. The di-sulfide bridges formed by cystein residues within the N-terminal half of the extracellular domain may result in two conformational models for the Ep-CAM molecule: a loop-like conformation (as suggested by Schon et al. [53]), and a conformation based on correct folding of the EGF-like repeats. The new data strongly points to the correctness of the latter model (Balzar et al., submitted). Black arrow indicates the potential proteolytic cleavage site at the position of Arg80.




are properly folded and reproduce the epitope profile of the respective repeat within the intact molecule (Balzar et al., submitted). Furthermore, as was demonstrated with extracellular domain deletion mutants, the repeats function as independent modules in adhesive interactions of Ep-CAM molecule (Balzar et al., submitted). This strongly suggests that the modular structure, analogous to the respective region of the nidogen rod-domain, is the conformation of the Ep-CAM molecule (Figure 2B).

Many transmembrane molecules with EGF-like repeats are involved in cell adhesion or signaling (Davis, 1990). Structural analysis of Ep-CAM suggested possible involvement of Ep-CAM in either cell-substrate or cell-cell adhesion (Simon et al., 1990). Ep-CAM was also frequently proposed to function as a growth factor receptor, but no actual experimental data has ever been presented to support this.

The sequence of the Ep-CAM molecule predicts the presence of three potential N-linked glycosylation sites (Figure 2A). It is likely that all three sites are used, and that the attached carbohydrates contain mannose oligosaccharide chains (Durbin et al., 1990; Schon et al., 1993). Blocking N-linked glycosylation using tunicamycin results in a single polypeptide chain of approximately 34 kDa (Thampoe et al., 1988). Since most carcinoma cell lines produce multiple Ep-CAM form of approximately 38, 40, and 42 kDa (Litvinov et al., 1994), the polypeptide backbone may be differently glycosylated. Whether the different Ep-CAM forms differ functionally has never been reported. Treatment of cells with O-glycanase does not cause changes in the molecular weight of the Ep-CAM molecule, suggesting that no O-linked glycosylation is involved (Thampoe et al., 1988).

The Ep-CAM molecule may exist as two additional post-translationally modified variants, namely a cleaved and a non-cleaved form. Pulse-chase studies have shown that Ep-CAM is synthesized as a 34 kDa protein, which is glycosylated to a 40 kDa glycoprotein. After longer chase periods a 32 kDa Ep-CAM form appears, which is derived from the 40 kDa form by proteolysis (Thampoe et al., 1988). As is depicted in Figure 2B, at the position arginine 80 of the Ep-CAM amino acid sequence a potential cleavage site is present for trypsin-related proteolytic enzymes (Szala et al., 1990; Bjork et al., 1993). After cleavage, the two fragments remain covalently cross-linked via disulphide-bridges under native conditions, whereas reduction of the disulphide-bridges generates a 6 kDa and 32 kDa peptide fragment. Epithelial and carcinoma derived cell lines reveal different degrees of cleavage (our unpublished results; Thampoe et al., 1988). Some cell lines contain only the 40 kDa non-cleaved Ep-CAM form, while others reveal both the 32 kDa and 40 kDa Ep-CAM protein. Moreover, the incubation of lysates from cell lines with only the 40 kDa Ep-CAM protein in the presence of lysates from cell lines having both Ep-CAM forms, results in the conversion of the 40 kDa non-cleaved protein into a 32 kDa molecule (Thampoe et al., 1988). Although cell-type specific cleavage might be a mechanism for generating specific differences in cell surface glycoproteins, the significance of cleavage for the function of Ep-CAM has never been demonstrated.

## The transmembrane and cytoplasmic domains

The transmembrane region contains 23 hydrophobic amino acid residues, and is followed by a relatively short 26-residue highly charged cytoplasmic domain. The cytoplasmic domain contains the internalization motif NPXY, which was identified in many cell surface receptors, such as the LDL receptor and mannose 6-phosphate receptor (Hopkins, 1992). The tyrosine residue present in the internalization motif is conserved in the Ep-CAM and GA733-1 cytoplasmic tails. Although GA733-1 can be phosphorylated at serine 303, the unique tyrosine residue that is present in the tails of both Ep-CAM and GA733-1 was never reported to be phosporylated (our unpublished results; Basu et al., 1995).

Besides the internalization motif, the relatively short cytoplasmic tail of Ep-CAM also contains two α-actinin binding sites (Balzar et al., 1998). Motif comparison

in the cytoplasmic tails of molecules interacting with α-actinin reveal no clear similarities, except for the arginine and lysine-rich consensus that can be found in the first Ep-CAM α-actinin binding site (RKKRMAK), and the α-actinin binding sites in ICAM-1 (RKIKK) and L-selectin (RRLKKGKKSKR) (Balzar et al., 1998). The involvement of the cytoplasmic domain for Ep-CAM function will be further discussed below.

## Ep-CAM mediated cell-cell adhesion

When Ep-CAM is expressed in L cells (murine fibroblasts incapable of intercellular adhesion), the resulting transfectants are capable of forming aggregates. The Ep-CAM molecules are present at the surfaces of interacting cells, concentrated at the cell-cell boundaries. The transfectants do not interact with parental L cells, which suggests that Ep-CAM is involved in homotypic adhesion (Litvinov et al., 1994). The adhesions mediated by Ep-CAM are relatively weak, as compared to some other adhesion molecules, such as classical cadherins. However, Ep-CAM adhesions are sufficiently strong to suppress the scattering of L cell transfectants in matrigel (Litvinov et al., 1994), or invasion of transfectants through reconstructed basement membrane (Basak et al., 1998). Based on the inhibition of metastasis of Ep-CAM transfected tumor cells in mice, as compared to control tumor cells (Basak et al., 1998), it may be claimed that Ep-CAM expression correlates with a better prognosis of carcinoma patients. Indeed, results of a study investigating markers for the assessment of nodal metastasis in laryngeal carcinomas revealed that a loss of Ep-CAM expression in the primary tumor correlated with nodal metastasis, high incidence of local recurrence, and poor prognosis (Takes et al., 1997).

When transfected in Ep-CAM-negative human epithelial cells, and expressed at average levels observed in carcinoma cell lines, Ep-CAM is capable to mediate aggregation in the absence of calcium (Litvinov et al., 1994a/b). When carcinoma cells are allowed to aggregate in the absence of calcium, the degree of aggregation can be reduced by the presence of mAb 323/A3 specific for Ep-CAM (Litvinov et al., 1994b). Furthermore, it has been shown that the aggregation of human fetal pancreatic cells can be blocked with anti-Ep-CAM mAbs KS1/4 and 323/A3 (Cirulli et al., 1998).

The cytoplasmic domain is required for Ep-CAM to mediate intercellular adhesion, since L-cell transfectants expressing Ep-CAM mutants lacking the complete cytoplasmic domain are not capable of forming aggregates, in contrast to the wild-type Ep-CAM transfectants. However, both L-cell transfectants expressing wild-type Ep-CAM or cytoplasmic domain deletion mutants are capable of binding to Ep-CAM coated solid phase, indicating that the extracellular domain is capable of forming Ep-CAM intercellular adhesions (Balzar et al., 1998). Because the cytoplasmic domain deletion mutant is not capable of inducing the aggregation of L-cell transfectants, this indicates that as long as the counter-receptor Ep-CAM molecule is "fixed", the extracellular domain is sufficient for adhesion. Thus, deletion of the cytoplasmic domain of the Ep-CAM molecule results in a protein that still has homophilic specificity, but is incapable of forming stable adhesions.

Ep-CAM is capable of associating to the actin cytoskeleton via the cytoplasmic tail. Treatment of Ep-CAM expressing cells with the actin de-polymerizing agent cytochalasin D results in the destruction of Ep-CAM mediated adhesions and internalization of the Ep-CAM molecule (Balzar et al., 1998). In contrast, agents disrupting other types of cytoskeleton (intermediate filaments or microtubuli) do not result in the internalization of Ep-CAM. Moreover, Ep-CAM molecules involved in cell-cell interactions may be discriminated from the remaining cellular pool by detergent solubility using the mild zwitterionic detergent CHAPS. It is also demonstrated that Ep-CAM interacts with the actin-based cytoskeleton via direct binding of the cytoplasmic domain to α-actinin (Balzar et al., 1998). Two domains within the cytoplasmic tail are capable of α-actinin binding, as probably both are required for interactions that secure the formation of cell-cell contact. Whether other molecules are involved in the association of Ep-CAM to the cytoskeleton needs to be further investigated.

In L-cells transfectants the opposing intercellular membranes are brought into a close proximity at Ep-CAM mediated cell-cell contacts, but no structures resembling junctional complexes, such as adherens junctions or desmosomes, were identified at these contacts (Balzar et al., 1999). In L cells co-transfected with Ep-CAM and E-cadherin both molecules localize at cell-cell contact sites, forming independent adhesions with no Ep-CAM detectable within morphologically distinguishable cadherin-mediated adherens junctions. In epithelial and carcinoma cell lines Ep-CAM and E-cadherin co-localized almost at the complete lateral membrane. However, no co-localization was observed between Ep-CAM and components of the tight junction (occludin and ZO-1), desmosomes (desmoplakins), or cell-matrix adhesions (β1-integrin) (Balzar et al., 1999). Ultra-structural analysis of the localization of Ep-CAM in normal colon tissue by immuno-electron microscopy confirmed the results of cell lines. In polarized epithelial cells of normal human colon, Ep-CAM was present at the lateral cell membrane including the adherens junction areas, but was fully excluded from the apical cell membrane, tight junctions, and desmosomes (Balzar et al., 1999).

## Function of Ep-CAM in epithelia

Inducing the internalization of Ep-CAM molecules from the cell surface by anti-Ep-CAM mAbs excluding Ep-CAM from the participation in adhesive interactions of epithelial cells, results in a decrease in the degree of aggregation (Litvinov et al., 1994b). Furthermore, disengagement of Ep-CAM mediated cell-cell interactions by Ep-CAM specific mAbs in human fetal pancreatic cells results in a significant increase in both insulin and glucagon gene transcription (Cirulli et al., 1998). This suggests that Ep-CAM internalization from the cell surface and/or functional inactivation of Ep-CAM by mAbs, may be a signaling event in the differentiation of pancreatic islet cells (Cirulli et al., 1998). In contrast, an increased expression of Ep-CAM, as demonstrated for the different stages of cervical intra-epithelial neoplasia, correlates with increased proliferation and a loss of markers for (terminal) differentiation (Litvinov et al., 1996). In normal and transformed human keratinocytes, the expression of Ep-CAM is also

inversely correlated with differentiation (Schon et al., 1995). Moreover, in transformed keratinocytes Ep-CAM directly correlates with cell proliferation (Schon et al., 1995). Thus, there is a clear connection between Ep-CAM and signaling cascades leading to a regulation of proliferation and differentiation.

Increased expression of Ep-CAM in cells interconnected by classic cadherins (E- or N-cadherin) has a negative effect on cadherin-mediated adhesions, whereas Ep-CAM mediated adhesions become predominant (Litvinov et al., 1997). Overexpression of Ep-CAM in cadherin positive cells causes no change in the total amount of cellular cadherin, but decreases the association of the cadherin/catenin complex with the cytoskeleton. As the Ep-CAM expression increases, the total amount of α-catenin decreases, whereas cellular β-catenin levels remain constant (Litvinov et al., 1997). The adhesion-defective mutant of Ep-CAM lacking the complete cytoplasmic domain has no effect on adhesions mediated by classic cadherins, suggesting that the cytoplasmic domain is involved in the regulation of cadherin-mediated adhesions. Negative effects on cadherin mediated adhesions by increased expression of Ep-CAM were also observed in L cell transfectants expressing both Ep-CAM and chimeras of E-cadherin, where the truncated cytoplasmic domain of cadherin was replaced by either complete or a fragment of (α-catenin (Winter et al., submitted). Although several important molecules present in the cytoplasmic complex of E-cadherin (such as (β-catenin and α-actinin) are excluded from the cytoskeleton anchor of the E-cadherin/α-catenin chimeras, the negative effect of Ep-CAM was still observed. This suggests that Ep-CAM affects the link between the α-catenin and F-actin by a yet unknown mechanism. The observed co-localization of Ep-CAM and E-cadherin at the lateral membrane in epithelial cells may be important for the modulating effect of Ep-CAM on cadherin-mediated junctions (Balzar et al., 1998).

Increased Ep-CAM expression correlates with proliferation, decreased cadherin-mediated adhesion, and a less differentiated phenotype suggesting that Ep-CAM regulates the strength of intercellular adhesion, and provides epithelial cells with flexible interconnections necessary for epithelial morphogenesis and tissue maintenance. This has been shown in at least one model, the ontogeny of the human pancreas. Developmentally regulated expression of Ep-CAM plays a morphoregulatory role during the development of pancreatic islets of Langerhans (Cirulli et al., 1998). Blocking of Ep-CAM by mAbs affected the transcription of genes encoding the pancreatic enzymes glucagon and insulin (Cirulli et al., 1998). The opposite, affected differentiation by forced expression of Ep-CAM, was shown for mammary gland epithelium. Human Ep-CAM ectopically expressed in the mammary glands of transgenic mice leads to ductal hyperplasia and affects the differentiation of both lobular and ductal cells (Balzar et al., submitted). In mammary glands of virgin transgenic female mice active secondary branching of ducts and enhanced proliferation (phenomena often observed in mice with forced expression of a growth factor relevant for mammary cell proliferation) was observed. For example, the ectopic expression of heregulin, a member of the neuregulin family of ligands of *erb*B receptor tyrosine kinases involved in signal transduction, induces similar mammary gland morphogenesis (Krane and Leder, 1996).





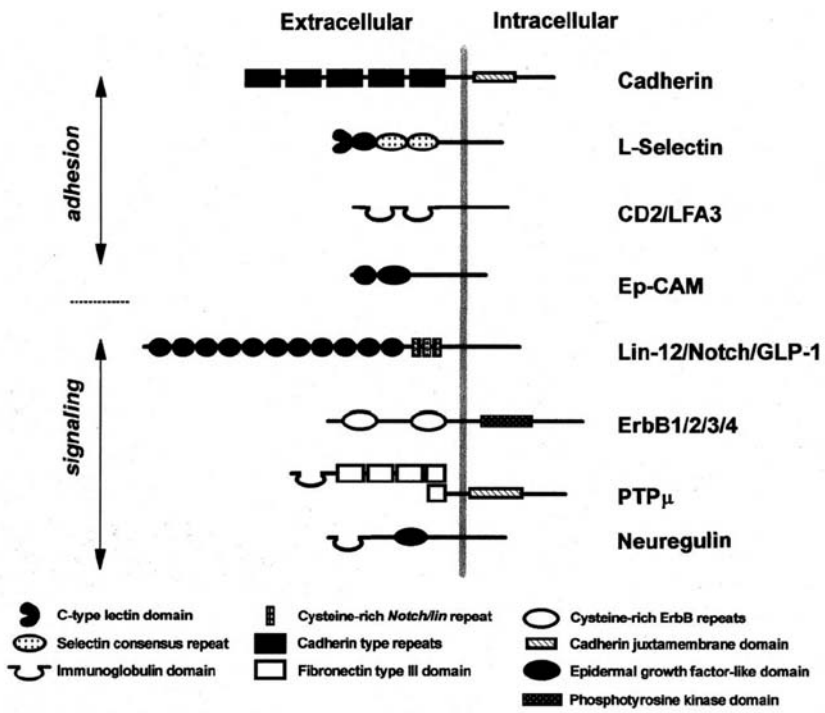
## Place of Ep-CAM among other adhesion molecules

Structurally Ep-CAM does not resemble any of the four major families of cell adhesion molecules, namely cadherins, integrins, selectins, and members of the immunoglobulin CAM super-family (Horwitz and Hunter, 1996; Aplin et al., 1998). The Ep-CAM extracellular domain contains EGF-like repeats, which are also present in selectins (Kansas et al., 1994), and members of the lin-12/notch/GLP-1 transmembrane receptor family (Artavanis-Tsakonas et al., 1995). Based on the presence of EGF-like repeats in the extracellular domain, Ep-CAM was proposed to function as a cell adhesion molecule (Simon et al., 1990), or a cell surface receptor capable of signal transduction (Fornaro et al., 1995). Despite the fact that the molecule is capable of mediating homophilic adhesive interactions, there is not sufficient evidence that Ep-CAM mediated adhesions are required for epithelial cell support. Other molecules, such as receptor protein tyrosine phosphatases (Brady-Kalnay et al., 1995), molecules involved in defining cell fate of the lin-12/notch/GLP-1 receptor family (Artavanis-Tsakonas et al., 1995), and molecules involved in juxtacrine signaling are capable of adhesive interactions as well. The border between intercellular adhesion and juxtacrine signaling, as two different functions, is becoming less clear, since many "classical" adhesion molecules (e.g. N-CAM and E-cadherin) were demonstrated to function as signaling morphoregulators (Cunningham, 1995). Figure 3 shows a comparison of Ep-CAM with other cell surface receptors participating in either adhesion or juxtacrine signaling indicating that some of the receptors share structural similarities with Ep-CAM. A secreted form, typical for most juxtacrine signaling molecules, is not reported for Ep-CAM. The recently reported data concerning the organization of Ep-CAM mediated adhesions suggest that it resembles more a typical adhesion molecule, being connected to actin microfilaments (Balzar et al., 1998). However, the exact role of Ep-CAM in epithelial cell functioning remains to be further investigated.

## Immunotherapy targeting Ep-CAM

The generation of mAbs for immunopathological diagnosis of cancer resulted in the discovery of antigens expressed by tumor cells (Koprowski et al., 1979; Oosterwijk et al., 1986). Many of these antibodies were specific for cell surface antigens of the tumor cells. Ep-CAM was one of the most immunogenic proteins to which antibodies were generated in mice immunized with carcinoma cells. Since Ep-CAM localizes at the cell surface of most carcinomas, the molecule is an attractive target for immunotherapy. Ep-CAM directed therapy against carcinomas started with treatment using unconjugated mAbs, which has resulted in some anti-tumor effects (Ragnhammar et al., 1993; Mellstedt et al., 1991; Weiner et al., 1993). However, after a decade of mAb therapy on solid tumors in patients, it can be concluded that naked mAbs generally lack the efficacy to eradicate solid established tumor masses (Lobuglio and Saleh, 1992). This lack of efficacy is believed to be caused by very poor localization of mAbs in larger tumors. The capacity of mAbs to eliminate single tumor cells in carcinoma patients may prevent the outgrowth of metastasis and could therefore be crucial for the survival of patients.

FIGURE 3 Schematic domain structure of Ep-CAM in comparison to other cell surface proteins involved in either cell-cell adhesion or transmembrane/ juxtacrine signaling.




Indeed, when Dukes C colorectal carcinoma patients with surgically resected solid tumor were treated for minimal residual disease with the Ep-CAM specific mAb 17-1A, this resulted in a 30% increase in the 7-year survival of 17-1A treated patients as compared to non-treated patients (Riethmuller et al., 1998). Since mAb 17-1A is a low affinity antibody, better immunotherapy results may be obtained using high affinity mAbs (Velders et al., 1995). However, it has been demonstrated that treatment with high affinity mAbs, such as GA733 and 323/A3, may cause damage to Ep-CAM positive normal epithelial tissues resulting in toxicity problems (Herlyn et al., 1991).

Recently, other strategies targeting Ep-CAM have been developed for the treatment of carcinomas, although most of them are only tested in pre-clinical studies. To provide better mAb tumour retention, high-affinity recombinant phage antibodies directed against Ep-CAM were developed (Roovers et al., 1998). Recombinant bispecific single chain antibodies specific for both Ep-CAM and the CD3/T cell receptor complex demonstrated (approximately 1000-fold) higher specific cytotoxicity against tumor cells in vitro (Kufer et al., 1997; Helfrich et al., 1998). Therefore, these bispecific single chain antibodies may result in improved therapy of minimal residual disease by retargeting activated T cells to micrometastatic carcinoma cells (Kufer et al., 1997). Activation of Ep-CAM specific T cells was also achieved both in vitro and in vivo by peptide vaccination (Ras et al., 1997) or DNA vaccination (our unpublished results). To overcome problems with respect to MHC-restricted target recognition by T-cells, and down-regulation of MHC molecules expression by tumor cells, chimeric T cell receptors specific for Ep-CAM were generated that were able to lyse tumor cells in vitro (Van Ratingen et al., submitted). Expression of these chimeric T cell receptors in cytotoxic T lymphocytes from patients by retroviral transduction, might be valuable for T cell based immunotherapy of carcinoma patients.

Since Ep-CAM has high expression levels in some normal human adult tissues (e.g. colon), one can expect toxicity as a side effect of a therapeutic approach. Indeed, infusion of carcinoma patients with high affinity mAbs against Ep-CAM (e.g. GA733) was accompanied by mild and short-lasting gastro-intestinal toxicity (Herlyn et al., 1991). Thus, appropriate pre-clinical models are required to study the efficacy and toxicity of newly developed strategies. Transgenic mice expressing human Ep-CAM in epithelial tissues might serve as such a model and have recently been generated (Balzar et al., submitted).

## Concluding remarks

Many classic cell adhesion molecules, i.e..integrins or selectins, have recently been identified as components of signaling cascades. Moreover, signaling from various cell adhesion molecules is regarded as a major mechanism of controlling processes as proliferation, differentiation, and (programmed) cell death (Edelman, 1986; Gumbiner, 1996). The function of CAM's is not only mechanically attach cells to adjacent cells and substrates, but also to respond to changes in the environment and adjust the biological response of cell through outside-in signaling. Since the adhesions mediated by Ep-CAM are relatively weak, as compared to cadherin-mediated adhesions or desmosomes, one may question their relevance for maintaining the epithelial tissue architecture. On the other hand, Ep-CAM does resemble "classic" adhesion molecules, since it associates to the cytoskeleton and forms independent adhesions (Balzar et al., 1998/1999). Although no true junctions are observed for Ep-CAM, the molecule is clearly capable of moving adjacent cell membranes together at the areas of reciprocal homotypic interactions.

The ability of Ep-CAM to regulate cadherin-mediated adhesions, tissue morphogenesis and the transcription of genes (as was found for the pancreatic enzymes glucagon and insulin) indicates that the molecule is involved in signal transduction regulating epithelial morphogenesis.

The (over-) expression of Ep-CAM correlates with both benign and malignant hyper- proliferation of epithelial cells. However, the exact role of Ep-CAM in tumor development remains unclear. Ep-CAM-mediated cell-cell adhesions prevent cell scattering, suggesting that the molecule might prevent metastasis. However, the negative

effect of Ep-CAM on cadherin-mediated adhesions may actually promote invasion and metastasis from carcinoma nodules. Thus, the dualistic role of Ep-CAM in tumor development requires further investigation.

Since Ep-CAM is expressed by most carcinomas, the molecule has attracted the attention in the field of cancer immunotherapy. Two important issues must be addressed for major improvements in Ep-CAM targeted immunotherapy. First, Ep-CAM expression is heterogeneous in carcinomas, which may cause partial eradication of the tumor after treatment. Second, since Ep-CAM is an epithelial differentiation antigen, and not a tumor-specific antigen, immunotherapy may have severe side effects causing toxicity to normal tissues. An increased interest for the biological properties of the Ep-CAM molecule (as can be observed from recent publications) will ultimately contribute to a better understanding of the function of Ep-CAM in development, maintenance, and tumorigenesis of epithelial tissues. Moreover, it will also contribute to development of new therapeutic strategies employing this antigen as a target.

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Chapter 3

# Ep-CAM affects the E-cadherin mediated adhesion

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## Abstract

The contribution of noncadherin-type, $Ca^{2+}$-independent cell-cell adhesion molecules to the organization of epithelial tissues is, as yet, unclear. A homophilic, epithelial $Ca^{2+}$-independent adhesion molecule (Ep-CAM) is expressed in most epithelia, benign or malignant proliverative lesions, and during embryogenesis. Here we demonstrate that ectopic Ep-CAM, when expressed in cells interconnected by classic cadherins (E- or N-cadherin), induces segregation of the transfectants from the parental cell type in co-aggregation assays and in cultured mixed aggregates, respectively. In the latter assay, Ep-CAM-positive transfectants behave like cells with a decreased strength of adhesion as compared to the parental cells. Using transfectants with an inducible Ep-CAM cDNA construct, we demonstrate that increasing expression of Ep-CAM in cadherin-positive cells leads to abrogation of adherens junctions. Overexpression of Ep-CAM has no influence on the total amount of cellular cadherin, but affects the interaction of cadherins with the cytoskeleton, since a substantial decrease in the detergent-insoluble fraction of cadherin molecules was observed. Similarly, the detergent-insoluble fractions of α- and β-catenins decreased in cells overexpressing Ep-CAM. While the total β-catenin content remains unchanged, a reduction in total cellular α-catenin is observed as Ep-CAM expression increases. As the cadherin-mediated cell-cell adhesions diminish, Ep-CAM-mediated intercellular connections become predominant. An adhesion-defective mutant of Ep-CAM lacking the cytoplasmic domain has no effect on the cadherin-mediated cell-cell adhesions. The ability of Ep-CAM to modulate the cadherin-mediated cell-cell interactions, as demonstrated in the present study, suggests a role for this molecule in development of the proliferative, and probably malignant, phenotype of epithelial cells, since an increase of Ep-CAM expression was observed in vivo in association with hyperplastic and malignant proliferation of epithelial cells.

## Introduction

Tissue and organ morphogenesis can be viewed as the result of interactions of various cell populations. One important type of intercellular interactions involved in the processes of tissue morphogenesis, morphogenetic movement of cells, and segrega-

tion of cell types, are adhesions mediated by cell adhesion molecules (Steinberg and Pool, 1982; Edelman, 1986; Cunningham et al., 1995; Takeichi et al., 1995; Gumbiner, 1996). Except for their direct mechanical role as interconnectors of cells and connectors of cells to substrates, cell adhesion molecules are also believed to be responsible for a variety of dynamic processes including cell locomotion, proliferation, and differentiation. There is also evidence that the adhesion systems within a cell may act as regulators of other cell adhesions, thereby offering a means of signaling that is relevant for rearrangements in cell or tissue organization (Edelman, 1993; Rosales et al., 1995; Gumbiner, 1996).

In many tissues, a critical role in the maintenance of multicellular structures is assigned to cadherins, a family of $Ca^{2+}$-dependent, homophilic cell-cell adhesion molecules (Takeichi et al., 1991; Gumbiner, 1996). In Epithelia this critical role belongs to E-cadherin, which is crucial for the establishment and maintenance of epithelial cell polarity (McNeil et al., 1990; Näthke et al., 1993), morphogenesis of epithelial tissues (Wheelock and Jensen, 1992; Larue et al., 1996), and regulation of cell proliferation and programmed cell death (Hermiston and Gordon, 1995; Hermiston et al., 1996; Takahashi and Suzuki, 1996; Wilding et al., 1996; Zhu and Watt, 1996). Expression of different types of classic cadherin molecules (Nose et al., 1988; Friedlander et al., 1989; Daniel et al., 1995), and even quantitative differences in the levels of the same type of cadherin (Steinberg and Takeichi, 1994) may be responsible for segregation of cell types in epithelial tissues. The phenotype of epithelial cells may be modulated by expression of combinations of different types of cadherins (Marrs et al., 1995; Islam et al., 1996). However, cadherins represent only one of the intercellular adhesions systems that are present in epithelia, along with adhesion molecules of the immunoglobulin superfamily, such as carcinoembryonic antigen (Benchimol et al., 1989) and others. The actual contribution of $Ca^{2+}$-independent non-junctional adhesion molecules to the formation and maintenance of the epithelial tissue architecture and epithelial cell morphology is not clear.

We have recently demonstrated that a 40-kD epithelial glycoprotein, which we have designated epithelial cell adhesion molecule (Ep-CAM) (Litvinov et al., 1994a), may perform as a homophilic, $Ca^{2+}$-independent intercellular adhesion molecule, capable of mediating cell aggregation, preventing cell scattering, and directing cell segregation. This type I transmembrane glycoprotein consists of two EGF-like domains followed by a cysteine-poor region, a transmembrane domain, and a short (26 amino acid) cytoplasmic tail, and is not structurally related to the four major types of CAMs, such as cadherins, integrins, selectins, and the immunoglobulin superfamily (for review see Litvinov, 1995). Ep-CAM demonstrates adhesion properties when introduced into cell systems that are deficient in intercellular adhesive interactions (Litvinov et al., 1994a). However, the participation of the Ep-CAM molecule in supporting cell-cell interactions of epithelial cells was not evident (Litvinov et al., 1994b).

Most epithelial cells types coexpress E-cadherin (and sometimes other classic cadherins) and Ep-CAM during some stage of embryogenesis. In adult squamous epithelia, which are Ep-CAM negative, de novo expression of this molecule is associated with metaplastic or neoplastic changes. Thus, in ectocervical epithelia, expression of Ep-CAM occurs in early pre-neoplastic lesions (Litvinov et al., 1996); most squamous carcinomas of the head and neck region are Ep-CAM positive (Quak et al., 1990), and basal cell carcinomas are Ep-CAM positive in contrast to the normal epidermis (Tsubura et al., 1992).

In many tumors that express Ep-CAM heterogeneously, an Ep-CAM-positive cell population may be found within an Ep-CAM negative cell population, with both cell types expressing approximately equal levels of cadherins, as illustrated in Figure 1A by a case of basal cell carcinoma. In glandular tissues such as gastric epithelium, which are low/negative for Ep-CAM, expression of Ep-CAM is related to the development of early stages of intestinal metaplasia (our unpublished observation). Even in tissues with relatively high Ep-CAM expression, such as colon, the development of polyps is accompanied by an increase in Ep-CAM expression (Salem et al., 1993). In intestinal metaplasia one may observe Ep-CAM-positive cells bordering morphological normal cells that are Ep-CAM negative (as illustrated in Figure 1B). Ep-CAM-positive cells bordering Ep-CAM-negative epithelial cells may also be found in some normal tissues such as hair follicles (Tsubura et al., 1992).

From the examples presented, an increased or the novo expression of Ep-CAM is often observed in epithelial tissues in vivo.Expression of an additional molecule that may participate in cell adhesion in the context of other adhesion systems may have certain effects on the cell-cell interactions. Therefore, we have investigated whether the increased/de novo expression of Ep-CAM in epithelial cells (a) has any impact on interactions of positive cells with the parental Ep-CAM-negative cells, and (b) modulates in any way intercellular adhesive interactions of cells interconnected by E-cadherin, which is the major morphoregulatory molecule in epithelia.

Here we demonstrate that expression of Ep-CAM by some cells in a mixed cell population expressing classical cadherins induces segregation of the Ep-CAM-positive cells from the parental cell population due to a negative effect on cadherin junctions caused by expression of Ep-CAM. The cadherin-modulating properties observed for Ep-CAM suggest a role for this molecule in the development of a proliferative and metaplastic cell phenotype, and probably in the development and progression of malignancies.

# Materials and Methods

## DNA Constructs

The SmaI-BglII fragment of human Ep-CAM cDNA was used for the wild-type Ep-CAM expression construct, as reported earlier (Litvinov et al., 1994a). Mutant Ep-CAM with a truncated cytoplasmic tail (Mu1) was generated by PCR amplification of the fragment of Ep-CAM cDNA corresponding to amino acids 1-289. The wild-type and mutant Ep-CAM cDNAs were cloned into pCEP4 and pMEP4 vectors (Invitrogen BV, Leek, The Netherlands) under control of cytomegalovirus or metallothionein II promotors, respectively. Both vectors used contain the Epstein-Barr virus's origin of replication and the EBNA-1 gene, which allow both episomal replication and self-support of the plasmid to large copy numbers in human cells. In murine cells, both vectors integrate into the cellular DNA.

## Cells and Transfections

The murine E-cadherin transfected L (LEC) cells (clone LUN.6), and the HCA clonal cell line isolated from the SV-40 immortalized normal epithelial cell line HBL-100 were recloned in our laboratory before transfections (provided by J. Hilkens, The Netherlands Cancer Institute, Amsterdam, The Netherlands). L cells (L929), colon carcinoma cell line (LS180), mammary carcinoma cells (MCF-7), and the pancreatic carcinoma cell line (CAPAN) were obtained from American Type Culture Collection (Rockville, MD). The normal human mammary-epithelium derived cell line (RC-6) and the squamous carcinoma cell line (U2) were cultured in DME with 10% FCS (provided by E. Schuuring, Leiden University, Leiden, The Netherlands), as were all cell lines. Cells were transfected using the DOTAP reagent (Boehringer Mannheim Corp., Mannheim, Germany) as described earlier (litvinov et al., 1994a). For murine cells, the stable clones obtained were either grown as isolated clones, or were pooled; human transfected cell lines were continuously cultered in the presence of selection marker (Hygromycin, 1 mg/ml; Boeringer Mannheim Corp.). 48 h before the experiments, cells were passaged into medium without hygromycin. To induce the expression of cunstructs under the control of the metallothionein promotor (pMEP4 vector), $CdCl_2$ was added to the culture medium at concentrations of 2-50 μM depending on the cell line.

## Cell Aggregation Assay

Cell aggregation assays were performed as described earlier (Litvinov et al., 1994a). The cells were detached with either TC treatment (Hank's buffer with 0.01% trypsin and 1 mM $CaCl_2$), or by 0.2% EDTA in Hank's buffer. The degree of cell aggregation was calculated as D = (N0 - Nt)/N0, where Nt is the number of remaining particles at the incubation time point t, and N0 is the initial number of particles corresponding to the total number of cells.

## Labeling of Cells and Sorting Experiments

For cell sorting experiments, the cells were labelled according to the manufacturer's protocol with fluorescent dyes (PKH-2 [green fluorochromo]. or PKH-26 [red fluorochrome]; Zynaxis Cell Science Inc., Malvern, PA), that incorporate the menbrane's lipid bilayer. The fluorochromes provide a stable labeling of living cells and do not interfere with with either cell surface proteins or with the cells behaviour and interactions (Horan et al., 1990; Litvinov et al., 1994a). Cell sorting/patterning experiments were performed as described elsewhere (Nose et al., 1988; Litvinov et al., 1994). Briefly, cells were dissociated with TC, washed in Dulbecco's PBS, labeled with one of the fluorochromes, washed three times in 50% FCS in DME, resuspended in DME containing 0.8% FCS and 1 mg/ml DNAse (Boehringer Mannheim Corp.), mixed at various ratios depending on the experiment, and allowed to aggregate as described for the aggregation assays. After 1-2 h of aggregation, the suspension of aggregates was analyzed under a confocal microscope (model MRC-600; Bio-Rad Laboratories, Carlsbad, CA). Images from different areas of the preparation were taken, and the number of cells of each color in the aggregates was determined. To study the segregation/patterning of cells in aggregates, the two cell types labeled with different fluorescent dyes were mixed at equal ratios, spun down, and allowed to aggregate in the pellet during the next 2 h at 37oC. The large aggregate formed was mechanically dispersed into smaller fragments, which were further cultured in suspension on a rotating platform (at 140 rpm). After 30 min and 24 h of culture, respectively, samples of aggregates were fixed with 4% formaldehyde in PBS/1 mM $CaCl_2$, and analyzed with confocal microscopy.

## Antibodies

Anti-Ep-CAM antibodies were 323/A3 (against human Ep-CAM; Litvinov et al., 1994) and G8.8 (against murine Ep-CAM; Nelson et al., 1996). The antibodies against epitopes in extracellular domains of human E-cadherin and human P-cadherin were obtained from Thamer diagnostica BV (clones HECD-1 and NCC-CAD-299, respectively; Uithoorn, The Netherlands). A mAb against an epitope in the extracellular domain of N-cadherin (clone GC-4) was obtained from Sigma Chemical Co. (St. Louis, MO). Antibody to the cytoplasmic domain of classical cadherins (clone CH-19; Sigma Chemical Co.) strongly reactive with N-cadherin and weak with other cadherin types, was used for immunoblotting experiments. Two mAbs against murine E-cadherin were used: one reactive with an epitope in the cytoplasmic domain (clone 36; Transduction Laboratories, Lexington, KY), and one reactive with the extracellular domain of the molecule (ECCD-2; Takara Shuzo Co., Shiga, Japan). Antibodies to α-catenin (clone αCAT-7A4; Zymed Laboratories, South San Francisco, CA) and β-catenin (clone 14; Transduction laboratories) both had cross-species reactivity. For immunoblotting experiments on immunoprecipitates, a polyclonal rabbit anti-serum to α-catenin was used (gift of J. Behrens, Max Delbruck Center for Molecular Medicine, Berlin, Germany).

## Immunofluorescence Microscopy

Cells growing on either glass slides or in multiwell chamber slides (Nunc, Naperville, IL) were fixed for 10 min in -20°C methanol, rinsed quickly in -20°C acetone, and allowed to dry. The preparations were blocked in 5% skim milk solutions in PBS for 30 min at 37°C, and indirect immunofluorescent staining was performed using a specific mAb and a goat anti-mouse IgG-FITC conjugate (Southern Biotechnologies, Birmingham, AL). The preparations were analyzed with a confocal microscope.

## Reflection Contrast Microscopy and Electron Microscopy

Both were performed as described (Prins et al., 1993). Cell aggregates were fixed with 2% paraformaldehyde/1.25% glutaraldehyde, postfixed with 1% Osmium tetroxide, embedded in Epon, and ultrathin sectioned. The preparations were examined, respectively, with a microscope equipped for epi-illumination (model Orthoplan; Leitz, Wetzlar, Germany), and an electron microscope (model CM10; Philips Electron Optics, Eindhoven, The Netherlands).

## Flow Cytometry

The expression of Ep-CAM was detected on living cells by using F(ab) fragments of mAb 323/A3 directly conjugated with 5(6)-Carboxy-fluorescein-N-hydroxy-succinimide ester (FLUOS), a FITC-like fluorochrome. The conjugation was performed according to the manufacturer's protocol (Boehringer Mannheim Corp.). To detect the cell surface expression of murine E-cadherin or human N-cadherin the mAbs ECCD-2 and GC-4 were used. The bound mAb was detected with an appropiate species-specific anti-IgG FITC conjugate (Southern Biotechnologies).

## Cell lysis and Cell Extraction with Detergents

Cells of various transfected cell lines were seeded at equal density on 10-cm Petri-dishes 48 h before lysis and cultured the last 24 h in either the presence or absence of $Cd^{2+}$ cations in the medium. To prepare total cell lysates, cell on the dishes were rinsed twice with ice-cold PBS (pH 7.4), and lysed in 1 ml boiling 1% SDS/10 mM EDTA. The extraction of detergent-soluble cadherins and catenins was performed as described by Hinck et al. (1994). Cells were rinsed three times with cold PBS, and 2 ml of cold extraction buffer (50 mM Tris/HCl, pH 7.0, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose, and protease inhibitor [complete; Boehringer Mannheim Corp.]) were added to the cells. Cells were incubated for 45 min on a shaker at 4°C, detached with a scraper, collected, and spun down in a centrifuge for 10 min at 15,000 rpm (5415C; Eppendorf scientific, Inc., Hamburg, Germany). The pellet was lysed with boiling 1% SDS/10 mM EDTA and then boiled for 5 min. The lysates obtained with boiling SDS were spun through a spin column (Quiashredder; Quiagen Inc., Hilden, Germany) to reduce the viscosity caused by DNA, and the preparations obtained were used for gel electrophoresis. The protein content was determined for each sample by measuring the optical density at 224 nm of a sample aliquot prediluted with 4% SDS.

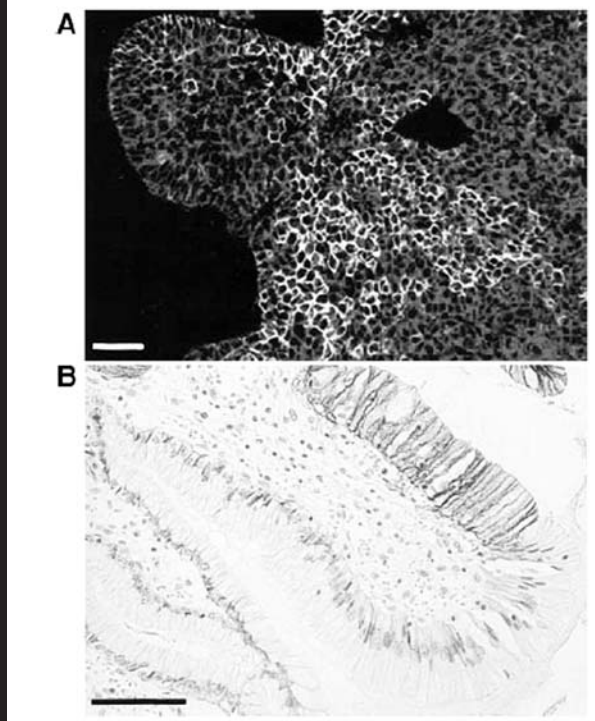




## Immunoprecipitation and Immunoblotting

Cells were lysed with extraction buffer (as described above), the lysates were clarified by centrifugation in a centifuge for 10 min at 15,000 rpm (5415C; Eppendorf Scientific Inc), and used for immunoprecipitation. 5 µg of a specific mAb was added to a lysate from $5x10^6$ cells in 1 ml, and incubated at 4°C in an end-to-end rotator for 1 h. 100 (l of 50% protein G-sepharose slurry (Pharmacia Biotech, Inc, Uppsala, Sweden) was then added to each tube, and the tubes were further incubated for 1 h. The immunoabsorbent beads were washes four times with 1 ml of the extraction buffer, and the precipitates were dissolved in Laemmli's sample buffer (1% SDS, 10% glycerol, 10 mM EDTA, 125 mM Tris/HCL, pH 6.8) containing 2% 2-mercapto-ethanol, boiled for 10 min, and used for further immunoblotting experiments. Immunoblotting was performed as earlier described (Litvinov et al., 1994), using the alkaline-phosphatase Protoblot system (Promega Corp., Madison, WI), or the enhanced chemiluminescent detection system (Amersham Intl., Little Chalfont, UK).

**FIGURE 1** Examples of Ep-CAM expression by some cells within an E-cadherin-positive cell population. (A) Heterogeneous expression of Ep-CAM in a basal cell carcinoma, as detected by immunofluorescent staining with mAb 323/A3 to Ep-CAM (green fluorescence); red fluorescence indicates the expression of E-cadherin (mAb HECD-1). (B) The de-novo expression of Ep-CAM in gastric mucosa in relation to the development of intestinal metaplasia; immunohistochemical staining with mAb 323/A3. Note the bordering of Ep-CAM positive and negative cells.

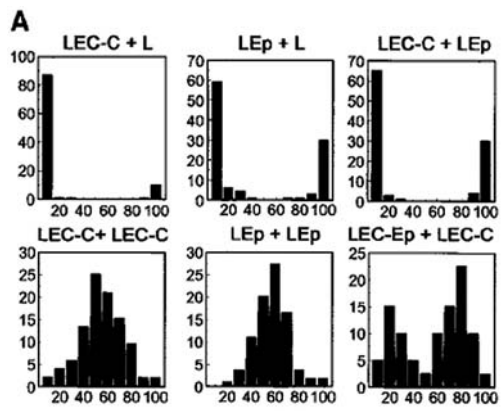


# Results

## Ep-CAM directs segregation within E-cadherin positive cells

To investigate to what extent the expression of Ep-CAM in epithelia modulates the interactions of Ep-CAM-positive cells with neighboring Ep-CAM negative cells, we have established a simple model: murine fibroblast L cells transfected with murine E-cadherin, and a derived cell line additionally supertransfected with Ep-CAM. The murine L cell fibroblasts have previously been used to demonstrate the adhesion properties of both E-Cadherin (Nagafuchi et al., 1987), and Ep-CAM (Litvinov et al., 1994); these cells are able to support the functional activity of both molecules and do not express endogenous cadherins or murine Ep-CAM (as was tested with a mAb specific for murine Ep-CAM; data not shown).

**FIGURE 2** Cell segregation directed by Ep-CAM. L cells were transfected with cDNA for E-cadherin (LEC) or Ep-CAM (LEp), and the E-cadherin transfectants were additionally supertransfected with either Ep-CAM cDNA (LEC-Ep) or blank vector (LEC-C). (A) Pairs of transfected cells were tested in co-aggregation assays: dispersed cells of two types (Type 1 + Type 2), each labeled with a different fluorescent dye, were mixed at equal concentrations. After 2 h of culturing in suspension, cell aggregates consisting of >10 cells were analyzed for the presence of cells of each type. The data is presented as percentage of aggregates (y-axis) containing the respective percentage of the Type 2 cells (x-axis). (B) Expression of E-cadherin and Ep-CAM in the transfectants, as determined by immunoblotting in total cell lysates using antibodies to E-cadherin and human Ep-CAM (323/A3), respectively.




LEC cells demonstrated all morphological changes related to E-cadherin expression reported for other E-cadherin transfectants of L-cells Nagafuchi et al., 1987; Chen and Öbrink, 1991; Wesseling et al., 1996). LEC cells were supertransfected with either Ep-CAM cDNA under the control of the constitutive cytomegalovirus promotor (LEC-Ep cells), or with the blank pCEP4 expression vector (LEC-C cells). These transfected cell lines were established without clonal isolation and represented a mix of >200 individual clones from each transfection. Additionally, L cells transfected only with the Ep-CAM cDNA were established (LEp cells).

When mixed in suspension, neither LEC nor LEp cells interacted with the parental L cells. The LEC and LEp cells also did not interact with one another, as was tested in coaggregation assays performed to exclude

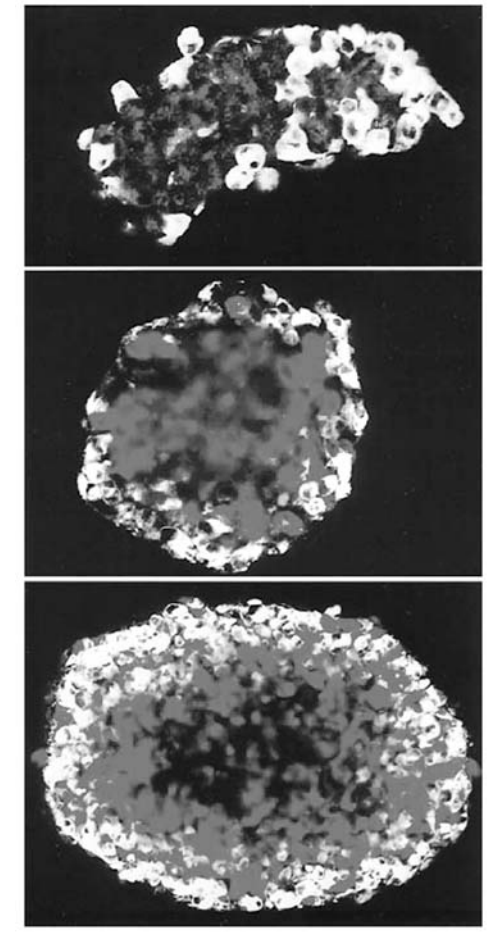




possible heterotypic interactions between Ep-CAM and E-cadherin (Figure2). LEC-C and LEC-Ep cells, when mixed, showed segregation in suspension coaggregation assays (Figure 2). Although some aggregates contained cells of both types, and the segregation could be described as partial only, the two cell types did show a clear preference for independent aggregation.

Immunoblotting revealed that LEC-C and LEC-Ep cells expressed approximately equal levels of E-cadherin molecules (Figure 2B). Since even relatively minor differences in the levels of cadherin expression may affect cell-cell interaction (Steinberg and Takeichi, 1994), we repeated this experiment with five pairs of cell lines obtained from several independent transfections. The degree of cell segregation varied (as estimated by the relative proportion if mixed aggregates formed), and correlated positively with the level of Ep-CAM expression at the surface of double transfectants (not shown).

**FIGURE 3** Segregation of Ep-CAM positive LEC cell transfectants from parental cells in multi-cellular aggregates. LEC-C and LEC-Ep cells, labeled with the fluorescent dyes PKH-26 and PKH-2, respectively, were mixed at a 1:1 ratio, sedimented, and allowed to form an aggregate. This aggregate, in which both cell types were represented in a random pattern, was mechanically dispersed, and the smaller aggregates obtained were further cultured in suspension for 24 h, fixed, and analyzed. The micrographs present optical cross-sections at the equatorial area of the aggregates after 24 h, as seen with a confocal microscope. The artificial colors were assigned to the cells depending on the color of the fluorochrome and the cell type: LEC-C (*red*); LEC-Ep (*white*) cells. The figures show similar cell patterning in different size aggregates in the range of <100 to ~1,000 cells. A dark area in the middle of some aggregates is an optically non-transparent zone.



When mixed as monocellular suspensions and sedimented together, LEC-C and LEC-Ep cells were able to establish connections in the pellet. The resulting large aggregate, formed by randomly distributed cells of both types, was mechanically dispersed into a number of smaller aggregates. After 24 h of culturing these aggregates in suspension, it was found that the LEC-C cells formed the tight core of the aggregates, with the LEC-Ep cells forming the external layer. This structure was observed for all aggregates irrespective of their size, with the latter ranging from 100 to more than 1,000 cells (Figure 3). When either LEC-C or LEC-Ep cells were mixed with the differentially labeled cells of self-type, no cell patterning was observed, indicating that segregation was unrelated to the labeling and other experimental procedures.

The positioning of cells in mixed structures (to the inner or outer layer, respectively) is determined by the relative strength of intercellular connections between the cells of each type (Steinberg and Pool, 1982; Foty et al., 1996). In this respect, the Ep-CAM transfectants of E-cadherin-positive cells interacted with the parental cells as did cells with a relatively decreased strength of cell-cell adhesion. In contrast to what could be expected from the expression of an additional intercellular adhesion molecule, the overall strength of intercellular adhesion among LEC cells seemed to become reduced upon the expression of Ep-CAM. Since Ep-CAM is a relatively weak adhesion molecule compared to E-cadherin (Litvinov et al., 1994a), it is highly suggestive that the E-cadherin-mediated cell-cell adhesions in LEC-Ep cells were negatively affected because of Ep-CAM expression.

## Expression of Ep-CAM suppresses the E-cadherin-mediated cell aggregation

Ep-CAM-mediated aggregation in suspension is rather slow, with ~40% of aggregation reached in 120 min for L cells with high levels of Ep-CAM expression (Litvinov et al., 1994a). In contrast, E-cadherin-mediated cell aggregation is relatively fast, as L cell transfectants expressing E-cadherin reach the plateau level of aggregation (50-80%) within 30 min, at which time the aggregation mediated by Ep-CAM is hardly noticable (not shown). If Ep-CAM is indeed able to negatively affect the E-cadherin-mediated cell-cell adhesion, the aggregation rates of E-cadherin/Ep-CAM transfectants should inversely correlate with the levels of Ep-CAM expression.

To investigate this, LEC cells with inducible Ep-CAM expression (LEC-MEp) were established by introducing the Ep-CAM cDNA under the control of an inducible metallothionein promotor. This promotor can give high levels of expression upon induction, but is leaky, with the construct being expressed to a certain level without induction with heavy metal ions. We selected two individual clones (LEC-MEp.6 and LEC-MEp.2) with different basal levels of Ep-CAM expression. Both the total number of Ep-CAM molecules (Figure 4A), as well as Ep-CAM expressed at the cell surface (Figure 4B), could be gradually induced in cells of isolated clones by increasing the Cd2+ concentrations in the medium ≤ 10 μM. A control cell line was prepared by transfecting a blank pMEP4 vector into LEC cells (LEC-MC cells).





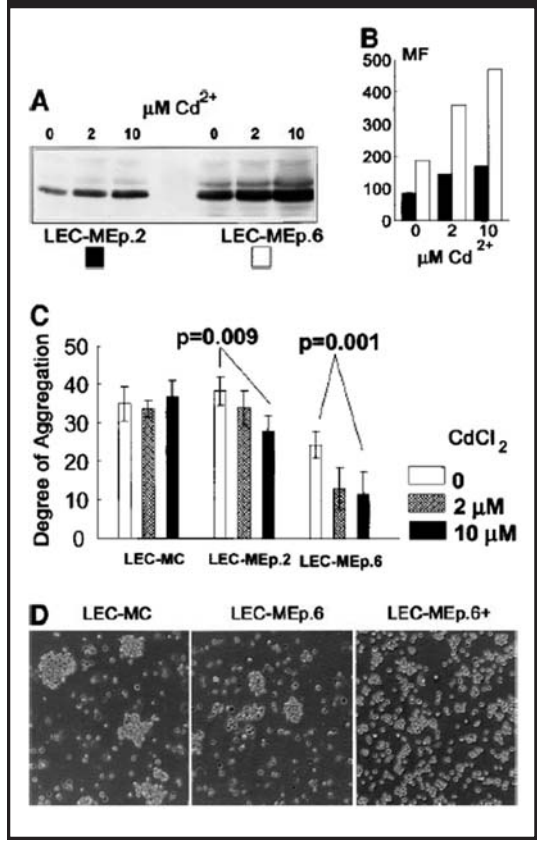



FIGURE 4 Effect of increasing expression of Ep-CAM on cell-cell interactions in L cell E-cadherin transfectants (LEC). LEC cells were super-transfected with Ep-CAM cDNA under the control of the methallothionein promotor (clones LEC-Mep.2 and LEC-Mep.6), or with blank vector (LEC-MC). Induction of Ep-CAM expression with CdCl2 for 24 h resulted in an increased total Ep-CAM (A, immunoblot of total cell lysates) and in an increased presence of Ep-CAM molecules at the cell surface (B, flow cytometry with anti-Ep-CAM F(ab)-FLUOS conjugate). Cells detached with TC treatment were allowed to aggregate in suspension for 30 min. in the presence of Ca2+, and the degree of cell aggregation was determined (C). The statistical significance of the observed differences in aggregation rates was determined using Student's t-test (p). Where indicated, cells were cultured for 24 h before the assay in the presence of CdCl2 in culture medium. The morphology of aggregates formed in 30 min. by LEC-MC and LEC-Mep.6 cells (the latter non-induced and induced with 10 (M CdCl2).

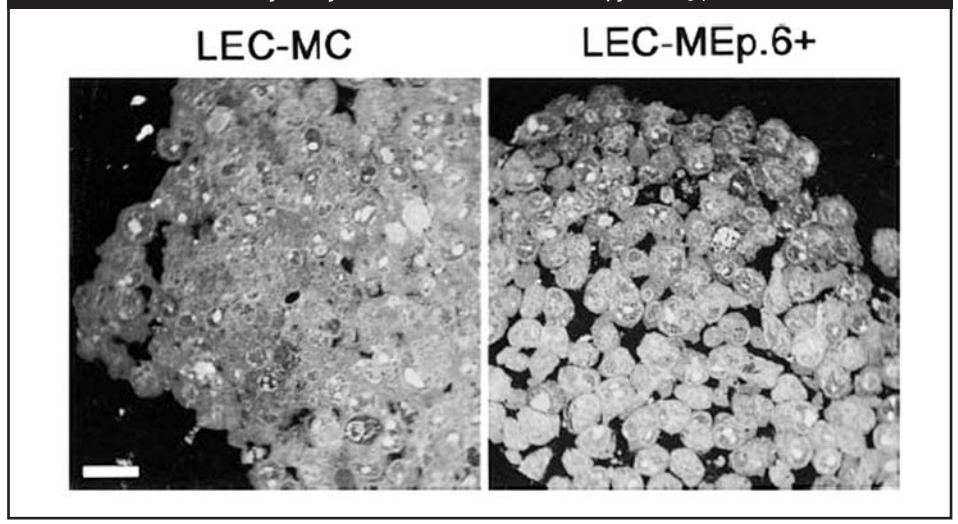
After being cultured for 24 h at different concentrations of $Cd^{2+}$, the cells were detached with EDTA and subjected to aggregation assays in the presence of $Ca^{2+}$ to allow both Ep-CAM and E-cadherin adhesions systems to participate in cell aggregation. The results showed that prior culturing in the presence of CdCl2 had no effect on the aggregation rate of the control LEC-MC cells, whereas the aggregation of Ep-CAM transfectants decreased in direct proportion the levels of Ep-CAM expressed (Figure 4C). The differences in aggregation were reproducible in independent experiments and were highly significant ($P < 0.01$; Student's t test).

Not only were the aggregation rates for cells expressing Ep-CAM reduced, but also the morphology of the aggregates was different from those formed by the control cells. Thus, most of the LEC-MC cells produced tight, large aggregates, whereas LEC-MEp.6 cells mainly formed small aggregates (Figure 4D). This was clearly related to the levels of Ep-CAM expressed by the cells, as the aggregates formed by LEC-MEp.6 cells

after induction of Ep-CAM expression were loosely interconnected and even smaller those formed by the non-induced cells.

Over time (≤ 24 h of suspension culture), even the induced LEC-MEp.6 cells were able to form some larger multi-cellular aggregates. However, the internal structure of these aggregates differed substantially from aggregates of LEC-MC cells, with the latter consisting of tightly interconnected cells, whereas the cells in LEC-MEp.6 aggregates had only sporadic connections (Figure 5). This argues against the hypothesis that the expression of Ep-CAM simply delayed, rather than inhibited, the E-cadherin-mediated cell aggregation, as the loose interconnection of cells in LEC-MEp.6 aggregates clearly points to a reduction in the number of the E-cadherin-mediated intercellular adhesions.

FIGURE 5 The internal structure of the large aggregates formed by LEC-MC and LEC-Mep.6 cells in 24 h of suspension culture. The expression of Ep-CAM in LEC-Mep.6 cells was induced with 10 μM CdCl2 for 24 h before the aggregation assay (+). The aggregates were fixed with 2% paraformaldehyde/1.25% glutaraldehyde, embedded into Epon, and ultrathin sectioned. Sections were analyzed by reflection contrast microscopy. Bar, 25 μm.




As was revealed by immunoblotting, the total number of cadherin molecules in transfectants was not affected by Ep-CAM expression or CdCl2 treatment, remaining approximately at the same level in LEC-MC and LEC-MEp cells of both clones. Also, the surface expression, as measured by flow cytometry, was almost identical (Figure 6).

## Ep-CAM affects the Moprphology of E-cadherin-transfected L cells

When seeded at low density, both Ep-CAM-positive clones LEC-MEp.2 and LEC-MEp.6 did not differ in morphology from the parental or control LEC-MC cells. However, when the cells were seeded at high density to form a monolayer culture, some morphological differences, which were especially pronounced between LEC-MC and

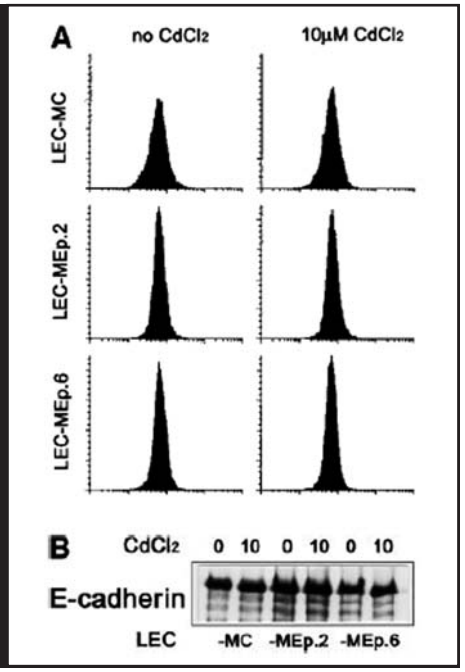




LEC-MEp.6 cells, appeared after 24 h. By that time, > 50% of all control cells had acquired an epithelioid morphology, with a clear interconnection of the lateral domains of neighbouring cells (Figure 7). In contrast, in cultures of LEC-MEp.6 cells, when cultured at the same high debsity in the absence of $Cd^{2+}$, <10% of all cells had the morphology observed in control cultures (as was estimated from direct cell counting). When cultured in the presence of 10 μM CdCl2, no cells with epithelial-like morphology were observed in LEC-MEp.6 cultures (Figure 7). Interactions among the cells seemed to be reduced to sporadic contacts, mainly involving filopodia-like structures.

The presence of $Cd^{2+}$ in the culture medium had no effect on the morphology of the control LEC-MC cells. The LEC-MEp.2 cells demonstrated a somewhat intermediate morphology when compared to the other two cell lines (not shown).

FIGURE 6 Expression of E-cadherin in blank vector (-MC) and Ep-CAM transfectants (clones -Mep.2 and -Mep.6) of LEC cells. The cells were cultured for 24 h in the presence of 10 μM $CdCl_2$ in culture medium. Cells were either (A) detached from the substrate with EDTA and analyzed in flow cytometry after staining with mAb ECCD-2 to murine E-cadherin, or (B) lysed with 1% SDS, and E-cadherin was detected in total lysates (5 μg protein/sample) by immuno-blotting with mAb 36.




## Expression of Ep-CAM Disturbs Association of E-cadherin with the Cytoskeleton

The data presented so far suggest that expression of Ep-CAM does not affect the number of E-cadherin molecules in cells, but does affect the ability of these molecules to form stable adhesions. As the differences between LEC-MC and LEC-MEp.6 cell lines were most clearly pronounced, we further investigated the changes of cadherin junctions in LEC-MEp.6 cells upon expression of Ep-CAM. Immunofluorescent staining revealed that in dense cultures of LEC-MC cells, most of the cell surface E-cadherin molecules were present in typical cadherin-type intercellular junctions and were absent from the free domains of the cell membrane (Figure 8). However, at the basal level of Ep-

CAM expression in in LEC-MEp.6 cells, only a fraction of the E-cadherin molecules was present in junctions, with some E-cadherin molecules being located at the free domains of the cell membrane. Induction of Ep-CAM for 24 h resulted in redistribution of E-cadherin, which was found all over the cell surface, and in a substantial reduction of structurally recognizable E-cadherin junctions (Figure 8).

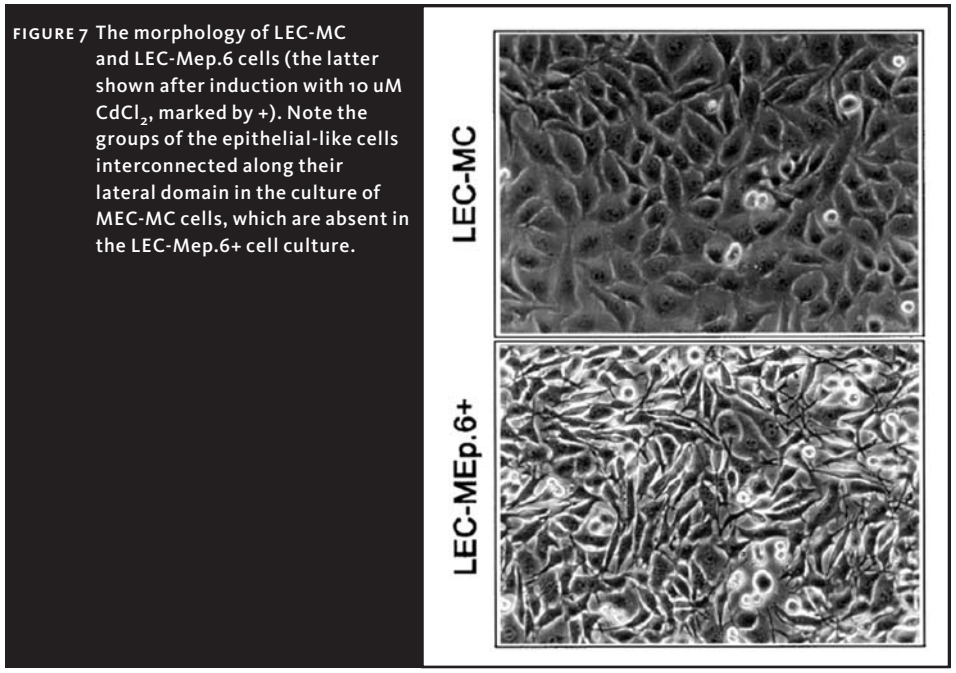



FIGURE 7 The morphology of LEC-MC and LEC-Mep.6 cells (the latter shown after induction with 10 uM $CdCl_2$, marked by +). Note the groups of the epithelial-like cells interconnected along their lateral domain in the culture of MEC-MC cells, which are absent in the LEC-Mep.6+ cell culture.

As was previously shown (Figure 6) by flow cytometry analysis and immunoblotting, both the total number of E-cadherin molecules and the cell surface fraction of E-cadherin were not changed upon induction of Ep-CAM, suggesting that the latter did not cause internalization or enhanced degradation of E-cadherin molecules, or suppression of their transportation to the cell surface. Rather, the observed changes in subcellular distribution of E-cadherin suggest some disturbance in the association of E-cadherin to the cytoskeleton, an association required for the E-cadherin junction formation (Nagafuchi and Takeichi, 1988). Indeed, the cytoskeleton anchored (Triton X-100-insoluble) fraction of cadherins was decreased upon the induction of Ep-CAM in LEC-MEp.6 cells, as can be seen in immunoblotting for E-cadherin in detergent-extracted cells (Figure 9A).

Interaction of E-cadherin molecules with the cytoskeleton is mediated by α- and β-catenins (Ozawa and Kemler, 1992; Jou et al., 1995), which are absent in L cells, but appear upon the transfection of E-cadherin (Nagafuchi et al., 1994). We compared the total content and detergent solubility of both catenins in LEC-MC and LEC-MEp.6 cells. Approximately similar levels of β-catenin were observed in control and LEC-





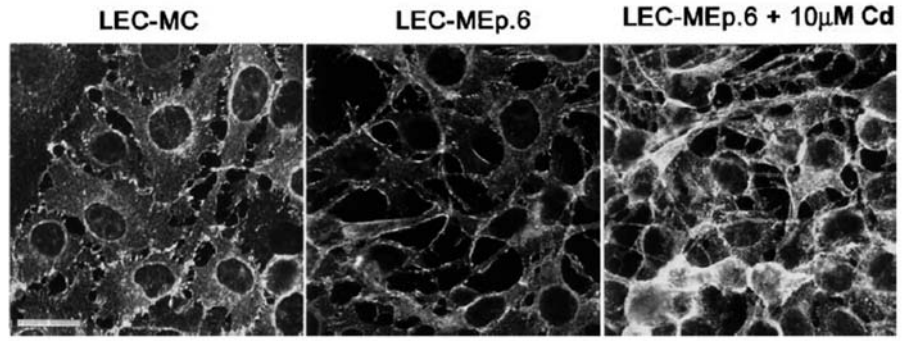



FIGURE 8 Subcellular localization of E-cadherin in LEC-MC cells and in LEC-Mep.6 cells. Ep-CAM in LEC-Mep.6 was induced by 10 μM $CdCl_2$ for 24 h before cell fixation. Immunofluorescent staining of methanol-fixed cells was performed as described in Materials and Methods using mAb ECCD-2 to murine E-cadherin. Bar, 10 mm.

MEp.6 cells regardless of the level of Ep-CAM expression in the latter. The detergent-insoluble fraction of β-catenin was, however, reduced in the induced LEC-MEp.6 cells.

In contrast to β-catenin, a reduction in both detergent-insoluble and total cellular fractions of α-catenin molecules was observed in induced LEC-MEp.6 cells (Figure 9A).

Apparently, the expression of Ep-CAM leads to a decrease in the cytoskeleton-anchored fraction of E-cadherin molecules. To investigate whether some catenins were still associated with the detergent-soluble fraction of E-cadherin molecules, the induced and non-induced LEC-MEp.6 cells were extracted with 0.5% Triton X-100, and immunoprecipitations were performed from the extracts using antibodies specific to E-cadherin or β-catenin (Figure 9B). Upon the induction of Ep-CAM in LEC-MEp.6 cells, there was a clear increase in the amount of β-catenin co-precipitating with the detergent-soluble E-cadherin molecules (Figure 9B). The reverse precipitations with anti-β-catenin mAb produced similar results, with increased co-precipitation of E-cadherin upon induction of Ep-CAM. However, when the immunoprecipitates of soluble E-cadherin were probed with anti-α-catenin antibody, no increased presence of (-catenin associated with this fraction of E-cadherin molecules was observed. The latter result was consistent with the observation of the decrease in total α-catenin in the cells. Additionally, a certain in α-catenin molecules co-precipitating with β-catenin was observed upon the induction of Ep-CAM (Figure 9B).

These results show that expression of Ep-CAM in cells results in accumulation of non-cytoskeletal-anchored E-cadherin/β-catenin complexes, and in a reduction of α-catenin molecules available for the formation of E-cadherin junctions. Since the observations were made after induction of Ep-CAM for 24 h, these soluble E-cadherin/β-catenin complexes may originate from either (a) dissociation of the pre-

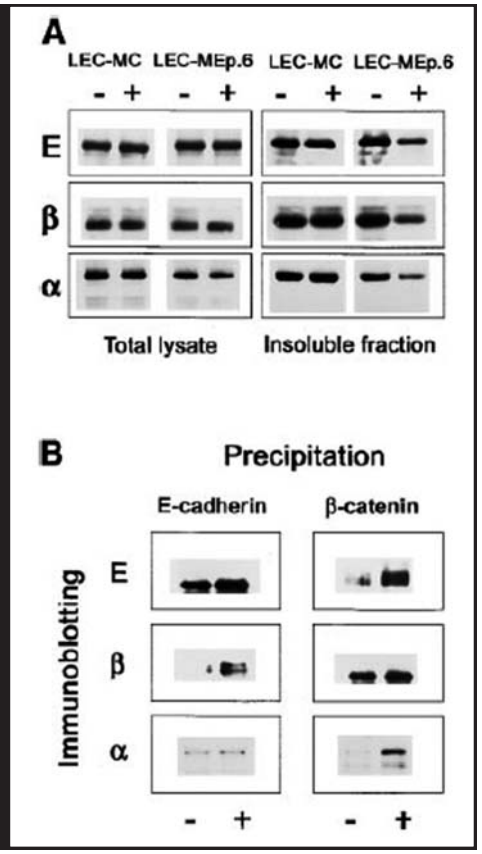



FIGURE 9 E-cadherin and catenins in LEC-MC and LEC-Mep.6 cells. (A) Cells were cultured in the absence (-) or presence (+) of $CdCl_2$ for 24 h, and lysed in 1% SDS either directly (Total lysate) or after extraction with 0.5% Triton X-100 (Insoluble fraction). Samples of cell lysates, equalized by protein content, were analyzed by immuno-blotting with antibodies to E-cadherin (mAb 36), β-catenin (mAb 14), or α-catenin (mAb 7A4). 5x more protein was taken for the extracted cell preparations. (B) LEC-Mep.6 cells cultured in the absence (-) or presence (+) of $CdCl_2$ were lysed in buffer with 0.5% Triton X-100, and immuno-precipitations were performed from the soluble fraction. The immuno-precipitates were analyzed in Western blotting with antibodies to E-cadherin (E) or catenins (α and β, respectively). A rabbit polyclonal serum was used for α-catenin detection. The precipitations were performed from an excess of the lysate; therefore, the total amount of an antigen precipitated by an antibody remains equal.

existing cadherin junctional complexes, or (b) may represent newly produced E-cadherin molecules that had formed a complex with β-catenin, but for which further anchorage to the cytoskeleton was not possible because of the non-availability of α-catenin. Experiments with short-time induction of Ep-CAM in LEC-MEp.6 cells (2, 4, and 6 h) provided no clear support to either of these options, since the levels of cellular Ep-CAM after 6 h of induction were only slightly higher than in non-induced cells, and were substantially lower than in cells induced for 24 h. After short-time induction, no substantial changes in either solubility of cadherins, or in the level of cellular (-catenin were observed (data not shown).

## Ep-CAM Affects Cadherin-mediated Junctions in Epithelial Cells

To investigate whether the expression of Ep-CAM in human epithelial cells affects cadherin junctions in a similar way to what has been observed for E-cadherin transfectants of L cells, we have chosen the immortalized mammary epithelial cell line HBL-100. This cell line expresses low levels of E- and P-cadherin, and relatively high levels of N-cadherin (as was tested in immunoblotting and flow cytometry with mAbs specific for each cadherin), and in contrast to all other cell lines of epithelial origin tested, contains no endogeneous Ep-CAM. Transfectants of a clonal cell line derived from HBL-100 origin, HCA, were obtained using episomal vectors that do not integrate into the genome and support themselves in transfected cells at high copy numbers. The results obtained with HCA/C (blank pCEP4 vector transfectants) and HCA/CEp (pCEP4/Ep-CAM transfectants) cells in coaggregation assays were similar to those obtained with LEC cell transfectants, with the HCA/CEp segregating partially from the parental cells. In mixed two-cell type aggregates, the cell patterning was similar to that observed in the LEC cell model (not shown).

Transfection with Ep-CAM affected the aggregation from monocellular suspensions of HCA cells. Afetr 30 min of aggregation in the presence of $Ca^{2+}$, 50±4 % aggregation was reached by HCA/C cells, and only 24±3 % by HCA/CEp cells. However, after 90 min the degree of aggregation for both cell lines was approximately equal, reaching 55%. This may be due to the formation of Ep-CAM-mediated intercellular adhesions, since after 90 min in the absence of $Ca^{2+}$ (allowing mainly Ep-CAM-mediated cell aggregation), the degree of aggregation for HCA/CEp cells was approximately the same, 47±4 % (only 8.3 % for contol HCA/C cells).

Despite the fact that after 90 min no substantial differences in aggregation extent were observed between the control and the Ep-CAM transfectants, the structure of the aggregates formed by the two cell lines differed substantially. As can be seen on

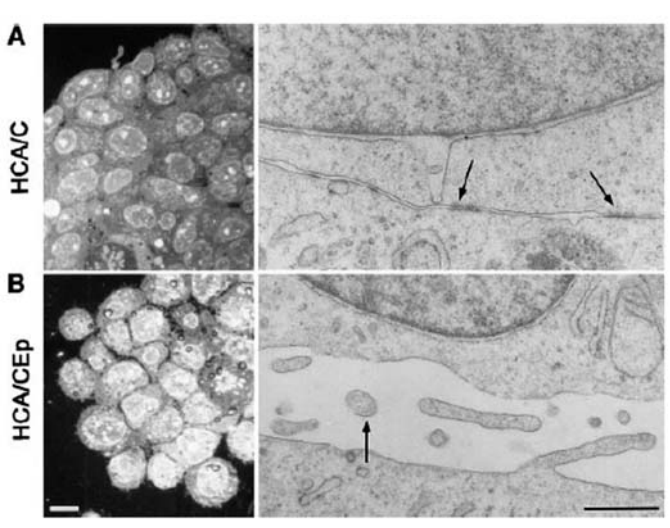



FIGURE 10 Ultra-structure of the aggregates formed by HCA/C and HCA/Cep cells. Reflection contrast micrographs (*left panel*) of cross-sections through the aggregates formed in 90 min by each cell type reveals a tight organization of the HCA/C aggregates and loosely interconnected cells forming the HCA/Cep aggregates. Electron microscopy (*right panel*) on the preparations shows the abundant presence of the adherens junctions between HCA/C cells (*arrows, top*). In contrast, microvilli (*arrow, bottom*) were found at the intercellular space between the cells of internal layers of HCA/Cep cells. In HCA/C cells, microvilli were present almost exclusively at the apical cell membrane of the outer layer of cells in the aggregate and not on the surface of the cells from internal layers.

cross-sections, the HCA/C aggregates were composed of tightly interconnected cells, in contrast to the HCA/CEp aggregates, which were formed by loosely interconnected cells (Figure 10). In the internal layers of the HCA/C aggregates, the membranes of the cells were interconnected by multiple adherens junctions (Figure 10), whereas in HCA/CEp aggregates the cell-cell contacts were rare and often did not contain morphologically distinguishable adherens junctions. The membranes of neighboring cells in these aggregates were mainly not in close proximity. In these intercellular spaces, microvilli were often observed (Figure 10), wich were present in HCA/C aggregates at the outer surface of the external layer of cells only.

To further investigate the effect of Ep-CAM on cell-cell contacts of HCA cells, the cells were also transfected with Ep-CAM cDNA under the control of the metallothionein promotor (Wt cells; Wt indicates wild-type Ep-CAM). This regulated the levels of Ep-CAM expression from ~50% to 350% of the levels observed in HCA/CEp transfectants (as estimated by flow cytometry on living cells; data not shown).

At the basal level of Ep-CAM expression, the morphology of Ep-CAM transfectants in cell culture was quite similar to the control cells transfected with the blank vector. However, induction of high levels of Ep-CAM resulted in loss of the polygonal morphology and in a more scattered phenotype, although the cells still remained attached to another (Figure 11). This change in morphology was observed for the entire cell population only at the highest (50 μM) concentration of Cd2+ tested, although some of these changes could be observed for some cells or groups of cells at lower concentrations of $Cd^{2+}$ (2-5 μM), with changes increasing proportionally to the Ep-CAM levels. The effect was reversible, and within 3 d of culture in the absence of the $Cd^{2+}$ in the medium, the cells regained their original morphology, consistent with the decrease of Ep-CAM levels back to that of non-induced cells (flow cytometry data; not shown).

Induction of Ep-CAM in cells did not affect either the total level of cadherins as was detected with immunoblotting using a pan-cadherin antibody (Figure 13), or the surface expression of N-cadherin (as was detected by flow cytometry; not shown). The surface expression of E- and P-cadherins in HCA cells was too low to be measured by flow cytometry. However, upon induction of Ep-CAM, the subcellular distribution of cadherins was changed: the typical junctional structures, including cadherins that were present in HCA cells (or control transfectants), were absent in induced (Figure 11), but not in non-induced Ep-CAM transfectants. The disturbance of cadherin's subcellular localization was suggested that the effect of Ep-CAM was similar to that observed for LEC cells. Indeed, the detergent solubility of the cadherins in HCA cells was increased after 24 h of Ep-CAM induction (see Figure 13). Induction of Ep-CAM had no effect on the level of total cellular β-catenin, but it negatively affected the level of total α-catenin (Figure 12); the detergent-insoluble fraction for both molecules was reduced in relation to the expression of Ep-CAM (Figure 12). All observations are based on 24-h induction of Ep-CAM expression.

FIGURE 11 Changes in sub-cellular distribution of cadherins in HCA cells upon expression of Ep-CAM. HCA cells transfected with inducible pMep4 vector, either blank (HMC) or containing the Ep-CAM cDNA (Wt), were induced with 50 μM $CdCl_2$ 24 h before fixation, fixed, and stained with anti-pan-cadherin mAb CH-19 (reacts mainly with N-cadherin, the major cadherin of HCA cells). Note the redistribution of cadherins from adherens junctions to free domains of cell membrane in Wt Ep-CAM transfectants, and a general shift of the cell phenotype to a more scattered one.

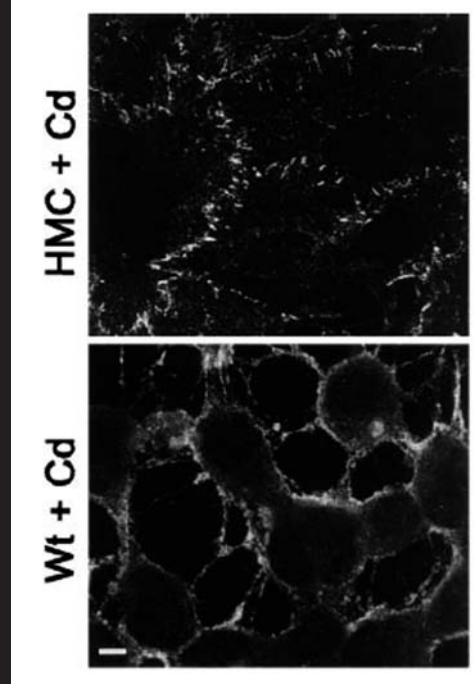
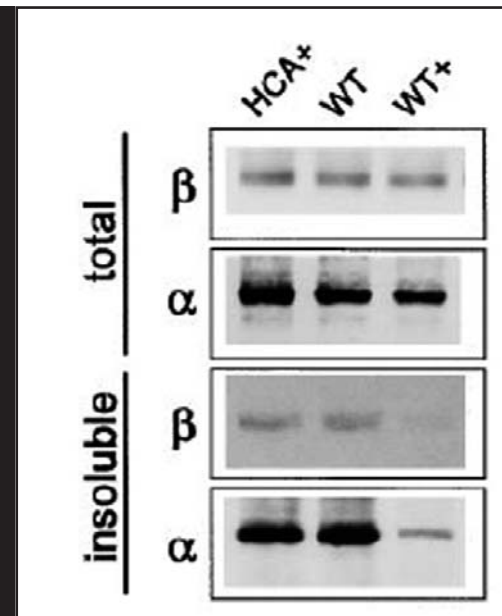


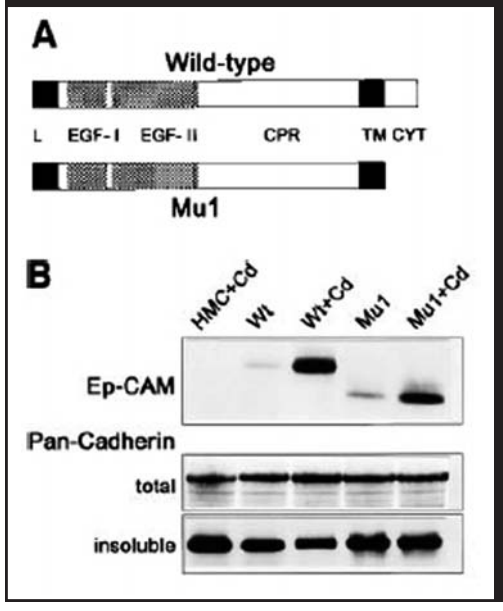

FIGURE 12 Detergent extractability of catenins in HCA cells and Wt Ep-CAM transfectants. Immunoblotting was performed on total cell lysates and on lysates of cells pre-extracted with 0.5% Triton X-100. The + indicates induction of cells with $CdCl_2$ for 24 h before lysis.



### The Cytoplasmic Domain of Ep-CAM Is Required to Affect the Cadherin-mediated Junctions

In both L and HCA cell models, we observed a decrease in cadherin-mediated adhesion and a reduction in cellular α-catenin upon the expression of Ep-CAM. Such changes in adherens junctions suggest that Ep-CAM induced certain intracellular effects, and we therefore investigated whether an Ep-CAM mutant lacking the cytoplasmic domain would be able to negatively affect cadherin-mediated adhesions. This mutated form of Ep-CAM (Mu1) was generated as shown in Figure 13. When transfected into L or HCA cells, Mu1 was expressed at the cell surface, similar to wild-type Ep-CAM, and was capable of mediating weak homotypic binding, but was not able to form stable adhesions (investigated in more detail in Balzar et al., 1998; Chapter 4). No effects produced by the wild-type Ep-CAM were observed in LEC cells transfected with Mu1 with respect to cell morphology, cell aggregation, and the number and extractability of the cadherin and catenin molecules (not shown). However, the transfections of Mu1 constructs into LEC cells never gave rise to a clonal cell line with the levels of Mu1 expression comparable to the wild-type Ep-CAM expression in LEC-MEp.6 cells, although the expression levels comparable to LEC-MEp.2 cells were achieved. This makes strict interpretation of the effects of Mu1 on E-cadherin in LEC cells difficult.
In contrast, when transfected into HCA cells under the control of an inducible promotor, Mu1 expression could reach levels similar to those of wid-type Ep-CAM expression in these cells (Figure 13). Nevertheless, no effect on cell morphology, nor on the internal structure of aggregates formed by the transfectants were observed upon expression of Mu1 in HCA cells, as compared to non-transfected cells or cells transfected with blank pMEP4 vector (HMC cells). Expression of Mu1 did not affect the total level of cadherins in HCA cells or their solubility, as did the wild-type molecule expressed at the same level.

FIGURE 13 The role of the cytoplasmic domain in the effect of Ep-CAM on cadherins.
(A) The structural maps of the wild-type and mutant (Mu1) Ep-CAM molecules. The leader peptide (L), EGF-like domains (EGF-I, EGF-II), cystein poor region (CPR), the transmembrane domain (TM), and cytoplasmic domain (CYT) are marked. The cytoplasmic domain is deleted in the Mu1 molecule. (B) HCA cells were transfected with either blank vector (HMC), or the wild-type Ep-CAM (Wt), or Mu1, under the control of a methallothionein promotor. Where indicated (+Cd), the cells were cultured in the presence of 50 μM $CdCl_2$ for 24 h before lysis. Aliquots of total cell lysates equalized by protein were probed in immuno-blotting using the Ep-CAM specific mAb. Content of N-cadherin in whole cell lysates and after extraction with 0.5% Triton X-100 was analyzed with anti-pan-cadherin mAb CH-19. Note that the expression of Wt Ep-CAM, but not of an approximately equal level of Mu1, affects the detergent solubility of cadherins.




## Discussion

$Ca^{2+}$ independent cell-cell adhesion molecules are present in epithelial and carcinoma cells along with the classic cadherins, and, although the data is scarce, it suggests a possible influence of the non-cadherin cell-cell adhesion molecules on cadherin-mediated cell-cell adhesion. Thus, the expression of non-cadherin adhesion molecules, such as protein Zero (Doyle et al., 1995) or N-CAM (Michalides et al., 1994), may cause augmentation of the cadherin-mediated adhesions.

In contrast to these observations, we demonstrate here that expression of Ep-CAM in cells interconnected by classic cadherins results in abrogation of the cadherin-mediated junctions in a direct correlation to the levels of Ep-CAM expressed. Despite the fact that Ep-CAM, being an adhesion molecule, provides additional intercellular connections to the cells, its expression leads to an overall decrease in the strength of interactions for cells interconnected by the classic E- and N-cadherins, as shown in this study. In modulating cadherin adhesions, Ep-CAM acted to some extent as an anti-adhesion molecule, if the overall strength of the intercellular adhesion in transfected cells is considered. This effect, however, differs from the previously demonstrated anti-adhesion effects of N-CAM-bearing polysialic acid (Rutishauser, 1992; Yang et al., 1992), since polysialylated N-CAM does not bear an adhesion function, and its action is more similar to the anti-adhesion affects of some other sialoglycoproteins, such as episialin (Wesseling et al., 1996) or CD43 (Ardman et al., 1992), which inhibit intercellular adhesion by not allowing the membranes of interacting cells to reach the proximity necessary to form adhesions. Ep-CAM not only weakens/abrogates the cadherin mediated adhesions, but replaces them with Ep-CAM adhesions becoming predominant (as was seen with HCA/Cep cell aggregation) to some extent.

The observed effects originate more likely from a possible existing coordination between the two adhesion systems rather than a simple antiadhesion effect of Ep-CAM. In epithelial cells a certain coordination between different adhesion systems was demonstrated for keratinocytes, where an increase of in E-cadherin induces down-regulation of β1-integrin (Hodivala et al., 1994). A similar coordination may exist between other systems involved in intercellular adhesion, such as Ep-CAM and cadherins, with E-cadherin in particular.

The molecular mechanisms behind the observed effect is not quite clear. Expression of Ep-CAM did not affect the expression nor the number of the cadherin

molecules, but did affect the formation of the cytoplasmic junctional complex of the cadherin molecules. In both murine fibroblasts and in human epithelial cells tested, a reduction in the total level of cellular α-catenin and an increased detergent solubility of cadherins were seen upon the expression of Ep-CAM. Cadherins that lost their connection to the cytoskeleton were mainly present as complexes with β-catenin. This argues the involvement of β-catenin in the modulating effect of Ep-CAM on cadherins, in contrast to the role suggested for β-catenin in regulation of cadherin junctions (for review see Gumbiner et al., 1993). However, *v-src* expression may shift cell-cell adhesion mediated by cadherins from a strong to a weak state without involvement of β-catenin (Takeda et al., 1995), which suggests the existence of alternative mechanism for regulating the cadherin junctions. Our observation indicate that the modulation of cadherin adhesions by Ep-CAM involves α-catenin, since the observed dissociation of cadherin junctions would require dissociation of α-catenin from its connection within the complex, anchoring E-cadherin molecule to the actin-cytoskeleton. α-Catenin was shown to be a link between the E-cadherin-β-catenin complex (Nagafuchi et al., 1991; Hirano et al., 1992; Hinck et al., 1994) and actin filaments, interacting with β-catenin on one side and with α-actinin (Knudsen et al., 1995) and probably actin filaments on the other side (Rimm et al., 1995).

We have recently demonstrated that Ep-CAM interacts with the actin based cytoskeleton via α-actinin, without the involvement of α- or β-catenins (Balzar et al., manuscript submitted for publication). A mutant Ep-CAM lacking its cytoplasmic domain did not affect the cadherin junctions. Therefore, the connection between Ep-CAM and α-actinin seems to be related to the negative effect on cadherin junctions. It is conceivable that over-expression of Ep-CAM may result in redistribution towards Ep-CAM of α-actinin molecules involved in the formation of cadherin junctions. It is not clear, however, to what extent or how the availability of α-actinin regulates the cellular levels of α-catenin, which become reduced upon induction of Ep-CAM. An alternative way by which E-cadherin junctions may be affected is through active signaling via the cytoplasmic domain of Ep-CAM, leading to down-regulation of α-catenin since the connection of Ep-CAM to the cytoskeleton via α-actinin is very similar to that of ICAM-1 (Carpen et al., 1992). The signal transduction via ICAM-1, resulting in phosphorylation of cortactin, was recently demonstrated (Durieu-Trautmann et al., 1994).

E-cadherin is involved in regulation of epithelial cell polarity (McNeil et al., 1990; Nathke et al., 1993), in morphogenesis of eoithelial tissues (Wheelock and Jensen, 1992; Larue et al., 1996), and in regulation of cell proliferation and programmed cell death in epithelia ( Hermiston and Gordon, 1995; Hermiston et al., 1996; Takahashi and Suzuki, 1996; Wilding et al., 1996; Zhu and Watt, 1996). In carcinomas, this molecule clearly acts as a suppressor of carcinoma cell invasion (Behrens et al., 1989; Chen and Obrink, 1991; Frixen et al., 1991; Vleminkx et al., 1991). The cadherin-modulating properties of Ep-CAM, as reported here, may play a role in epithelial cell proliferation, and possibly in tumor progression. This hypothesis is supported by the observed association of Ep-CAM with actively proliferating or premalignant cell populations, respectively,





such as proliferating cells in hair follicles (Tsubura et al., 1992), cells of preneoplastic lesions in the uterine cervix (Litvinov et al., 1996), colonic polyps (Salem et al., 1993), or in intestinal metaplasia in gastric mucosa (this article). The previously reported association of Ep-CAM expression with a poor prognosis in breast carcinoma patients (Tandon et al., 1990), and our recent similar observations in patients with head and neck cancer (Takes et al., 1997) as well as the increase in Ep-CAM expression from low to high grade bladder carcinomas (Zorzos et al., 1995), indicate a possible role for Ep-CAM in tumor progression.

The data presented in this paper demonstrate an interaction between Ep-CAM and classic cadherin-based adhesion systems and suggest a cross-talk between adhesions systems as a mechanism through which the strength of the intercellular adhesion among epithelial cells may be regulated.

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Chapter 4

# Expression of Ep-CAM shifts the state of cadherin adhesions from strong to weak without involvement of β-catenin

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## Abstract

Various adhesion molecules play an important role in defining cell fate and maintaining tissue integrity. Therefore, cross-signaling between adhesion receptors should be a common phenomenon to support the orchestrated changes of cells' connections to the substrate and to the neighboring cells during tissue remodeling. Recently, we have demonstrated that the epithelial cell adhesion molecule Ep-CAM negatively modulates cadherin-mediated adhesions in direct relation to its expression levels. Here, we used E-cadherin/ α -catenin chimera constructs to define the site of Ep-CAM's negative effect on cadherin-mediated adhesions. Murine L-cells transfected with either E-cadherin/ α -catenin fusion protein, or E-cadherin fused to the carboxy-terminal half of α-catenin, were subsequently supertransfected with an inducible Ep-CAM construct. Introduction of Ep-CAM altered the cell's morphology, weakened the strength of cell-cell interactions, and decreased the cytoskeleton-bound fraction of the cadherin/catenin chimeras in both cell models. Furthermore, expression of Ep-CAM induced restructuring of F-actin, with changes in thickness and orientation of the actin filaments. The results showed that Ep-CAM affects E-cadherin-mediated adhesions without involvement of β-catenin by disrupting the link between α-catenin and F-actin. The latter is likely achieved through remodeling of the actin cytoskeleton by Ep-CAM, possibly through pp120.


## Introduction

Various adhesion molecules that mediate cell's interactions with the substrate and the neighbouring cells are recognized to play an important role in defining the cell fate, differentiation, and other biological characteristics [1,2]. Data are accumulating that adhesion receptors do function not only as physical interconnectors between various cell types and substrates, but represent an important group of signal-transducing modalities, participating in tissue morphogenesis [3-7].

Cross-signaling between adhesion receptors is likely to be a common phenomenon, being partly responsible for the orchestrated changes in the number and composition of cellular adhesion receptors during cell migration or differentiation. For example, in keratinocytes undergoing terminal differentiation and stratification, a decrease of β1-integrin expression is evidently related to an upregulation of E-cadherin, and is negatively controlled by the formation of E-cadherin-mediated adhesions [8 -10]. Furthermore, the flexible and rapid changes of N-cadherin activity in migrating cells of the neural crest were shown to be controlled by signals from β1 and β3 integrins [11]. Recently, β1-integrin expression was also reported to result in a decrease of total cadherin and α-catenin protein levels, together with redistribution of cadherin and α-catenin from the detergent insoluble fraction to the detergent soluble fraction [12]. In mammary epithelial cells, blocking of β1 integrin was demonstrated to suppress the metastatic potential of cells, likely by upregulating E-cadherin adhesions [13]. Lampe et al. [14] demonstrated the subtle interactions in keratinocytes between α3β1, adhesion to laminin-5, and Rho signaling, which promotes intercellular communication mediated by gap junctions, while Braga described the impinge of the Rho family of small GTPases on the regulation of cadherindependent adhesion and epithelial morphology [15].

We have previously demonstrated that the epithelial cell adhesion molecule Ep-CAM negatively affects the cadherin-mediated adhesions in direct proportion to its expression levels [16]. Ep-CAM is a calcium-independent homophillic cell adhesion molecule of 39-42 kDa [17,18] which is expressed by the majority of epithelial tissues, excluding the adult squamous epithelium and some epithelium-derived cell types, such as hepatocytes [19,20]. However, de novo expression of Ep-CAM can be observed for these cell types as well, during active cell proliferation in embryogenesis, or at the early stages of tumorgenesis [19,21].

Ep-CAM does not structurally resemble any of the major families of the adhesion molecules (cadherins, selectins, integrins, or cell adhesion molecules of the Ig superfamily). It is a type I transmembrane glycoprotein, consisting of an extracellular domain, containing two EGF-like repeats, and a short cytoplasmic domain of 26 amino acids in which two binding sites for α-actinin are present (for review see Balzar et al. [22]). α-Actinin is required to stabilize the intercellular adhesions mediated by Ep-CAM, by connecting Ep-CAM to F-actin [23]. We have demonstrated that the cytoplasmic domain of Ep-CAM is required for its negative effect on cadherins [16]. Upon overexpression of Ep-CAM, it was observed that cadherin adhesions dissociate, which leads to accumulation of detergent-soluble E-cadherin/β- catenin complexes, and to a decrease in total cellular α-catenin [16]. This may be the result of the E-cadherin/β-catenin complex dissociating from α-catenin anchored to F-actin, or, alternatively, the total

preexisting E-cadherin/β-catenin/α-catenin complex is eliminated from the surface and destined for degradation via internalization, and the observed cytoplasmic E-cadherin/β-catenin complexes represent newly synthesized proteins.

We used in this study cell lines transfected either with E-cadherin or with chimera proteins composed of E-cadherin directly linked to α-catenin [24]. Supertransfection of Ep-CAM in these cell lines had a dramatic effect on (1) morphology, (2) the aggregation ability of cells, (3) on subcellular localization, and (4) on the degree of detergent extractability of chimera proteins. Both tested fusion molecules were affected by expression of Ep-CAM, independent of the involvement β-catenin present in the cadherin adhesions. Therefore, we suggest that Ep-CAM expression affects the link between α-catenin and F-actin within the E-cadherin-mediated adhesion complex as a result of the Factin network being reconstructed, possibly through pp120.

# Materials and methods

## Cell culture

Murine fibroblast L-cells (clone L929) were from the American Type Culture Collection (Rockville, MD). L-cells transfected with either fusion protein Eα or EαC (see below) were developed earlier by Dr. A. Nagafuchi [24]. Selected clones were supertransfected with full-length Ep- CAM. All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin (all from GibcoBRL, Gaithersburg, MD) in a humidified, 5% CO2, 37°C incubator. Although cells were stably transfected, 1 mg/ml G418 and 1 mg/ml Hygromycin B were added to each eighth passage.

## Constructs and transfections

L-cells transfected with a full-length E-cadherin (EL cells [17]), or Eα or EαC constructs [24]. were used. The latter are hybrid molecules from E-cadherin and α-catenin (see Table 1 and Figure 1A). In the Eαhybrid, the cytoplasmic tail of E-cadherin was excised downstream of amino acid 657, and the remaining domains were fused to a full-length α-catenin (amino acids 1-906). EαC was composed likewise, except that only the C-terminal half of α-catenin (amino acids 509-906) was used in the hybrid (Figure 1A). The constructs in the pBATEM vector were a kind gift from Dr. A. Nagafuchi [24]. and transfected clones were selected at 1 mg/ml G418.

Human Ep-CAM cDNA in pMep4 vector (Invitrogen, Leek, The Netherlands), with metallothionein promoter, was used to supertransfect EL, EαL, EαCL as well as parental L-cells (see table 1). Control cell lines were transfected with an empty vector. DOTAP transfection reagent was used according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany). The transfectants were selected at 1 mg/ml Hygromycin B (Boehringer Mannheim) as previously described [23].

**TABLE 1** Nomenclature of transfected cell lines.

| CONSTRUCTS | CELL LINES |
|---|---|
| pJΩ/ E-cadherin<br>pMep4/ - | EL |
| pJΩ/ E-cadherin<br>pMep4/ wt Ep-CAM | EL-Ep |
| pBATEM/ Eα<br>pMep4/ - | EαL |
| pBATEM/ Eα<br>+ pMep4/ wt Ep-CAM | EαL-Ep |
| pBATEM/ EαC<br>pMep4/ - | EαCL |
| PBATEM/ EαC<br>+ PMEP4/ WT EP-CAM | EαCL-EP |

## Antibodies

Depending on the experimental settings, E-cadherin and its derivatives were detected by either monoclonal antibody (mAb) 36, recognizing the juxtamembrane site of the cytoplasmic domain of E-cadherin (Transduction Laboraties, Lexington, KY), or mAb ECCD2, which has its epitope in the extracellular domain (Thamer Diagnostica BV, The Netherlands). mAb against β–catenin and pp120 (both mouse) was from Transduction Laboratories. mAb 323/A3 against human Ep-CAM was from Centocor Europe B.V., Leiden, The Netherlands. Alexa-488-conjugated phalloidin was from Molecular Probes Europe B.V., Leiden, The Netherlands. Other antibodies were to: (lysosomal membrane proteins), LAMP1 (1D4B), and LAMP2 (ABL-93) (both were rat mAbs from Hybridoma Bank). FITC-labeled lectin from Helix Pomatia Agglutinin (HPA) was from Sigma, St. Louis, MO.

## Cell dissociation and aggregation assay

For cell aggregation assays, cells growing in monolayers were washed with ice-cold 25 mM HEPES in Hanks' solution and detached by either 1.0% EDTA or 0.25% trypsin/ 0.1% EDTA; $10^6$ single cells were allowed to aggregate in 2 ml of normal DMEM supplemented with 1% Nutridoma- SP in the presence of 1 mM $CdCl_2$ during 2 h under gentle gyration (75 rpm, 37°C, 5% $CO^2$).

For cell adhesion reformation assays, cells growing in monolayers were detached with 0.25% trypsin/0.1% EDTA and dissociated by gentle pipetting to monocellular suspension; $10^6$ single cells were allowed to aggregate overnight in 2 ml of DMEM supplemented with 1% Nutridoma-SP. The aggregates were resuspended 15 times. Fractions before and after resuspension were fixed with 0.5% glutaraldehyde. Aggregates were measured in a cell counter (Casy TCC; Schärfe Systems, Reultingen, Germany).

## Flow cytometry

Eighty percent density cultures were induced overnight with 10 μM $CdCl_2$, detached with 0.1% EGTA in Hanks' buffer on ice, and counted; $10^6$ cells were incubated with 0.1 μg of the primary antibody for 30', the Alexa-488- labeled respective IgG-specific conjugate (Molecular Probes Europe B.V.) for 30', and propidium iodide solution (1 μg/ml) for 15'. Fluorescent intensity was measured for $10^4$ events, using a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA).

## Detergent extraction, lysis of cells, and Western blot analysis

Overnight induced (10 μM $CdCl_2$) cell cultures were rinsed with ice-cold 1 mM $CaCl_2$/1 mM $MgCl_2$ in phosphate- buffered saline (PBS) and extracted with 50 mM NaCl/10 mM Tris-HCl (pH 7.0) 1 mM $CaCl_2$/1 mM $MgCl_2$/ 300 mM sucrose/complete protease inhibitor (Boehringer Mannheim)/1% Triton X-100. Cells were scraped and gently shaken on ice for 45 min. Samples were collected and spun down (15 min, 15,000 g). The detergent insoluble fraction was separated from supernatant (the detergent-soluble fraction) and further lysed in hot 1% sodium dodecyl sulfate (SDS)/10 mM EDTA. For total lysates, the cells were lysed in hot 1% SDS/10 mM EDTA immediately after rinsing.

Protein concentration in lysates was measured using Bio-Rad *Dc* Protein Assay (Bio-Rad Lab, Hercules, CA). Samples of protein lysates were separated by SDS- polyacrylamide gel electrophoresis and were transferred electrophoretically onto PVDF membrane (Millipore, Bedford, MA). The membranes were blocked with 5% skim milk in PBS for 1 hr, incubated with a primary monoclonal antibody for 2 h, washed for 1 h in PBS/0.05% Tween 20, and incubated for 1 h with an anti-mouse IgG horseradish peroxidase (HRP) conjugate (Transduction Laboratories). The blots were developed using the ECL detection substrate (Amersham Pharmacia Biotech, Buckinhamshire, UK).

## Immunofluorescent microscopy

Overnight induced (10 μM $CdCl_2$) cell cultures were rinsed three times with ice-cold PBS/1 mM $CaCl_2$/1 mM $MgCl_2$, fixed either with cold methanol (15 min, -20°C) or with paraformaldehyde (as previously described [23]). Fixed cells were rehydratated with PBS, blocked with 5% skim milk in PBS, incubated with a primary monoclonal antibody for 2 h, washed with PBS, and incubated for 1 h with goat IgGs against antibody isotypes (PickCell Laboratories BV, Amsterdam, The Netherlands), conjugated with Alexa-488 or Alexa-546 (Molecular Probes Europe, Leiden, the Netherlands). Stained cells were analyzed by confocal microscopy (Zeiss LSM 510; Jena, Germany). For analysis of the cytoskeleton, cells were permeablized with 0.3% Triton X-100 and incubated for 1 h with 0.5 mM Alexa- 488-conjugated phalloidin (Molecular Probes).

**FIGURE 1** E-cadherin/α-catenin fusion proteins.
(A) Schematic representations of the full-length E-cadherin and the α-catenin molecules. The binding domains (amino acid numbers) for β-catenin (b), α-actinin (a-A), pp120 (p120), and F-actin (F-a) are indicated. The two lower schemes represent the fusion molecules Ea and EaC.
(B) Immunoblotting of cell lysates of L-cells transfected with blank vector (L), full-length E-cadherin (EL), the E a construct (Eα L), or the EαC construct (EαCL). The 120-kDa fulllength E-cadherin, 220-kDa Eα and 145-kDa EαC molecules are indicated. Note that only the cells with the full-length E-cadherin express β-catenin.
(C) Detection by immunoblotting of Ep-CAM in the blank vector transfectants (EαL and EαCL) and Ep-CAM cDNA transfectants (EαL-Ep and EαCL-Ep). "+" indicates overnight incubation of 10 μM $CdCl_2$; "-", no induction.

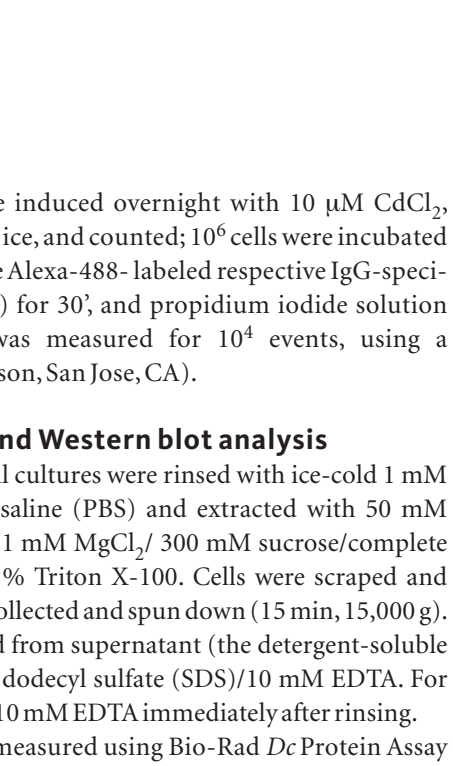



**FIGURE 2** The cell morphology and aggregation capacity are changed after Ep-CAM introduction.
(A) The mock transfectants EαL and EαCL show the tightly interconnected phenotype expected for E-cadherin-mediated cell-cell contacts, in contrast to the Ep-CAM-transfected cells EαL-Ep and EαCL-Ep. These cells are more loose and less spread, which was even enhanced upon induction with 10 μM $CdCl_2$ (EαL-Ep and EαCL-Ep Magnification, 200x.
(B) An enlarged detail of the cells shown in A.
(C) The results of aggregation of EαCL and EαCL-Ep are shown. The graph represents the degree of 2-h aggregation in the presence of calcium after EDTA detachment. Aggregates are grouped by diameter size: single cells, small aggregates (20-40 μm), and large aggregates (40-140 μm)
(D) The panel shows the overnight aggregation in DMEM/Nutridoma-SP after trypsin/EDTA detachment (cells/RS, resuspended) and subsequent 15' resuspension. Aggregates are grouped by diameter size: single cells, and small and large aggregates.

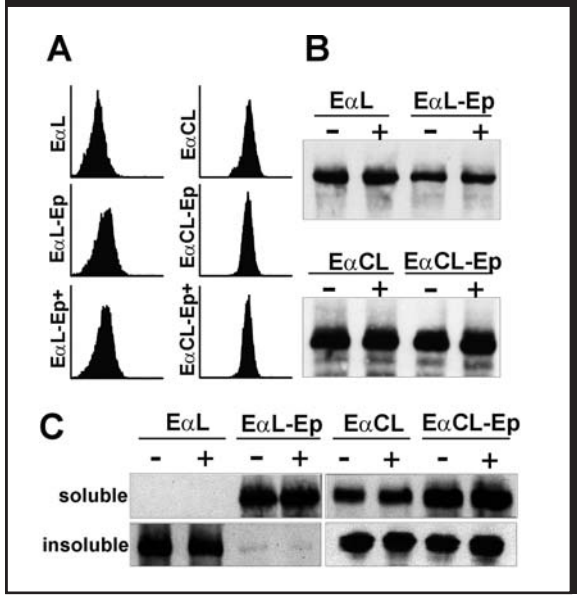



FIGURE 3 Surface expression of the Eα and EαC fusion proteins after Ep- CAM introduction. (A) Flow cytometry analysis of living cells with the E-cadherin monoclonal antibody ECCD2 in mock and Ep-CAM transfectants. *x*-axis represents the mean fluorescence; *y*-axis represents the number of cells. (B) Western blots stained for E-cadherin in EαL and EαL-Ep, as well as in EαCL and EαCL-Ep. (C) Differences in cytoskeleton-bound and "free" E-cadherin/α–caterin are represented as Triton X-100-soluble and insoluble fractions. Both soluble and insoluble fractions of the two cell lines were stained for the E-cadherin construct. Upregulation of the soluble fraction is found in both EαL-Ep and induced EαL-Ep, as well as in EαCL-Ep and induced EαCL-Ep+, while the insoluble fraction is decreased. Everywhere: "+" indicates overnight incubation of 10 μM $CdCl_2$; "-", no induction.

# Results

To investigate the molecular basis of the negative effect of Ep-CAM on E-cadherin-mediated adhesions [16], L-cells were transfected with two chimerized molecules, Eα and EαC, obtained by fusing E-cadherin and α-catenin, and supertransfected with Ep-C AM (see material and methods section). Nomenclature of all transfected cell lines is summarized in Table 1. As shown in Figure 1A, in both constructs the binding site for β-catenin was excluded from the Ecadherin sequence. The binding site for β-catenin was still present in the α-catenin fragment within the Eα construct, but not in the EαC construct. A potential α-Actinin-binding site of α-catenin is present in Eα, but not in EαC. L-cells stably transfected with the full-length E-cadherin were used as a control (EL; see Table 1).

Selected clones of L-cells transfected with Eα or EαC constructs showed expression of both constructs, and were recognized by both anti-E-cadherin and anti-α-catenin mAbs (see also Nagafuchi et al. [24]). Figure 1B shows the full-length E-cadherin (120 kDa) and the fusion proteins in the transfectants with their corresponding molecular weights (200 and 145 kDa, respectively). A protein with a molecular weight similar to the wild-type α-catenin was detected with anti-α-catenin antibody in Eα transfectants, but it may represent a degradation product originating from the hybrid molecule, as was reported earlier [24].





## Regulated expression of Ep-CAM in EαL and EαCL cell transfectants

L-cells, EL (full-length E-cadherin transfectants), EαL, and EαCL cells were supertransfected with either the pMep4 vector only, or pMep4 containing the full-length Ep-CAM cDNA (Table 1). The pMep4 vector contains the metallothionein promotor, which can be induced by divalent heavy metal ions (i.e., $Cd^{2+}$ or $Zn^{2+}$). At least 10 selected clones were tested for Ep-CAM expression of Ep-CAM and the $CdCl_2$ inducibility by Western blotting of total lysates (Figure 1C). The cDNA transcription is under control of the methallothionein promoter and transcription can be enhanced by bivalent cations, and a stepwise expression of a molecule can be achieved by the following: (1) the mock transfection (promoter only), (2) the transfection with "normal" transcription Ep-CAM cDNA, and (3) the transfection after $CdCl_2$ incubation resulting in enhanced transcription of Ep-CAM cDNA. Membrane expression of Ep-CAM, and its increase upon CdCl2 induction, was confirmed by flow cytometry (results not shown).

## Ep-CAM affects cell-cell interactions in E-cadherin, Eα,and EαC transfectants

Both Eα and EαC constructs did promote intercellular adhesion in L-cell cultures, as was observed by the changes in cell morphology of the respective transfectants (Figure 2A and B). Introduction of Ep-CAM in both EαL and EαCL cells resulted in a change of cell culture morphology (Figure 2A and B): upon expression of Ep-CAM the number of cell-cell interaction sites, as well as the areas of the cell membrane involved in contact, became reduced. This effect was more pronounced upon enhancing expression levels of Ep-CAM with 10 μM $CdCl_2$. On average, Ep-CAM transfectants (both EαL-Ep and EαCL-Ep) showed more membrane protrusions resembling filopodia. These membrane protrusions were often observed in the full-length E-cadherin transfectants, but not in cells transfected with the chimera constructs. None of the changes were observed in the mock transfectants (EαL and EαCL) of both cell lines, either in the presence or absence of $CdCl_2$, providing evidence that the effects were specifically related to the expression of Ep-CAM.

To evaluate to what degree cell-cell interactions were affected in the cultures of the Ep-CAM-expressing transfectants, the cells were subjected to an aggregation assay. To leave the E-cadherin and Ep-CAM molecules intact, cells were detached by using EDTA. Both EαL-Ep and EαCL-Ep cells were able to aggregate in the presence as well as in the absence of calcium, although to a different extent (Figure 2C). The parental cells E_L and E_CL did not aggregate in the absence of $Ca^{2+}$. This confirms participation of Ep-CAM in cell-cell adhesion of the transfectants.

The aggregates formed by Ep-CAM transfectants in the presence of $Ca^{2+}$ differed in morphology from those formed by their respective parental cell lines (not shown). No large aggregates were found to be formed by EL-Ep cells, whereas the opposing was observed for EL cells. No differences in structure of aggregates or aggregation rate, however, were observed between EαCL and EαCL-Ep. This suggested that the adhesions mediated by the chimeras were not affected by Ep-CAM.

Detaching cells with trypsin/EDTA damages E-cadherin but not Ep-CAM molecules, and therefore the reformation of cadherin-mediated adhesions from intracellular pooled cadherins in the presence of fully active Ep-CAM is measured in such an aggregation assay. The cadherins are fully recovered after 10 h, as was seen by immunoblotting for the intact and trypsin-digested cadherins (not shown). Cells aggregating for an extended time after trypsin/EDTA digestion allows the cadherin adhesion to be reformed when the Ep-CAM mediated adhesions are already established. Overnight aggregation of EL and EL-Ep in the presence of $Ca^{2+}$ showed a similar aggregation profile as was observed with EDTA detachment (Figure 2D). EαCL and EαCL-Ep both showed enhanced capability to aggregate compared to EL and EL-Ep and their aggregation profile was almost identical. However, the aggregates formed by EαCL-Ep cells could easily be dissociated by pipetting in contrast to the aggregates formed by EαCL.

Reformation of E-cadherin-mediated cell-cell contacts by intracellularly pooled proteins was more affected by Ep-CAM expression after trypsin/EDTA digestion, compared to the same cells detached with only EDTA. Newly assembled E-cadherin-mediated adhesions in EαCL-Ep differ in the presence of Ep-CAM from those established in parental cells. Despite the equal aggregation rate of EαCL and EαCL-Ep cells, the adhesion is much weaker in Ep- CAM transfectants.

## Expression of Ep-CAM results in internalization of Eα and EαC molecules

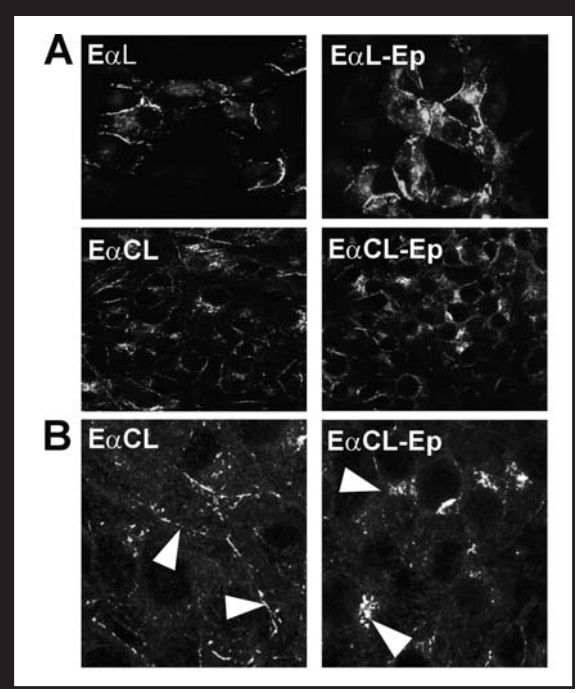
Since expression of Ep-CAM results in the abrogation of E-cadherin-mediated adhesions, the total expression level of the Eα and EαC molecules was analyzed in the presence of different levels of Ep-CAM. Flow cytometry analysis showed no decreased membrane expression of both Eα and EαC fusion proteins (Figure 3A). Upregulation of Ep-CAM by $CdCl_2$ induction did not affect the expression level of Eα, neither did the addition of $CdCl_2$ itself to the control cell lines. The expression levels of Eα or EαC proteins in total lysates remained equal upon low and high Ep-CAM expression when Western blotted and probed with E-cadherin (Figure 3B) and α-catenin (not shown) antibodies.

It has been shown for E-cadherin that a distinction can be made by Triton X-100 extraction between the cytoskeletonanchored fraction and the fraction stored in cytoplasmic depots, or floating at the cell membrane [25-27]. Therefore, to discriminate functional E-cadherin molecules (meaning E-cadherin that is anchored to the actin skeleton) from the nonfunctional pools, the cells were extracted with 1% Triton X-100. Western blotting showed increasing amounts of extractable Eα and EαC, in direct proportion to Ep-CAM expression levels (Figure 3C).

Immunofluorescent stainings confirmed that Ep-CAM abrogates the Eα and EαC chimera-mediated adhesions. To track down the subcellular distribution of both constructs, EαL and EαCL as well as EαL-Ep and EαCL-Ep were stained for E-cadherin. In EαL and EαCL cells, typical E-cadherin "spike-like" intercellular membrane staining was observed, representing adherens junctions [28-30]. Upon Ep-CAM introduction, this staining pattern turned into a diffuse cytoplasmatic staining, whereas Ep-CAM was localized on the membrane (EαL-Ep and EαCL-Ep; Figure 4A). Several previous studies demonstrated that Ep-CAM was localized mainly at the membrane, where it is involved in intercellular homotypic adhesions [17,23,30].

Judging from the fluorescent intensity of staining for E-cadherin in various cell compartments, most of the Eα molecules in EαCL-Ep cells can be observed in the perinuclear zone, while in EαCL it is still present at cell-cell junctions (Figure 4B, indicated by arrows). Double staining for lysosomal membrane glycoprotein (LAMP1 or LAMP2) and E-cadherin in the EL and EαCL-Ep cells did not show any colocalization (results not shown). To investigate whether the intracellular staining reflects newly synthesized proteins, cells were probed with lectin from Helix Pomatia (HPA), but no colocalization was found for the EαC protein and HPA. (results not shown). Since HPA binds to glycosilated groups of synthesized proteins in the *trans* Golgi network, the intracellular staining for E-cadherin/α-catenin molecules probably does not reflect their de novo synthesis. The perinuclear presence of the molecules probably represents an internal (storage) compartment for proteins internalized from the plasma membrane [31]. but remains to be studied.

**FIGURE 4** Intracellular localization of Ea and EaC hybrids.
(A) EαCL and EαCL-Ep stained for E-cadherin show a different localization pattern. Upon Ep-CAM expression (EαCL-Ep), the membranous spike-like expression observed in EaCL cells has shifted to a diffuse intracellular pattern.
(B) Intensity for E-cadherin staining was calculated to a rainbow pallet; black represents no fluorescence and white the highest fluorescent intensity. Arrowheads indicate the highest intensity of E-cadherin-positive staining at cell-cell boundaries in EαCL cells, while most E-cadherin staining was found in the cytoplasm in EαCL-Ep cells.



## Ep-CAM expression results in rearrangement of the cadherin-mediated junctions

Since the cell morphology and aggregation assays demonstrated that Ep-CAM can affect EαC molecule in EαCL-Ep cells, we investigated the effect of Ep-CAM on the catenin-like molecule pp120. pp120 can bind E-cadherin at the juxtamembrane domain and is capable of regulating cadherin-mediated adhesions [32]. In Figure 5A, cells are stained for pp120 (green) and E-cadherin or EαC (red). Colocalization was observed for EL and EαCL. Upon Ep- CAM introduction, E-cadherin was still present at the plasma membrane in EL-Ep; colocalizing with pp120; however, more pp120 was found in the cytoplasm of the cells, compared to EL. In EαCL-Ep cells, EαC and pp120 molecules colocalized to a lesser extent than in EαCL, and some cells showed cytoplasmic pp120. Western blots probed for pp120 showed substantial differences between EL/EL-Ep and EαCL/ EαCL-Ep (Figure 5B). An upregulation in pp120 expression levels was detected in EαCL-Ep compared to EαCL, while a slight decrease of its total cellular level was found in EL-Ep compared to EL. That EαC, in contrast to full-length E-cadherin, does not increase the amount of pp120 remains unexplained. In contrast to the full-length E-cadherin molecules, EαC chimeras are functionally challenged, since several known and

FIGURE 5 Localization of components of the cadherin junction.
(A) Double stainings for pp120 (green) and E-cadherin (red) in L-cells expressing full-length the E-cadherin (EL) or the EαC chimera (EαCL) upon Ep-CAM expression (EL-Ep and EαCL-Ep). From each picture a box with zoom is placed next to the scanned overview.
(B) Western blot of total cell lysates of parental L-cells (L), L-cells expressing full-length E-cadherin (EL), L-cells expressing the EαC fusion protein (EαCL), and these cells, transfected with Ep-CAM, were probed for pp120 and E-cadherin (E-cad). Expression of Ep-CAM was induced by addition of $CdCl_2$ (+).

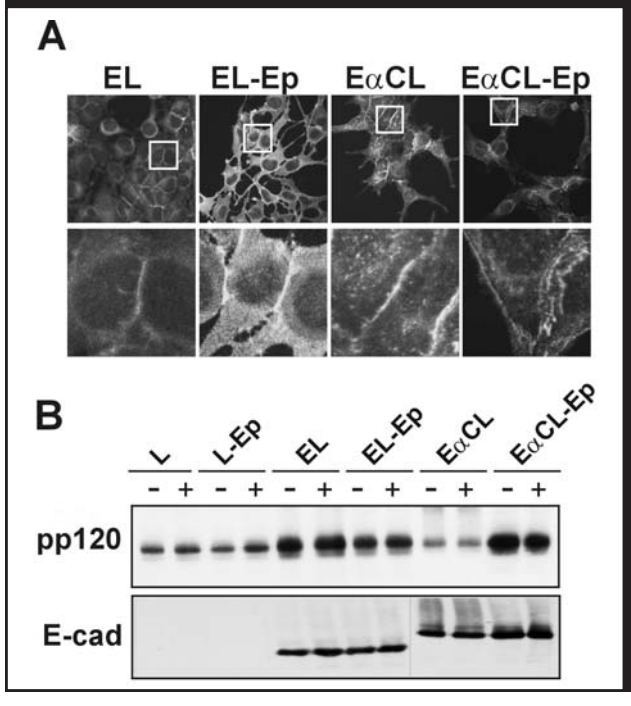
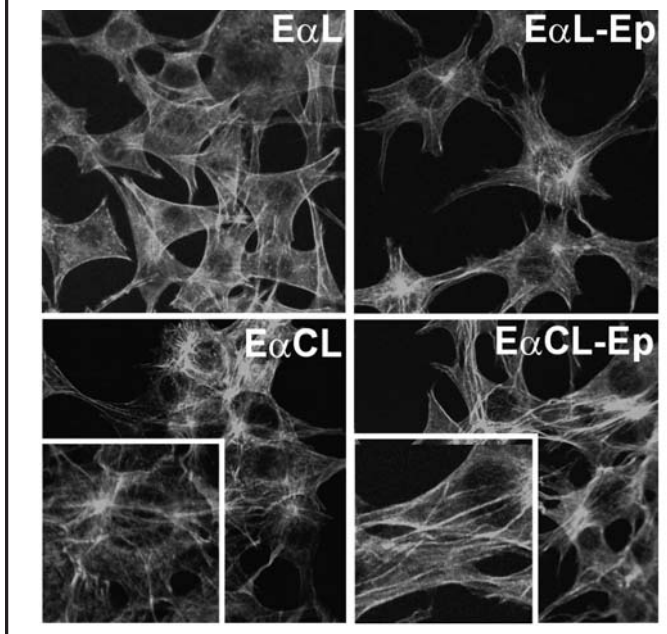



FIGURE 6 Cytoskeletal changes upon expression of Ep-CAM. Actin stainings of L-cells expressing the fusion proteins (EαL and EαCL) show an altered actin cytoskeleton upon Ep-CAM expression (EαL-Ep and EαCL-Ep). Cortical actin has disappeared and actin fibers are condensed in both Ep-CAM transfectants (EαL-Ep and EαCL-Ep) compared to the mock transfectants (EαL and EαCL).




unknown regulatory molecules are unable to dock, thereby leaving normal regulation and feedback mechanisms incomplete.

In summary, pp120 colocalized with both E-cadherin and EαC, also when Ep-CAM was expressed. Ep-CAM expression increased the total expression levels of pp120, but $CdCl_2$-upregulated expression of Ep-CAM did not result in additional increased pp120 expression levels.

## Ep-CAM expression results in cytoskeletal rearrangements

Proper anchoring of the chimera constructs would require at least binding of the α–catenin domain to F-actin or to other unknown anchor points. To investigate whether the overexpression of Ep-CAM is accompanied by the rearrangements of the cytoskeleton, the transfectants were stained with fluorochrome-conjugated phalloidin (Figure 6).

Interestingly, transfection of Eα or EαC constructs had different effects on the organization of F-actin in L-cells. The Eαconstruct in L-cells induced the formation of a very diffused cytoskeleton with some cortical actin; the transfection of the EαC construct in L-cells induced an astral arrangement of actin filaments. Introduction of Ep-CAM in both EαL and EαCL clearly changed the actin organisation. In EαL-Ep, Ep-

CAM expression led to the disappearence of the cortical actin, which was replaced by fibers concentrating in the area of the nucleus. In the EαCL-Ep cells, enlongated stress fibers along the cell body were observed. Although understanding the mechanism requires further investigation, Ep-CAM expression is likely to result in signaling events leading to rearrangement of the F-actin network.

# Discussion

Cell-cell adhesions in epithelial cells, primarily those formed by E-cadherin, are closely involved in regulation of cell proliferation, differentiation, and migration [7,33]. We have previously demonstrated that expression of Ep-CAM, another epithelial cell-cell adhesion molecule, is capable of abrogating the cadherin-mediated cell-cell junctions [16]. This study aimed to find the interaction site in E-cadherin complexes that is affected by Ep-CAM expression. Using hybrid molecules of E-cadherin and α-catenin, several mechanisms regulating cadherins can be bypassed, since several binding domains are no longer present (Figure 1). Expression of Ep-CAM did affect the junctions mediated by the shortest E-cadherin/ α-catenin construct. Ep-CAM introduction changed cell morphology of EαL and EαCL cells to a more loose and less spread phenotype of EαL-Ep and EαCL-Ep cells (Figure 2A and B). The chimera EαC lacks binding sites for α-actinin, Shc, and β-catenin, and therefore for IQGAP1 [34-39]. Thus, it is likely that the key for regulating E-cadherin complexes by Ep-CAM must be found either at the juxtamembrane domain of E-cadherin, or at the carboxy-terminal half of α-catenin. This may demonstrate a pathway for Ep-CAM to regulate E-cadherinmediated junctions, which excludes β-catenin and regulation of its binding to E-cadherin of α-catenin.

Despite that pathways involving β-catenin are recognized as the main regulating pathways for the E-cadherinmediated adhesion, it is not unlikely that E-cadherin can be affected via molecules other than β-catenin [40-42]. Ecadherin- mediated adhesions can exist in a weak or a strong state, and it has been proposed that the shift of E-cadherin mediated adhesion from the weak to strong state does not require β-catenin [41]. It was shown that a weak state of cadherin-mediated adhesion in EαC-transfected L-cells was easily destroyed by repetitive pipetting, concomitant wit increased levels of tyrosine phosphorylation of proposedly cytoplasmic ezrin, radixin, and/or moesin proteins [43].

Results presented here show that repetitive pipetting of Ep-CAM-transfected EαCL cells, EαCL-Ep, can destroy the cell-cell adhesions in aggregates in the presence of $Ca^{2+}$, suggesting that E-cadherin-mediated contacts had shifted from a strong to a weak state upon Ep-CAM expression. E-cadherin that was attracted from intracellular pools during overnight aggregation in DMEM/Nutridoma-SP did reduce the number of large aggregates in EL, compared to EL-Ep, a phenomenon observed between EαCL and EαCL-Ep as well (Figure 2D). The E-cadherin may be pooled intracellularly, and can be recycled to the membrane and be available for junction formation [31]. Resuspension of EL and EαCL resulted in a slight reduction of large aggregates, but in EL-Ep and EαCL-Ep the reduction is substantional. The increase in small aggregates in resuspended EαCL-Ep may be accounted for by the Ep-CAM-Ep-CAM cell adhesions taking over.

In this study, actin stainings showed that expression of Ep-CAM led to cytoskeletal rearrangements in all cell lines described, although the exact character of F-actin rearrangements varied between the studied models. Data have been accumulating, showing that RhoA, Rac1, and Cdc42 mediate cytoskeletal dynamics and thereby regulate cadherin activity [44,45]. Adaptor protein Shc or adaptor molecule IQGAP1, an effector of Rac1 and Cdc42, are unlikely to affect Eα or EαC molecules upon activation of Ep-CAM, since the interaction epitopes are absent in the chimeras [34,38,41]. However, an inverse correlation between RhoA activity and noncadherin-bound pp120 was demonstrated for fibroblasts and CHO cells [46,47]. Furthermore, it was shown that selective uncoupling of pp120 from E-cadherin destroys the strong adhesion mediated by E-cadherin [32,48]. The binding site for pp120 is available in the fusion proteins Eα and EαC [24,46]. From our results, it is possible to conclude that signaling from Ep-CAM to E-cadherin involves pp120. Upon Ep-CAM expression, both E-cadherin and EαC fusion molecules were detected intracellularly. Total levels of pp120 are elevated in EαCL-Ep cells, but not in EL-Ep cells compared to their control cells (EαCL and EL), and more pp120 is found to be in the cytoplasm. These data suggest involvement of pp120 upon Ep-CAM expression, thereby changing RhoA activity.

In summary, E-cadherin-mediated cell-cell adhesions are abrogated by expression of Ep-CAM, without involvement of the regulating molecule β-catenin. Ep-CAM affects the E-cadherin-mediated adhesion indirectly by disrupting the link between α-catenin and F-actin, likely by modulating the organization of the latter, involving pp120. Studies are currently being performed to examine which molecules are involved in the communication pathway between E-cadherin and Ep-CAM.

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Chapter 5

# Downregulation of Ep-CAM induces growth retardation and terminal differentiation of squamous carcinoma cells

## Abstract

The squamous carcinoma cell lines UMSCC2 and -22B, derived from a head and neck carcinoma, are both highly proliferative in vitro and tumorigenic in vivo. These cells express high levels of Ep-CAM, a cell adhesion molecule of normal simple, transitional and pseudostratified epithelia, but not of squamous epithelia, where its expression is associated with preneoplastic changes or malignancy. To investigate the role of Ep-CAM in cell-cell adhesion, anti-sense mRNA constructs for Ep-CAM were introduced in UMSCC2 and -22B cells.

Transfectants expressing the anti-sense constructs showed a remarkable morphological change resembling squamous/terminal differentiation. As compared to the mock transfectants, the antisense construct has markedly suppressed the number of Ep-CAM molecules in cells. Suppression of Ep-CAM resulted in relocation of E-cadherin and β-catenin to the cell-cell junctions and anchorage of the E-cadherin junctional complex to the cytoskeleton. Retardation of cell proliferation and expression of involucrin and cytokeratin 13, markers for terminal and squamous differentiation respectively, were observed.

These results suggest that suppression of Ep-CAM expression in carcinoma cell lines augments E-cadherin mediated adhesions, and promotes the expression of squamous differentiation markers.

## Introduction

Tissue development and remodelling, as well as various physiological and pathological processes, are governed by cell-cell adhesion and interactions of cells with the extracellular matrix and basement membrane.

E-cadherin is one of the major constituents of the adherens junctions formed in epithelial cells. Via α-catenin and β-catenin it is associated with the actin cytoskeleton, and in this way mediates cell-cell adhesion, the rigid structure of epithelial tissues, and

epithelial cell polarity (for review see Gumbiner, 2000). In various malignant tissues, a causal relationship exists between loss of E-cadherin mediated adhesions and malignancy [StCroix et al., 1998; Perl et al., 1998]. Loss of either E-cadherin expression or function renders the cell more invasive and tumorigenic, in particular in squamous carcinoma [Takeichi et al., 1995; Schipper et al., 1991].

Another epithelial cell-adhesion molecule, Ep-CAM, is expressed in nearly all epithelia like simple, transitional and pseudostratified, but not in mature squamous epithelium (for review see Balzar et al., 1999; Winter et al., 2003). In squamous epithelium, it is de novo expressed upon malignancy, from the early stages on, as we have shown for cervical tissues. With tumor progression, more areas of the tissue are expressing Ep-CAM, indicating the association with proliferation, while differerentiation is lost or decreased [Litvinov et al., 1996]. Expression of Ep-CAM is associated with a proliferative cellular phenotype. Studies in liver development showed that Ep-CAM is downregulated upon maturation of cells [De Boer et al., 1999]. Previously, we have demonstrated that in vitro introduction of Ep-CAM by transfection with human Ep-CAM, abrogates the E-cadherin mediated adhesions, resulting in decrease of total cellular α-catenin expression levels [Litvinov et al., 1997]. These results suggest the existence of an inverse correlation between Ep-CAM and E-cadherin mediated adhesions.

These E-cadherin suppressing properties of Ep-CAM indicate that upon malignant transformation of cells, Ep-CAM may contribute to both the proliferative epithelial as well as the malignant phenotype. Furthermore, a developmentally regulated expression and function of Ep-CAM was found in pancreatic islet ontogeny, showing an inverse correlation between expression of Ep-CAM and functional differentiation of cells [Cirilli et al., 1998]. Moreover, a phenomenon of Ep-CAM regulating tissue development and differentiation was demonstrated in the mammary gland of transgenic mice overexpressing Ep-CAM [Balzar et al., 2001]. Compared to non-transgenic littermates, the mammary glands of transgenic mice show a hyperplastic phenotype after termination of lactation, probably because of inhibited involution. Likely, as a regulator of cadherins, Ep-CAM is crucial in tissue development, normal or abnormal with respect to cell growth, differentiation and motility.

In this study we present evidence that suppression of Ep-CAM expression by antisense constructs results in augmentation of E-cadherin mediated adhesions, and the cells tend to differentiate towards a squamous, terminal differentiation state, as shown by expression of cytokeratin 13 and involucrin respectively.





# Materials and Methods

## Cell culture

The cell lines used in this study, UMSCC-2 and UMSCC-22B derived from a head and neck squamous carcinoma (U2 and U22B, University of Massachusetts Squamous Carcinoma Cell line clone 2 and 22B), were kindly provided by Dr. E Schuuring (Dept. of Pathology, Groningen, the Netherlands). Parental and transient transfected cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (all purchased from GibcoBRL, Gaithersburg, MD) in a humidified incubator at 37°C with 5% $CO_2$.

## Constructs

The 150 bp antisense construct of Ep-CAM was derived from a PCR product of 150 bp from the wild type human Ep-CAM (nt 141 - 272, including the ATG; Genbank accession number M76124). The PCR product used in this study was cloned into two inducible expression vectors: the pIND/pVgRXR system for transcient expression, or into pTracer/cmv2, for tracing the construct in transfected cells by expressing green fluorescent protein (GFP, all from Invitrogen, San Diego CA).

For transient expression, the PCR product was cloned in the antisense orientation into the multiple cloning site of *pInd*, and the resulting construct was cotransfected with pVgRXR for transient expression. Transcription of the antisense Ep-CAM was induced upon 24 hrs incubation with MuristeroneA (Invritrogen).

To locate and trace the construct, the PCR product was cloned in the antisense orientation into the pTracer/cmv2 according to the manufacturer's recommendations.

## Transfection

For all transfections, cells were plated in 6-wells plates and grown over-night to a 30% confluency with mostly single cells. Cells were incubated with 15 (g DNA construct, for 2 days using Fugene6$^{TM}$ transfection reagent (Boehringer) according to the manufacturer's recommendations.

The pInd/pVgRXR transfected clones were selected by neomycin (G418, Gibco BRL), the pTracer transfected cells were not selected, but traced with GFP. To enhance transcription of the construct in the pInd/pVgRXR system, 25 μM MuristeroneA (MuA) was added 72 hours prior to experiments.

## Detergent extractions, cell lysis, and Western blot analysis

Cells were grown to 75% density, left on ice for 10 minutes and rinsed with ice-cold PBS/1mM $CaCl_2$ /1mM $MgCl_2$. For extraction, cells were incubated with 0.5% Triton-X-100 in extraction buffer (50 mM TrisHCl, pH7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 300 mM sucrose, 1 tablet complete protease inhibitor (Boehringer Mannheim), gently scraped from the dish, collected and rotated for 30 minutes at 4°C.

The samples were collected and spun down (15 minutes, 15000 rpm). The detergent insoluble fraction (pellet) was separated from the supernatant and further lysed in 100°C SDS buffer (1% SDS, 10 mM EDTA, 10 mM TrisHCl pH7.4, 1 tablet complete protease inhibitor). For total protein lysate, cells were lysed in hot-SDS buffer immediately after rinzing. Protein concentrations in lysates were measured using Bio-Rad $D_C$ Protein Assay (Bio-Rad Lab., Hercules, CA), for equalized loading of the individual samples.

Lysates were size fractionated by SDS/PAGE and semi-dry blotted onto polyvinyl difluoride membrane (Millipore, Bedford, MA). Filters were blocked for 1 hour in 5% skim milk, incubated with a primary antibody for two hours, washed in PBS/ 0.05% Tween-20, and incubated for 1 hour with an anti-mouse IgG HRP-conjugate (Transduction Laboratories, Lexington, KY). The filter was developed with ECL detection substrate (Amersham Pharmacia Biotech, Buckinghamshire, UK).

### Immunofluorescence Microscopy

Cell cultures transfected with the pIND vector were incubated 72 hours with 25 μM MuA to induce transcription. Then they were rinsed with ice-cold PBS / 1mM $CaCl_2$/1mM $MgCl_2$ and fixed with either cold methanol (15 minutes, -20°C) or 4% paraformaldehyd (as previously described; Balzar et al., 1999b) and air-dried. Fixed cells were re-hydrated with PBS, blocked with 5% skim milk in PBS, incubated with a primary antibody for 2 hours, followed by 1 hour incubation with fluorchrome-conjugated IgGs. Cells were examined by confocal microscopy (Zeiss LSM 510, Jena, Germany). For analysis of the cytoskeleton, pTracer transfected cells were incubated for 1 hour with 4% paraformaldehyde, and membranes were permeabilized using 0.5% TritonX-100/PBS and incubated with TRITC-labeled phalloidin (Molecular Probes Europe B.V., Leiden, the Netherlands).

### Antibodies

Ep-CAM was detected with mAb 323/A3 (Centocor Europe BV, Leiden, NL), E-cadherin with mAb HECD-1 (Thamer Diagnostica BV, Uithoorn, NL), β-catenin with clone 14 (Transduction Lab., Lexington, KY), involucrin with clone SY5 (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), and ck13 with clone 1C7 (Thamer Diagnostica BV). For cytoskeletal stainings, TRITC-labeled phalloidin was used (Sigma-Aldrich).

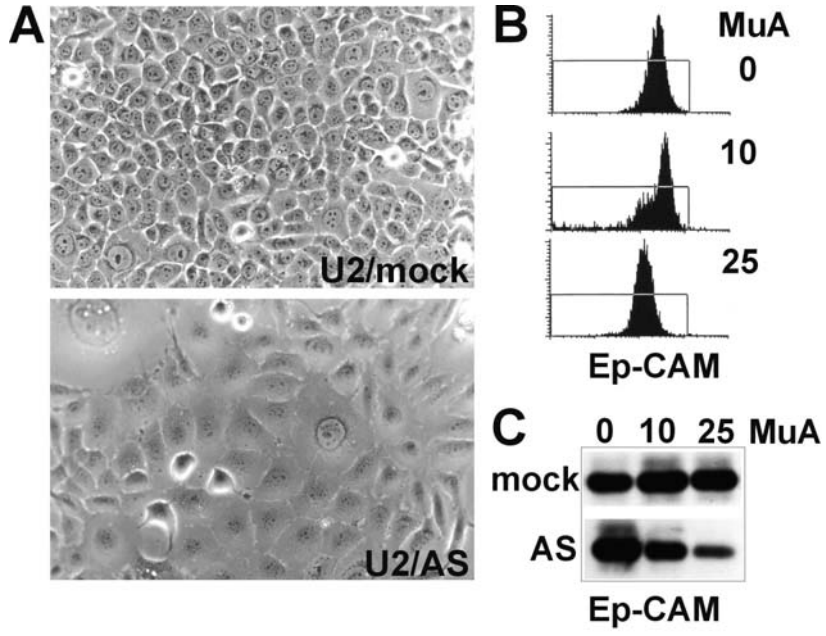
## Results

Ep-CAM is de novo expressed during tumorgenisis in squamous carcinomas of the head and neck tissues [Winter et al., 2003]. The carcinoma cell lines UMSCC-2 and -22B (U2 and U22B) were derived from tumors and are highly proliferative in vitro, as well as highly tumorigenic in vivo. They display relatively high (U2) or intermediate (U22B) levels of Ep-CAM expression, and express various types of classic cadherins at the sites of cell-cell contacts, of wich mainly E-cadherin.





**FIGURE 1** A: Phase contrast microscopic photographs of transciently transfected U2 cells. The highly proliferative and tumorgenic mock transfectant cells (U2/mock) are small and have relatively little cytoplasm. Upon downregulation of the Ep-CAM expression by 72 hrs incubation with MuA, the antisense transfectant cells (U2/AS) are larger with relatively more cytoplasm and are more polarized (U2/AS).
B: Flow cytometric analysis of Ep-CAM expression in Ep-CAM antisense transfected U2 cells and transcription induction by 0, 10, or 25 μM MuA incubation for 72 hours. C: Westen blot analysis for Ep-CAM expression in total lysates of mock transfected and Ep-CAM antisense (AS) transfected U2 cells after 72 hour incubation with 0, 10, or 25 μM MuristeroneA.




### Morphological changes upon Ep-CAM downregulation

The squamous carcinoma cell lines U2 and U22B used in this study, show relatively high (U2) or intermediate (U22B) levels of Ep-CAM expression. Introduction of the 150 bp antisense Ep-CAM construct in U2 cells resulted in a pronounced effect on the morphology of these cells (Figure 1A). The U2/AS cells were much larger as compared to mock transfectants, with more cytoplasma and more polarized orientation. Furthermore, in U2/AS cells, vacuoles were present, and an increased cell death was observed.Confluency was substantionally delayed in the U2/AS, suggesting a reduced proliferation rate (data not shown).

Cell surface levels of Ep-CAM in mock, *pInd*-transfected and MuA induced transfected U2 cells were measured quantitatively by flow cytometric analysis (Figure 1B). Using the *pInd* system, transcription of the antisense construct was induced with 0, 10 or 25 μM MuA incubation. Upon 10 μM MuA, the Ep-CAM expression on the cell

membrane was decreased. This effect was more pronounced upon induction with 25 μM MuA. These results show that Ep-CAM surface expression can be reduced by this antisense construct and the reduction is induced dose dependently.

Samples of equalized protein concentration were Western blotted and showed a dose-dependent reduction in expression levels for Ep-CAM upon MuA induction (Figure 1C). No effects were observed for the mock transfected cells.

## Augmentation of adherens junctions in cells with decreased level of Ep-CAM

Whether the reduction in surface expressed Ep-CAM has any effect on the E-cadherin expression was tested in Western blotting. 80% confluent cell cultures were overnight incubated with the antisense construct with or without incubation with 25 μM MuA (*pIND*-system). Samples were equalized for protein concentration and Western blotted. Figure 2A shows that the total levels of Ep-CAM may be reduced, but the total amount of E-cadherin expression remained unchanged (Figure 2A).

However, immunofluorescence staining showed an increased presence of E-cadherin at the cell membrane in the U2/AS as compared to the parental cell line (Figure 2B). Relatively more Ep-CAM was present in the cytoplasm and the amount of Ep-CAM at the cell membrane was decreased. Most striking was the induction of classical E-cadherin mediated adhesions. The observed staining pattern resembled the typical 'spike-like' cadherin staining pattern. The reduced staining intensity was the result of the E-cadherin molecules being engaged in cell-cell adhesions, i.e. the binding epitope for the antibody was no longer available. In paralel, a substantial portion of the β-catenin was detected at the cell membrane in the typical spike-like staining. For proper E-cadherin mediated adhesions, the presence of β-catenin is required in the adherens juntion complex. Immunofluorescent staining of the parental cells showed the presence of β-catenin at the cell membrane and in the cytoplasm, in U2/AS a punctate staining and relocation of β-catenin along the cell membrane was observed. The β-catenin was mostly present in the 'spike-like' pattern at the cell membrane, i.e. probably in cadherin-mediated junctions. The cytoplasmic pools of β-catenin (ready for degradation?) were more or less reduced. Reduced expression levels of Ep-CAM augmented the presence of β-catenin at the cell membrane, indicating that E-cadherin and β-catenin are structurally involved in cell-cell adhesions in U2/AS.

## Downregulation of Ep-CAM induces terminal differentiation

To distinguishes between cytoskeleton bound and non-bound E-cadherin, cell cultures were extracted with detergent. Western blotting of lysates with equal amounts of total protein showed that the expression of the insoluble fraction of E-cadherin (i.e. cytoskeleton bound Kinch et al., 1995; Skoudy 1995; Francis et al., 1997) increased dramatically in the Ep-CAM suppressed cells (Figure3A).

Western blotting for the terminally differentiation marker involucrin showed an increased expression level in the Ep-CAM antisense transfectants, suggesting that the





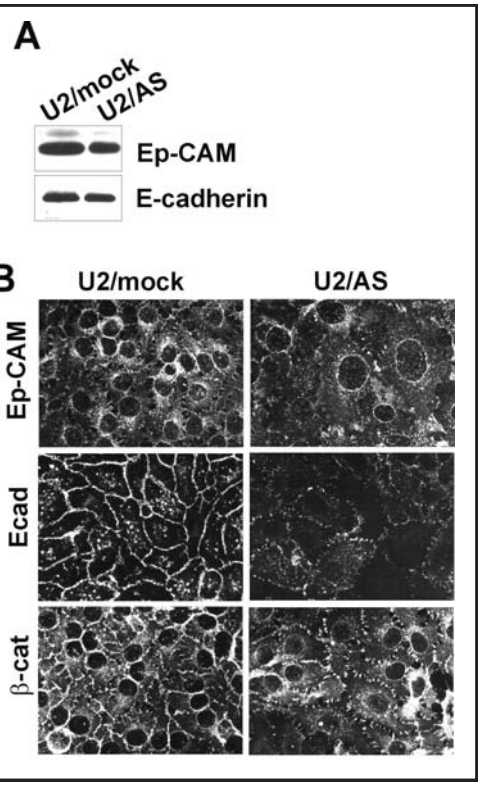



FIGURE 2 A: Western blot analysis for total lysates of transiently transfected, 72 hours 25 μM MuA incubated U2 cells (pInd vector). Upon decreased Ep-CAM expression in U2/AS, the total amount of E-cadherin remained equal in both U2/mock and U2/AS.
B: Immunofluorescent stainings of transiently transfected, 72 hours 25 μM MuA incubated U2 cells (pInd vector). Confluent cell cultures were stained for Ep-CAM, E-cadherin (Ecad), and β-catenin (β-cat).

suppression of Ep-CAM in these cell has forced the cells from proliferation towards differentiation state. Immunofluorescence microscopical analysis showed that both the terminally differentiation marker involucrin and the squamous differentiation marker cytokeratin 13 (ck13) were observed in U2/AS cells, while immunostaining for involucrin and ck13 was absent in the parental cells (Figure 3B).

These results indicate that upon downregulation of Ep-CAM, a more differentiated phenotype can be induced in squamous carcinoma cells. The cells resemble in a way the normal differentiation of squamous cells in vivo.

## Cytoskeletal rearrangements upon Ep-CAM downregulation

Previously, we demonstrated that Ep-CAM expression can shift the balance of GTPase activity within epithelial cells from Rho to Rac activity (Winter et al., unpublished results). The cytoskeletal rearrangments were studied upon downregulation of Ep-CAM in U2 and U22B cells. U22B cells display a more organized phenotype and are therefore more suitable for cytoskeleton studies. Therefore, the results for U2 cells are not shown.

The U22B cells were transfected with the mock or antisense Ep-CAM pTracer/cmv2 plasmid construct. so transfectants can be identified with confocal

microscopy by GFP. TRITC-conjugated phalloidin stainings of both U2 and U22B mock transfectants, showed two different cytoskeleton compostions as opposed to Ep-CAM antisense transfectants (Figure4, results for U2 not shown). In U22B, most actin was observed in the cortical areas of the cell where junctions are formed with neighbouring cells, while stress fibers formation is induced upon Ep-CAM suppression. These observations suggest that the balance of GTPase activity is shifted upon either lack of Ep-CAM expression, presence of E-cadherin expression, or both.

# Discussion

While in normal adult squamous epithelium Ep-CAM expression is absent, in cervical intraepithelial neoplasia de novo expression was detected which is correlated with the severity of the lesion [Litvinov et al., 1996]. The increased expression of Ep-CAM was associated with activated proliferation, as was shown by co-expression of the proliferation marker Ki-67 [Litvinov et al., 1996]. Furthermore, overexpression of Ep-CAM was observed in carcinomas derived from simple epithelium [Balzar et al.,

FIGURE 3 A: Cells were extracted with TritonX-100 to separate the cytoskeleton bound portion from the non-cytoskeleton bound fraction. The triton-insoluble fraction represents the cytoskeleton bound fraction. Western blot analysis for the insoluble E-cadherin and involucrin in transiently transfected, 72 hours 25 μM MuA incubated U2 cells (pInd vector). Upon decreased Ep-CAM expression in U2/AS, both cytoskeleton bound E-cadherin, as well as the squamous differentiation marker involucrin are increased.
B: Immunofluorescent stainings of transiently transfected, 72 hours, 25 μM MuA incubated U2 cells (pInd vector). Confluent cell cultures were stained for cytokeratin 13 (ck13) and involucrin.

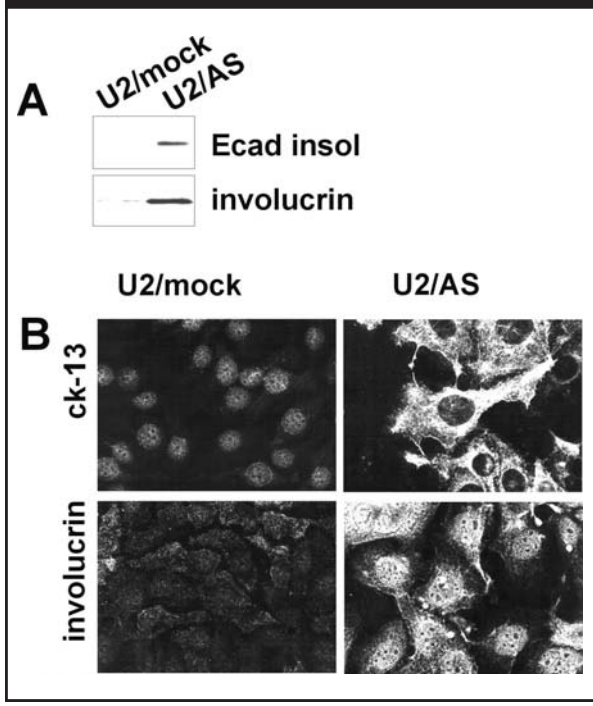
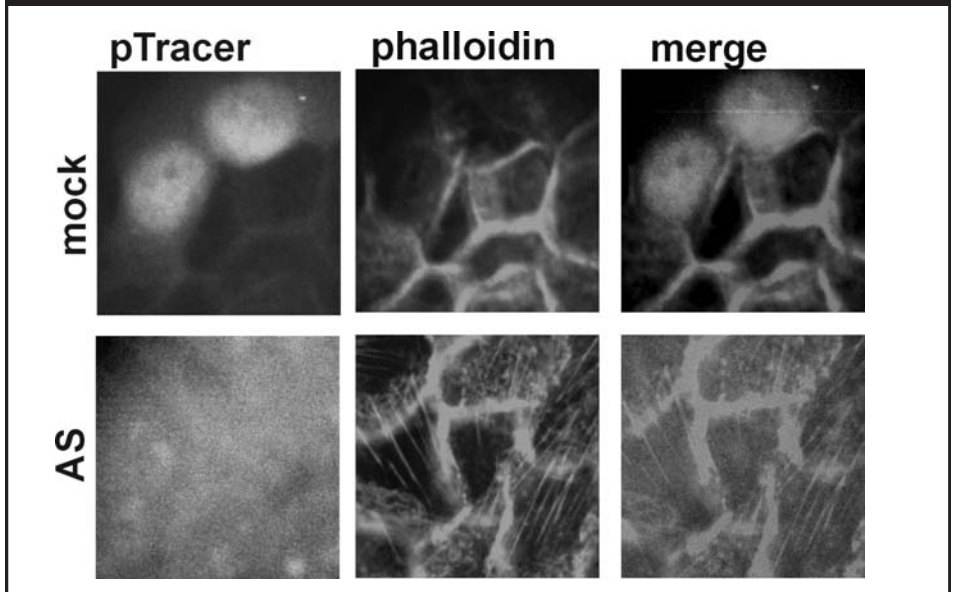









FIGURE 4 Actin staining of U22B cells, stable transfected with mock or Ep-CAM antisense (AS). Using the pTracer plasmid system, positively transfected cells appear as green cells. Actin was stained by incubation with TRITC-labelled phalloidin incubation (red). In U22B / mock transfectants, merely cortical actin was observed. Upon downregulation of the Ep-CAM expression (AS), long and thick stress fibers are formed.




1999; Winter et al., 2003]. Therefore, the question was raised, whether the malignant phenotype could be reverted in vitro by downregulating Ep-CAM in a squamous carcinoma cell line.

The transfection of Ep-CAM antisense constructs in U2 cells resulted in decreased Ep-CAM expression, affecting altered cell morphology. Immunofluorescent stainings showed that downregualtion of Ep-CAM resulted in the reappearance of E-cadherin and β-catenin in cell-cell adhesions. The total level of expression of E-cadherin remained equal in both mock and antisense transfection models, but extraction experiments showed an increased level of cytoskeleton-bound E-cadherin, confirming that E-cadherin is involved in adhesions when Ep-CAM expression is downregulated.

Another important phenomenon, is the 're-differentiation' capacity of the cells upon Ep-CAM downregulation. Positive staining for terminal and squamous differentiation markers involucrin and cytokeratin 13 (ck13) were vitually absent in mock transfectants, but were clearly expressed in the antisense transfectants. From our results it is unclear whether the expression of ck13 and involucrin is induced by cell-cell adhesions mediated by E-cadherin, or simply due to the absence of Ep-CAM expression. Further research is required to detect similar effects in vivo.

Immunofluorescent stainings for cytoskeleton structure showed a difference

between the mock and antisense transfected cells, and it is generally known that the family of small GTPases regulate the structure of the epithelial cytoskleton. The induction of stress fibers in U22B/AS cells is in line with observations by others in epithelial cells when Rho activity is induced [Braga et al., 200a; Takaishi et al., 1995]. Stress fiber formation suggests Rho activity in the cells, and the cell status must have changed upon Ep-CAM suppression. As previously described, Ep-CAM and E-cadherin expression and the activity of the small Rho GTPases are tightly regulated. Rac activation caused disassembly of cadherin junctions in normal human keratinocytes in a time and concentration dependent manner [Williams et al., 1997]. Transfection of constitutively active Rac, V12Rac, in MCF10A carcinoma cells decreases E-cadherin activity, as well as Ras transformation decreased cell-cell adhesion in several cell types (for review see Braga et al., 2000b). On the other hand, Rho activity is required for the establishment of E-cadherin based cell-ell adhesion [Braga et al., 200a,b; Takaishi et al., 1995]. GTPases are involved in cytoskeletal rearrangements upon signals for proliferation, cellular adhesions and migration [Braga et al., 2000a; Williams et al., 1997]. In Drosophila, decreased p120 activity results in accumulation of Rho1 at sites of cadherin based adhesions [Magie et al., 2002]. In vitro, α-catenin and p120 co-immunoprecipitate with Rho1 [Magie et al., 2002]. Ep-CAM expression may shift the GTPase balance within cells towards Rac activity [Winter et al., unpublished results]. and Ep-CAM (indirectly) inhibits binding of pp120 to E-cadherin [Winter et al., 2003]. Downregulation of Ep-CAM with antisense constructs is likely to shift the GTPase activity balance from Rac activity to Rho activity. We have demonstrated that E-cadherin mediated adhesions can be shifted from a strong to a weak state and vice versa, requiring actin cytoskeletal rearrangements [Winter et al., 2003]. These rearrangements are likely to involve Ep-CAM expression, since Ep-CAM is capable of shifting E-cadherin adhesions when truncated E-cadherin is chimerized to α-catenin [Noren et al., 2000]. In the chimerized Eα molecules where α-catenin is directly linked to the extracellular and transmembranal domein of E-cadherin, the binding site for pp120 in E-cadherin is still present, allowing Rho and pp120 to regulate cytoskeleton and E-cadherin junctions [Anastadiadid et al., 2000; Nagafuchi et al., 1994; Winter et al., 2003]. Our results of the cytoskeletal stainings of mock and antisense transfected U22B cells indicate that downregulation of Ep-CAM by antisense constructs may not only augment E-cadherin cell-cell contacts, but also affects the downstream processes. The balance of the GTPase activity has shifted from Rac activity to Rho activity.

In summary, results presented here show the possibility to downregulate Ep-CAM expression in carcinoma cell lines and thereby reversing the malignant phenotype. Both restoration of E-cadherin mediated adhesions and squamous differentiation are observed in this model when Ep-CAM transcription is downregulated. The future strategies of treatment of epithelial cancer may include the use of antisense contructs. The treatment of colorectal cancer has been shown clinically effective by administration of antibodies (edecrolomab) directed against Ep-CAM [Riethmuller et al., 1998]. Further research is needed to establish whether the use of antisense constructs is more targeted and more effective.





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Chapter 6

# Cadherins are regulated by Ep-CAM via activation of phosphaditylinositol-3 kinase

*Mol Cell Biochem 2007, 302:19-26*
*Winter MJ, Cirulli V, Biaire IB, Litvinov SV.*

## Abstract

The cross-signaling between (cell) adhesion molecules is nowadays a well accepted phenomenon and includes orchestrated cellular changes and changes in the microenvironment. For example, Ep-CAM is an epithelial adhesion molecule that prevails in active proliferating tissue and is suppressed in a more differentiated state of the cell. E-cadherin adhesion complexes are typical for the advanced and terminal differentiated cell status. During normal proliferation, E-cadherin is not suppressed. We have demonstrated the effect of overexpression of Ep-CAM on E-cadherin, which probably affects the connection of cadherins and F-actin. Phosphatidylinositol 3-kinase (Pi3K) participates in various regulating mechanisms, for example in signaling to nuclei, vesicle transport, and cytoskeletal rearrangements.

The effect of Ep-CAM on E-cadherin mediated junctions as well as the involvement of Pi3K in regulating adherens junctions, led us to investigate the potential interaction between Pi3K and Ep-CAM. Introduction of Ep-CAM in the epithelial cells caused abrogation of N-cadherin mediated cell-cell adhesion, which could be inhibited by Pi3K inhibitor LY294002. Moreover, the Pi3K subunit p85 was precipitated with Ep-CAM from cell lysates, and this complex showed kinase activity. The Pi3K activity shuttled from N-cadherin to Ep-CAM.

From our results, we conclude that Ep-CAM cross signaling with N-cadherin involves Pi3K, resulting in the abrogation of the cadherin adhesion complexes in epithelial cells.

## Introduction

The maintainance of tissue integrity is a complex and dynamic process, requiring a balance between a variety of signaling pathways. Responding to external signals requires many secondary pathways within the cells in order to respond accurately and in a coordinated fashion [Alahari et al., 2002]. Disturbances in these pathways could lead to dramatic dysfunctioning of the cell, for example carcinogenesis. Diagnosis of *early*

pathological development is very difficult. First, insight and understanding of the disturbed regulating pathways is required.

Ep-CAM is an adhesion molecule that is expressed in all simple, pseudostratified epithelia, but not in mature squamous epithelia. It is strongly associated with cellular proliferation *in vitro* as well as *in vivo* [Litvinov et al., 1996]. Tumors originating from epithelial cells over-express or express *de novo* Ep-CAM. *De novo* expression is already detected in the precursors of carcinomas of squamous epithelium, where Ep-CAM expression serves as an early marker [Litvinov et al., 1996; Winter et al., 2003].

Phosphatidylinositol 3 kinase (Pi3K) is an ubiquitous enzyme involved in many intracellular signal transduction pathways. It participates in various regulating mechanisms, for example in cytoplasmic- to nuclear signaling, vesicle transport, cytoskeletal rearrangements and CD28-B7 outside-inside signaling [Alahari et al., 2002; Cefai et al., 1996; Fukui et al., 1998]. Important target molecules are the small Rho GTPases, which are involved in cytoskeletal organisation. Cytoskeleton (re-) arrangement results in signaling to integrins and cadherins. Ras/GTP, activated by growth factors, promotes Pi3K activity and thereby inhibits apoptosis [Marshall 1996]. The Pi3K protein consists of an 85 kDa and a 110 kDa subunit. The p85 subunit is recognized as being the regulatory subunit, while the p110 subunit employs the actual kinase activity that converts plasma membrane lipid $PIP_2$ into $PIP_3$.

E-cadherin adhesion complexes are typical for the advanced and terminal differentiated cell status. The Ep-CAM expression in epithelia inversely correlates with cadherin-mediated junction complexes. In vitro studies demonstrated that cadherin-mediated adhesions are abrogated upon Ep-CAM introduction, probably by affecting the connection between cadherin adhesion complexes and F-actin [Litvinov et al., 1997; Winter et al., 2003]. This results in reversing the E-cadherin-induced epithelial phenotype of L-cells into a mesenchymal phenotype [Litvinov et al., 1996,1997]. Our *in vivo* studies revealed a regulated expression of Ep-CAM in pancreatic islet ontogeny [Cirulli et al., 1998]. During maturation of the pancreatic islet-like cells, Ep-CAM expression decreases and simultaneously the (endocrine) differentiation increases. Pi3K was demonstrated to negatively regulate the differentiation in human foetal pancreatic cells [Ptasznik et al., 1997]. In cells expressing cadherin, cytoskeletal rearrangements are observed upon Ep-CAM introduction [Winter et al., 2003]. These rearrangements may be the result of involvement of small GTPases or Pi3K.

The results described here, show that signaling between Ep-CAM and cadherins involves activation of Pi3K.





# Material and methods

## Cells, constructs and cell culture

Human breast epithelial cells HBL100 (clone HCA, kindly provided by Dr. Hilkens, The Netherlands Cancer Institute, Amsterdam. The Netherlands) were transiently transfected with full length human Ep-CAM cDNA that was subcloned into the pMep4 vector (Invitrogen BV., Leek, the Netherlands), using the HindIII/Bgl restriction sites [Litvinov et al., 1997]. The contruct is placed under the control of the methallothionin promotor, which can be induced by divalent heavy metal ions (e.g. $Cd^{2+}$or $Zn^{2+}$). This allows a stepwise expression of Ep-CAM. Transfection was performed with FugeneTM6 Transfection Reagent (Boehringer Mannheim, Mannheim, Germany) and selected by 1 mg/ml Hygromycin B (Boehringer Mannheim), as described earlier [Litvinov et al., 1997].

For total-Akt and phospho-Akt blots, cells were serum starved overnight, and re-administered 2% foetal bovine serum during 8 hours in the presence or absence of $CdCl_2$ or 25 μM LY294004 (LY) before lysing. $CdCl_2$ induces the transcription of Ep-CAM, LY294004 inhibits the Pi3K activity.

## Antibodies and chemicals

The monoclonal antibodies anti-N-cadherin (clone #32), anti-E-cadherin (clone #36), anti-α-catenin, anti-β-catenin and anti-p85 were obtained from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against total-Akt and phospho-Akt are from Cell Signaling Tech. (Beverly, MA). The monoclonal antibody 323/A3, directed against Ep-CAM, was kindly provided by Centocor B.V., the Netherlands. Secondary antibodies for immunoblotting were labeled with HRP (Tansduction Laboratories) and for immunofluorescent stainings isotype specific antibodies were labeled with either Alexa-488, Alexa-546 (Molecular Probes Europe BV, Leiden, the Netherlands) or Cy-5 (PickCell, Amsterdam, the Netherlands).

For immunoprecipitations, Protein G Sepharose beads from Amersham were labeled (Amersham Pharmacia Biotech, Buckinhamshire, UK). At least 16 hours prior to fixation or lysis of cells, 25 μM LY294002 (Biomol Research Laboratories Inc., Plymouth Meeting, PA) was added to the culture medium to inhibit Pi3K. Ep-CAM expression in transfected cells was enhanced by overnight incubation of 10 or 25 μM $CdCl_2$.

## Immunoprecipitation

Cells were grown to 80% density and overnight induced (25 μM $CdCl_2$). The procedure was followed as described previously [Balzar et al., 1998], using TritonX100, β-octyl glucopyranoside or CHAPS as detergents.

## Detergent extraction, total lysates and Western Blot analysis

For extraction, overnight induced (25 μM $CdCl_2$) cell cultures were rinsed with ice-cold 1 mM $CaCl_2$/ 1 mM $MgCl_2$ in PBS and extracted with 1% TritonX-100 or 50 mM

CHAPS in extraction buffer as described previously [Balzar et al., 1998]. The samples were collected and spun down (15', 15000 rpm). The detergent-insoluble fraction of cells was separated from supernatant (the detergent-soluble fraction), and further lysed in hot 1% SDS/ 10 mM EDTA. For total lysates, the cells were lysed in hot lysis buffer immediately after rinsing off the culture medium with ice-cold 1 mM $CaCl_2$/ 1 mM $MgCl_2$ in PBS.

Protein concentrations in homogeneous lysates were measured using Bio-Rad $D_C$ Protein Assay (Bio-Rad Lab., Hercules, CA). For all Western blots, 10 μg of homogenous lysate was loaded per lane of the 10% SDS-gel, representing equal protein load per lane while avoiding control stainings [Balzar et al., 1998; Litvinov et al., 1997; Winter et al., 2003]. Proteins were separated by SDS/PAGE, and transferred electrophoretically onto PVDF-membrane (Millipore, Bedford, MA). The membrane was stained as described previously [Litvinov et al., 1997]. The blots were visualized using the ECL detection substrate (Amersham).

### Pi3K activity assay

The Pi3K activity of anti-p85 immunoprecipitates was analyzed as described previously [Ptasznik et al., 1997].

Briefly, aliquots of cell lysates normalized for protein content were incubated with anti-Ep-CAM antibodies and absorbed onto protein A-Sepharose and washed. The reaction was carried out for 10 min in a buffer that also contained 10 μg of PI (Avanti Polar Lipids, Alabaster, AL) and 10 μCi [γ-32P]. ATP (6,000 Ci/mmol; DuPont/NEN, Wilmington, DE). After the incubation, the reaction was stopped with methanol plus 2.4 N HCl (1:1, vol/vol), and lipids were extracted, and analyzed by thin-layer chromatography.

### Confocal Microscopy

Cells at 80% density and overnight induced (25 μM $CdCl_2$) were washed with ice-cold 1 mM $CaCl_2$/ 1 mM $MgCl_2$ in PBS, and fixed during 15 minutes with -20°C methanol and allowed to dry. Cells were blocked with 5% skim milk in PBS, the primary monoclonal antibody was incubated for 1 hour, followed by an hour incubation with an fluorochrome labeled secondary antibody.

For actin staining, cells were fixed for 1 hour in 4% paraformaldehyd, permeabilized with 0.3% TritonX-100 [Balzar et al., 1998]. and incubated with TRITC-labeled phalloidin (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). After drying, cells were mounted and examined by confocal microscopy (Zeiss LSM 510, Jena, Germany).

## Results

### Pi3K switches between Ep-CAM and N-cadherin

The breast epithelial cell line HBL100 was transiently transfected with pMep4 plasmids containing either wt Ep-CAM cDNA, HME, or empty vector, HMC. cDNA transcription under control of the methallothionin promotor can be enhanced by incubation with divalent cations. This enables a 3-step increase in expression level by 1) the





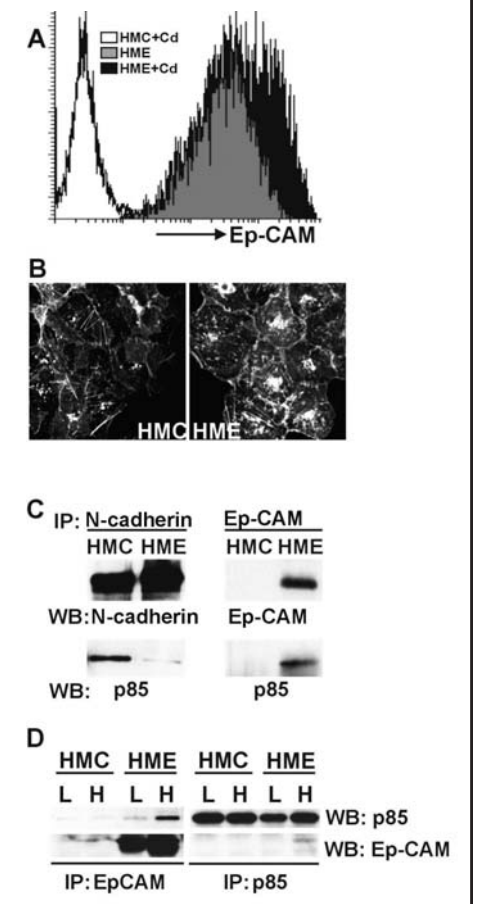


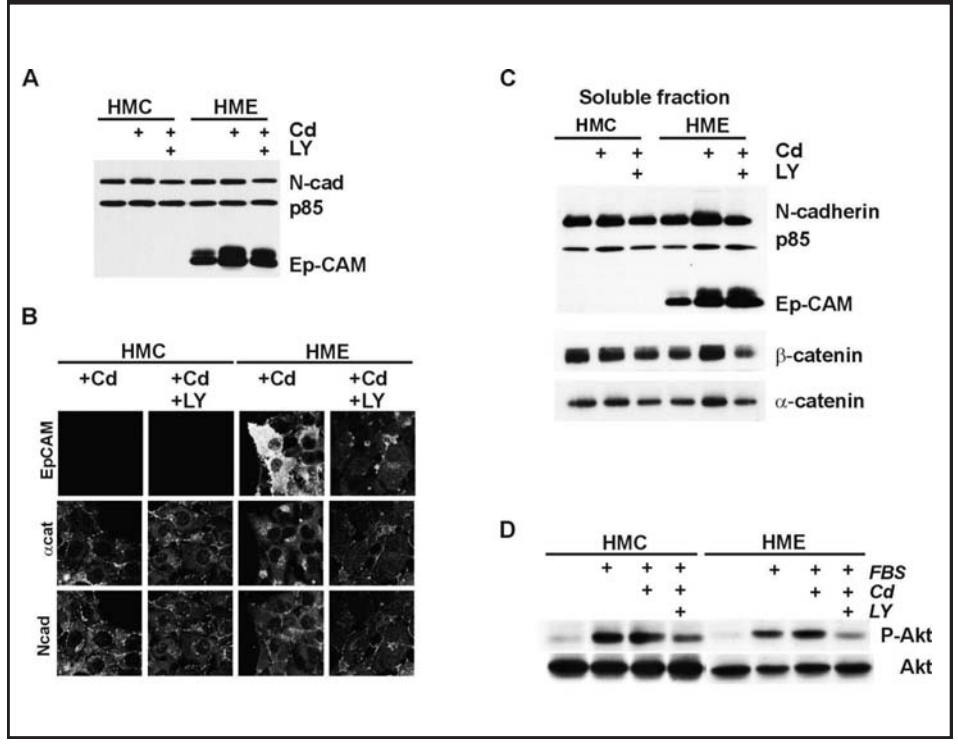

**FIGURE 1** Expression of Ep-CAM in HbL100 cells.
A: Flow cytometry analysis of mock (HMC) and Ep-CAM transfected HbL100 cells (HME). The mock transfected cells, HMC, did not express Ep-CAM at the cell surface (white). The Ep-CAM expression was detected in HME (grey), which was increased upon overnight incubation with $CdCl_2$ (black)
B: F-actin staining of overnight $CdCl_2$ induced HMC and HME cells, using TRITC-conjugated phalloidin. In HME cells, the actin cytoskeleton is concentrated in the cortical area, in contrast to HMC cells, where more fibers can be found
C: 10 μg of homogenous lysate was loaded per lane of the 10% SDS-gel, representing equal protein load per lane. Western blotting total lysates of HMC and HME and probed for N-cadherin, Ep-CAM and the Pi3K subunit p85. Cell cultures were lysed after overnight incubation with CdCl2 and LY. P85 shuttled between N-cadherin and Ep-CAM. In HMC cells, p85 precipitated with N-cadherin. Upon Ep-CAM introduction, HME cells, p85 now precipitated with Ep-CAM.
D: Immunoprecipitation for Ep-CAM and p85 on high (H) or low (L) density cell cultures, and Western blotted for Ep-CAM and p85. P85 and Ep-CAM only co-precipitated in HME cells from high densed cultures.

mock transfectant (promotor only, HMC), 2) transfectant with 'normal' transcription (HME), and 3) transfectant with enhanced expression (HME + 25 μM $CdCl_2$) [Balzar et al., 1998]. Cell surface expression of Ep-CAM was measured by flow cytometry analysis (Figure 1A).

Cross-signaling between E-cadherin and Ep-CAM has previously been demonstrated to involve the connection between cadherin-mediated cell-cell junctions and F-actin [Balzar et al., 1998; Winter et al., 2003]. Cytoskeletal rearrangements are observed upon Ep-CAM introduction in cells expressing N-cadherin (Figure 1B). In HME cells, the actin cytoskeleton is concentrated in the cortical area, in contrast to HMC cells where more fibers can be found.

Co-immunoprecipitation for N-cadherin (Figure 1C) or Ep-CAM showed that the Pi3K regulatory subunit, p85, shuttles between cadherin and Ep-CAM; most p85 is

FIGURE 2 Abrogation of N-cadherin mediated adhesions.
A: 10 μg of homogenous lysate was loaded per lane of the 10% SDS-gel, representing equal protein load per lane. Western blotting of total lysates of CHAPS extracted HMC and HME cell cultures after $CdCl_2$ and/ or LY incubation, stained for N-cadherin, Ep-CAM and p85. In HME cells, LY incubation decreased the high Ep-CAM expression levels.
B: Immunofluorescent staining of HMC and HME for Ep-CAM, α-catenin and N-cadherin after incubation with $CdCl_2$ and LY. Ep-CAM expression in HME cells, abgrogated the presence of N-cadherin and α-catenin at the cell-cell boundaries. In these same HME cell, inhibition of Pi3K by LY, resulted in re-location of N-cadherin and α-catenin at the cell-cell boundaries
C: Western blotting of the soluble fraction of CHAPS extracted HMC and HME cell cultures after $CdCl_2$ and/ or LY incubation, stained for N-cadherin, Ep-CAM, Pi3K subunit p85, β-catenin and α-catenin. The soluble fraction represents the cadherins and catenins that are not bound the cytoskeleton, i.e. not complexed in cell-cell adherens junctions. In HME cells, the soluble fraction of Ep-CAM increased upon LY incubation, while the soluble fractions of β- and α-catenin decreased.
D: Western blotting for total Akt and phosphorylated Akt (P-Akt) in HMC and HME total lysates after serum starvation and subsequent addition of FBS, $CdCL_2$ and LY. In serum starved HMC and HME cells (first lanes of both) there was hardly any phos-phorylated Akt detectable. Addition of FBS induced the p-AKT in HMC and HME, and upon increased expression of Ep-CAM (CD induced), more P-AKT was detected in HME. This increase in P-Akt in HME was only partially partially blocked by LY, although the levels of P-Akt in HME appeared to be decreased more than in HMC.




complexed with N-cadherin in HMC cells, while in Ep-CAM expressing HME cells, the precipitation complex with N-cadherin has 'lost' most of the p85 molecules. Immunoprecipitation with Ep-CAM only precipitated molecules in the Ep-CAM expressing HME cells, where p85 has appeared in the complex. Interestingly, performing the immunoprecipitation for Ep-CAM on high (95%) or low (15%) density cell cultures, p85 and Ep-CAM only co-precipitated in HME cells from high density cultures (Figure 1D).

## Effect of Ep-CAM on N-cadherin can be blocked by LY

Western blotting of total lysates of HMC, HME, and HME + $CdCl_2$ cells showed that the amount of the regulatory subunit of Pi3K, p85, had not changed in these cells (Figure 2A). Pi3K inhibitor LY294004 (LY) incubation resulted in a slight decrease of total N-cadherin in both HMC + $CdCl_2$ and HME + $CdCl_2$. Ep-CAM expression was strongly upregulated in HME + $CdCl_2$ cells, but upon LY incubation the total expression of Ep-CAM had decreased.

Immunofluorescent staining for N-cadherin and α-catenin showed that these molecules were present at the cell-cell boundaries in HMC, most likely in cadherin-mediated adhesion complexes (Figure 2B). The cadherin mediated adhesion complex had turned more diffuse in HME cells, where Ep-CAM was expressed. Upon LY incubation, the N-cadherin and α-catenin re-located to the cell plasma membrane, showing the 'spike'-like staining pattern in HME + $CdCl_2$/LY, while Ep-CAM had left the cell-cell boundaries.

Ep-CAM transcription and expression in cell cultures (80% monolayer) were induced with 10μM $CdCl_2$. Cells were subsequently incubated with 25 μM Pi3K inhibitor LY. Cells were CHAPS detergent extracted before lysing them, allowing discrimination between the soluble and insoluble proteins. Figure 2C shows the soluble fraction that represents the cadherins and catenins that are not bound the cytoskeleton, i.e. not complexed in cell-cell adherens junctions. As a lysate, the soluble fractions of CHAPS extracted cells (i.e. non-bound to F-actin) were Western blotted and stained for N-cadherin, p85, Ep-CAM, β-catenin and α-catenin. Per lane, 10 μg of each homogenous lysate was loaded, representing equal protein load per lane while avoiding control stainings. Figure 2C demonstrates that the N-cadherin, β-catenin and α-catenin levels had increased upon $CdCl_2$ enhanced Ep-CAM (Figure 2C). Only the soluble fraction of cadherins and catenins increased while the total amount remained unchanged (not shown, see [Litvinov et al., 1997]). The increased proteins in the soluble fraction indicates that more cadherin junctional complexes are disconnected from the cytoskeleton upon Ep-CAM introduction, and not the result of increased cadherin and catenin expression (the total amount of cadherins and cadherins did not change). By blocking Pi3K activity by LY incubation, levels of soluble N-cadherin, β-catenin and α-catenin decreased, although Ep-CAM expression levels were still high.

It is plausible that Ep-CAM, which is associated with proliferation both *in vitro* and *in vivo* [Litvinov et al., 1996], regulates epithelial cell differentiation via the

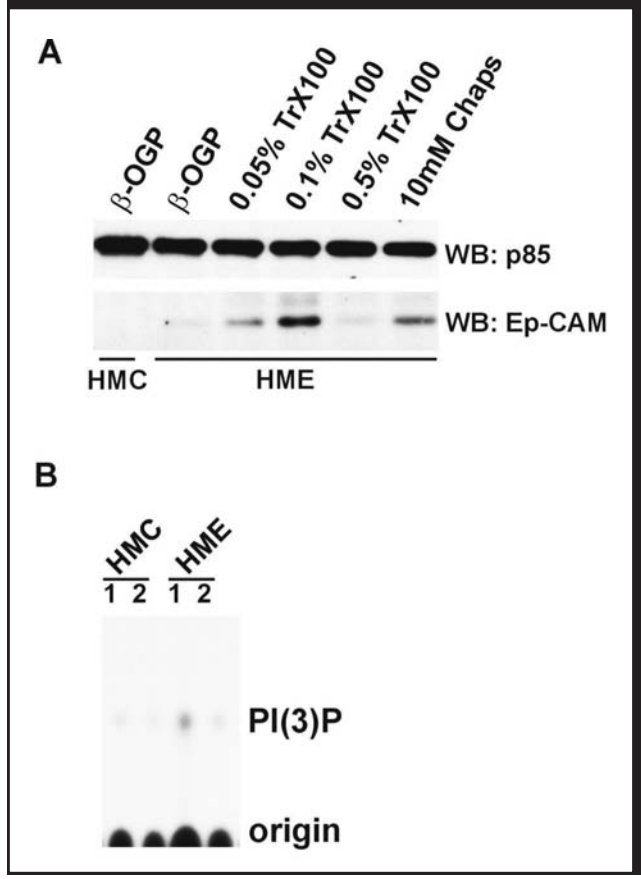



FIGURE 3 Ep-CAM and Pi3K in signaling.
A: 10 μg of homogenous lysate was loaded per lane of the 10% SDS-gel, representing equal protein load per lane. Immunoprecipitation with Pi3K subunit p85 in lysates of several detergents, and blotted for p85 and Ep-CAM. OGP: β-octyl glucopyranoside. Ep-CAM is present in the precipitated complex
B: Kinase activity assay for precipitates from HMC and HME. The formation of PI3P in HME cells is the result of kinase activity in the Ep-CAM/p85 complex (lane 1; precipitate with anti Ep-CAM antibody), while no $PIP_3$ was formed when precipitated with a control antibody (lane 2; anti-IgG).

Pi3K/Akt signaling pathway. To validate the active state of Pi3K, the level of phosphorylation of Pi3K substrate, Akt, was measured. Serum-starved HMC and HME were Western-blotted for total Akt and phosphorylated Akt (P-Akt, Figure 2D). Total lysates of both HMC and HME hardly showed any P-Akt. Addition of 2% serum had activated Pi3K, as can be concluded from the increase in P-Akt per 10 μg in both HMC and HME. Incubation with $CdCl_2$, which enhanced the expression of Ep-CAM in HME, increased the level of P-Akt in HME, as compared to serum-induced HME cells. This increase of Pi3K activity could partially be blocked by LY, although the levels of P-Akt in HME appeared to have decreased more than in HMC. Since equal protein concentrations were blotted and the total levels of Akt did not change, this demonstrates Pi3K activity as a result of Ep-CAM expression.

### Pi3K is associated with Ep-CAM

Immunoprecipitations with the Pi3K regulatory subunit, p85, using different cell lysing buffers, demonstrated that Ep-CAM is present in the precipitated complex (Figure 3A).

It remained unclear whether this Ep-CAM/p85 complex included the Pi3K subunit p110 that employes the kinase activity. Therefore, a kinase activity assay was performed (Figure 3B): The Ep-CAM/p85 complex was precipitaed with the anti-Ep-CAM antibody and subsequently incubated with 32-P labeled ATP and PI. If the Ep-CAM/p85 complex would have kinase activity, this would result in the formation of PI3P, while no PI3P was formed when precipitated with a control antibody.

## Discussion

Cross-signaling between cadherins and other adhesion molecules is a common phenomenon [Alahari et al., 2002]. Ep-CAM expression is associated with proliferation and inhibits epithelial differentiation [Cirilli et al., 1998; De Boer et al., 1997; Litvinov et al., 1997]. In a previous study we showed that the communication between Ep-CAM and E-cadherin does not involve the regulatory molecule β-catenin [Winter et al., 2003]. Ep-CAM abrogated the E-cadherin-mediated adhesion complex by disrupting the connection between α-catenin and the actin cytoskeleton. An identical response to Ep-CAM introduction was found for N-cadherin [Balzar et al., 1998]. Since this is not the result of a physical interaction, one or more other molecules have to be involved [Winter et al., 2003]. In the present study, we show that cross- signaling between Ep-CAM and classical cadherins involves the Pi3K signaling pathway. This was already suggested by the fact that Rac/Pi3K activation induced disassembly of cadherin- mediated adhesions. The same findings have been described for Ep-CAM expression [Braga et al., 2000; Litvinov et al., 1994; Potempa et al., 1998]. As previously described, both Ep-CAM and p85, the regulatory subunit of Pi3K, are capable to bind α-actinin, although under different conditions [Balzar et al., 1998; Shibasaki et al., 1994]. Our results demonstrating co-precipation of Ep-CAM and p85 suggest for the first time that these molecules do interact. Furthermore, our results indicate that this complex has kinase activity. Both Ep-CAM and E-cadherin mediated adhesions are localized at the basal-lateral membrane and mediate cell-cell contacts by themselves. Ep-CAM also regulates cadherin-mediated adhesions, which might be modulated by shuttling of p85. Nelson et al. reported that Pi3K activity is required for cell-cell contact induced proliferation, which in line with our results [Nelson and Chen 2002].

Pi3K activation promotes the cell entering the S phase of the cell cycle and Pi3K activation promotes cellular changes that are characteristic of premalignant transformation [Klippe et al., 1998]. Ep-CAM is associated with proliferation both in vitro and in vivo and was de novo expression was demonstrated in adult squamous carcinomas [Litvinov et al., 1996; Winter et al., 2003a]. Akt has been shown to repress transcription

of the E-cadherin gene. Secondly, cells expressing constitutively active Akt produce the transcription factor Snail that represses the expression of the E-cadherin gene [Grille et al., 2003]. Ep-CAM and E-cadherin expression are reversely correlated in epithelial cells and forced expression results in abrogation of E-cadherin-mediated adhesions [Litvinov et al, 1996; Winter et al, 2003b]. Therefor, it is not unlikely that Ep-CAM is involved in de Pi3K/Akt signaling pathway regulating epithelial cells upon external signals.

Signaling pathways downstream from Pi3K are implicated in cell dissociation and scattering in MDCK cells [Royal et al., 1997]. Furthermore, forced Ep-CAM expression in transgenic mice induced budding and secondary branching of the mammary glandular tree in virgin females [21]. MAPK and Pi3K activation by Ras is required for HGF/SF-induced adherens junction disassembly in MDCK cells [Potempa et al. 1997; Royal et al., 1997], and Pi3K activity promotes invasiveness in epithelial breast cells [Keely et al., 1997, 1998]. Ep-CAM promotes invasiveness in Ep-CAM transfected HBL100 cells in matrigel (our unpublished data) and the relationship with the the small GTPases in this process is under investigation.

Both Ep-CAM and p85, the regulatory subunit of Pi3K, can bind α-actinin, although under different experimental conditions [Balzar et al., 1998; Shibasaki et al., 1994]. Recently, p85 was found to bind to a specific domain in membrane-bound ezrin, a plasma membrane- microfilament linker. Moreover, Gautreau and co-workers stated that survival signaling by ezrin is regulated through the Pi3K/Akt pathway [Gautreau et al., 1999]. ICAM-2 clustering induced ezrin tyrosine phosphorylation and enhanced threonine phosphorylation , concomitant with an increase in p85/ Pi3K association in Jurkat T cells [Perez et al., 2002]. It is possible that in the complex of Ep-CAM and Pi3K ezrin is also present sustaining a survival in cells that have down-regulated E-cadherin mediated adhesion.

Potempa and Ridley found that the disassembly of cadherin junctional complexes in migrating HGF/SF stimulated MDCK cells could be blocked by Pi3K inhibitors LY or PD98059 incubation as well as by micro-injected dominant negative MAPKK1 [Potempa et al., 1998]. However, activation of Pi3K or p42/p44 MAPK was not sufficient to induce a disruption of adherens junctions. Wennström and Downward reported similar results in EGF stimulated COS-7 cells [Wennstrom et al., 1999]. They postulated that the ability of LY to inhibit the EGF-R/Ras/Raf/MEK pathway is due to basal Pi3K activity that contributes to Ras activation upon weak stimuli (e.g. low concentration of EGF) [Wennstrom et al., 1999]. Pi3K activity downstream from Ras can only be induced by high concentration of EGF induced Ras or oncogenic Ras. The basal Pi3K activity may be provided by Ep-CAM expression.

A role for Ep-CAM as a morphoregulatory molecule becomes more plausible. Ep-CAM expression implicates proliferation and differentiation, as previously demonstrated [Cirulli et al., 1998; De Boer et al., 1999; Litvinov et al., 1997; Winter et al., 2003a]. Pi3K physically interacts with activated growth receptors, transducing survival signals. The signaling between Ep-CAM and Pi3K has its concequences for cellular





motility and/or invasive capacity. Disturbed regulation of these molecules may result in increased metastatic capacity, possibly by affecting the small Rho GTPases and thereby inducing cytoskeletal rearrangments.

It can be concluded from the results described here that Pi3K can act downstream from Ep-CAM. The question of a potential feedback loop from Ep-CAM to Pi3K remains to be studied. Our first experiments do not eliminate the possibility. E.g. expression of Ep-CAM deletion mutants incapable of adhesion, results in altered p85 expression levels (unpublished observations). These preliminar observations would be in line with conclusions from other research groups. For example, Akt, acting downstream of Pi3K, is activated by the formation of E-cadherin-mediated junctions [Pece et al., 1999], while formation of E-cadherin-mediated junctions was demonstrated to be inversely correlated to Ep-CAM expression [Litvinov et al., 1997]. Although the mechanism of a putative regulation loop needs further investigation, such a mechanism may explain the observations of both a *de novo* Ep-CAM expression in cervical carcinoma [Litvinov et al., 1996]. and the amplification of the gene encoding for Pi3K subunit p110α in cervical and ovarian carcinomas [Ma et al., 2000; Shayesteh et al., 1999].

The experiments reported here demonstrate that Pi3K can act downstream from Ep-CAM. Abnormal signaling between these molecules may play a crucial role in the biology of some epithelial tumors.

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Chapter 7

# The epithelial cell adhesion molecule Ep-CAM as a morphoregulatory molecule is a tool in surgical pathology

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## Abstract

Cell adhesion receptors (CAMs) are actively involved in regulating various cell processes, including growth, differentiation, and cell death. Therefore, CAMs represent a large group of morphoregulating molecules, mediating cross-talk between cells and of cells with their environment. From this perspective, CAMs do contribute to cells and tissue organization, and in diseased tissue, to the disease development and biological characteristics. Therefore, observed changes in expression patterns of adhesion molecules may contribute to establish a diagnosis. A distinct shift in expression patterns in neoplastic epithelium has been described, for example for cadherins, integrins, and CD44. A relatively novel cell CAM, Ep-CAM, was first reported to be a pan-carcinoma antigen, although it is rather a marker of epithelial lineage. Several antibodies directed to Ep-CAM have been generated, and many epithelial tissues and their neoplastic appendages have been studied. This article outlines the results of these studies. Based on the results of these studies, we conclude that Ep-CAM immunohistochemistry can be a useful tool in the diagnosis of disturbed epithelial tissues.

## Introduction

Four major families of cell adhesion molecules (CAMs) are recognized on the basis of their structure: integrins, selectins, CAMs of the immunoglobulin gene (IgG-like) super family, and cadherins. Also other types of molecules with adhesion properties have been reported, for example, syndecans, CD44, and Ep-CAM. Nowadays, CAMs defined as morphoregulatory molecules that affect cellular processes, based on data about inside-out and outside-in signaling and signal transduction pathways. During embryogenesis, but also in tumor development, the maturation and differentiation of epithelial cells is regulated by signals within the epithelium and between epithelia and other tissues. Every tissue type and state of maturation can be defined by specific expression patterns of adhesion molecules. Changes in expression patterns of one or several adhesion molecules may suggest altered tissue differentiation or maturation. In other words, a disturbed tissue maintenance may be concomitant by ectopic or overexpression of adhesion molecules, and this can be used as a tool in surgical pathology. For example, in epithelial tissues many studies have been conducted to study the morphoregulatory role of E-cadherin [1]. A tumor suppressor function for E-cadherin has been proposed frequently, and has been demonstrated in breast and gastric cancer. Inactivation of E-cadherin is an early and crucial step in the formation of lobular carcinoma *in situ*, as a precursor of invasive lobular breast cancer and hereditary gastric cancer [2-6]. Furthermore, co-expression of E-, N-, and P-cadherin was demonstrated for several breast tumors, but unique expression patterns were distinguishable for each type of tumor [7]. Here we analyze in detail the biological significance and diagnostic value of the expression changes of a novel adhesion receptor, Ep-CAM.

## Ep-CAM

Ep-CAM has first been identified as a tumor-specific antigen on several carcinomas of different origin. Several independent studies generated different antibodies directed against the tumor-specific molecule expressed on carcinomas (figure 1). In

TABLE 1 Known antibodies directed against the epithelial adhesion molecule Ep-CAM.

| ANTIBODY | EPITOPE | REFERENCE |
|---|---|---|
| AUA1 | EGF-like domain I | Durbin et al., 1990 [66] |
| Ber-EP4 | EGF-like domain I | Latza et al., 1990 [67] |
| CO 17-1A | EGF-like domain I | Herlyn et al., 1979 [68] |
| C215 | EGF-like domain I | Bjork et al., 1993 [69] |
| ESA, EGP-2, EGP40 | Not established | Simon et al., 1990 [70] |
| FU-MK-1 | Not established | Watanabe et al., 1993 [22] |
| GA733-2 | EGF-like domain I | Szala et al., 1990 [71] |
| HEA125 | Not established | Momburg et al., 1987 [20] |
| K928 | Not established | Quak et al., 1992 [72] |
| K931 | EGF-like domain I | Copper MP, 1993 [73] |
| KSA, KS-1, KS1/4 | EGF-like domain I | Varki et al., 1984 [74] |
| MM104 | Cysteine-poor region | Schön et al., 1993 [75] |
| MH99 | EGF-like domain I | Mattes et al., 1983 [76] |
| MOC31 | EGF-like domain I | Myklebust et al., 1991 [77] |
| MT201 | Not established | Naundorf et al., 2002 [78] |
| VU-1D9 | EGF-like domain I | Tsubura et al., 1992 [53] |
| 2G8 | EGF-like domain II | unpublished data |
| 311-1K1 | Cysteine-poor region | Helfrich et al., 1994 [79] |
| 323/A3 | EGF-like domain I | Edwards et al., 1986 [80] |

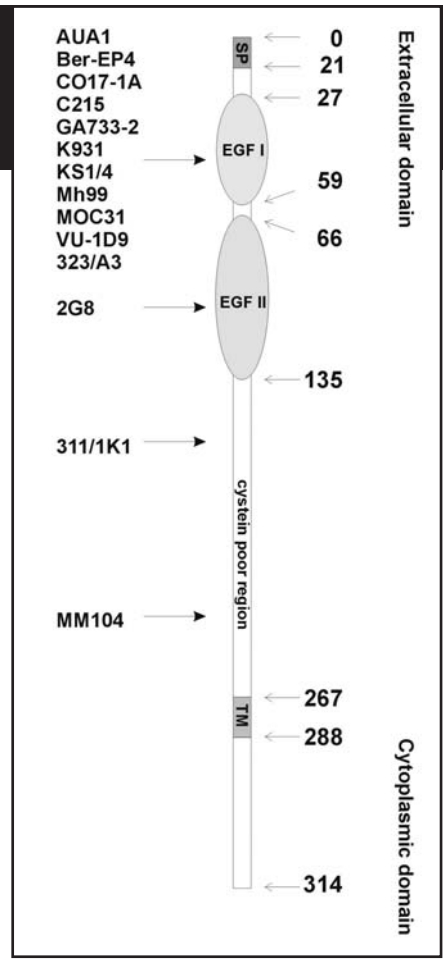



FIGURE 1 Schematic composition of Ep-CAM. SP, signal peptide; EGF, EGF-like domain; TM, transmembrane. The numbers indicate the amino acid residues that mark the regions in the molecule.

addition, the corresponding cDNA had been independently cloned by a number of groups [8-11]. Therefore, the molecule was first known by many different names, ie, the human pan-antigen epithelial glycoprotein EGP40, CO17-1A antigen, KSA1/4, ESA, GA733-2, MOC31, Ber-EP4, and so forth (table 1). In the early 1990s, the reports on the carcinoma antigens and the cloning of the cDNA were combined and it became clear that the described molecules were virtually identical. Initial studies on the characteristics of the molecule revealed that the molecule is a marker of epithelial lineages.

It is a type I transmembrane glycoprotein, not structurally related to one of four the major families of the adhesion molecules. The molecule consists of an extracellular domain containing two epidermal growth factor (EGF)- like repeats, and a short intracellular domain of 26 amino acids in which two binding sites for α-actinin are present for linkage to the actin cytoskeleton (Figure 1)[12]. It is a relatively small protein that is highly conserved during evolution and mediates calcium-independent homotypic cell-cell adhesions [13,14]. It is normally expressed at the basolateral membrane of cells by the majority of epithelial tissues, except in adult squamous epithelium and some specific epithelium cell types, such as hepatocytes [15-17]. Based on the properties of this molecule, it was renamed epithelial cell adhesion molecule, Ep-CAM [15,16]

Further studies revealed that in murine fibroblaststransfected with Ep-CAM, the expression of Ep-CAM is associated with proliferation [15,16]. On (over-) expression of Ep-CAM, cadherin adhesions dissociate, which leads to accumulation of detergent soluble E-cadherin/β-catenin complexes, and to a decrease in total cellular α-catenin [18]. This suggests that during cell division, the strong, tight E-cadherin-mediated cellular adhesion is abrogated, while the weaker intercellular adhesion mediated by Ep-CAM still holds the cell in place [18,19]. After the proliferative phase, Ep-CAM expression declines and higher levels of E-cadherin mediate intercellular adhesions and direct cellular differentiation. Based on a large study on the expression of Ep-CAM, the possibilities to target Ep-CAM for immunotherapy were explored [20]. Patients with Dukes' C colorectal carcinoma who had undergone curative surgery, were treated in a monotherapy in the adjuvant setting with edrecolomab, the murine IgG2a monoclonal antibody that recognizes and binds with low affinity to Ep-CAM. After 7 years of follow-up, the edrecolomab-treated group had a 32% reduction in mortality, and a 23% reduction in recurence, as compared to the observation arm [20]. More clinical trials are ongoing.

Ep-CAM expression is believed to be an early marker for (pre-) malignancies [21]. Immunohistological stainings of dysplastic colon cells showed overexpression of Ep-CAM. Not only the basolateral membrane was Ep-CAMpositive, apical positivity was also observed. For mature squamous epithelium, a *de novo* expression has bee described in weak, mild, and severe dysplasia [21]. Because it is important to diagnose (pre-) malignancies at early stages, Ep-CAM immunohistochemistry can be of use to diagnose aberrant tissue morphology.

## Ep-CAM in Various Malignant Tissues

As mentioned, throughout the last 3 decades many antibodies were raised against a widely detected tumor antigen that later was designated Ep-CAM. Several histological studies of expression patterns of Ep-CAM were conducted on different tissues, but because of the variety of names for antibodies and types of studies a comprehensive overview of the results is lacking. These are listed below and summarized in table 2. The findings for Ep-CAM expression patterns in adult tissue, premalignancy, and malignancy will be discussed.

### Head and Neck Region

In the squamous epithelium of the oral cavity expression of Ep-CAM is a reliable marker for the development of neoplasia. Weak, mild, and severe oral mucosal dysplasias displayed high expression levels of Ep-CAM in dysplastic basal and suprabasal cells, whereas normal epithelial cells are Ep-CAM-negative [22].

In glottic squamous epithelium, Ep-CAM [using monoclonal antibody (mAb) 323/A3]. was expressed in all dysplastic areas with the border of the expression corresponding to the border of the dysplasia. In all dysplasia a full layer expression of Ep-CAM was observed, indicating complete dysplasia of the epithelium (Sjogren EV, unpublished results).

In invasive tumors, a strong heterogeneity in Ep-CAM expression within and between tumors was observed. Besides proliferation, this heterogeneity was found to correspond to keratinization, with keratinizing areas being low or negative in Ep-CAM expression (Sjogren EV, unpublished results).

Nodal metastases and their corresponding primary tumors of head and neck squamous carcinoma were examined for gene expression [23]. The expression of most genes involved in tumorigenesis, for example E-cadherin, was similar in primary

tumors and metastases. Surprisingly, Ep-CAM expression was detected less frequently in metastases, compared to the corresponding primary tumor, suggesting involvement in metastasis. To identify high-risk patients having small numbers of disseminated tumor cells in early tumor stages, a reverse transcriptasepolymerase chain reaction assay for Ep-CAM expression that detects a single tumor cell within normal cells was successfully developed [24]

## Esophagus

The squamous epithelium of the esophagus is clearly negative for Ep-CAM, while the columnar epithelium in Barret's esophagus displays a diffuse and low expression pattern for Ep-CAM.25 In biopsies of Barret's esophagus a heterogeneous pattern of Ep-CAM staining is present. Within several patients, the expression of Ep-CAM (mAb KS-1) differed among various regions of the columnar esophageal epithelium of the intestinal type [26].

Preliminary data of Kumble and collegues [26] showed high expression levels of Ep-CAM (mAb KS-1) in four tested adenocarcinomas of the esophagus. The authorshypothesized that Ep-CAM is positively correlated with the progression to adenocarcinoma of the esophagus.

## Gastric

In normal gastric epithelium no Ep-CAM expression can be observed, only in the basal layer of crypts. However, with the development of intestinal metaplasia, a strong up-regulation of Ep-CAM expression is observed in all cases studied with 323/A3 (De Boer CJ, unpublished results). Ep-CAM expression appeared throughout thecrypts and is constitutive to the foveola. The authors found that expression of Ep-CAM can already be detected on some cells on the border of normal and metaplastic cells that have no metaplastic phenotype yet, and suggested that expression of Ep-CAM may be an early event in the development of gastric metaplasia that corresponded completely with increased proliferation as measured by an increase in Ki-67-positive cells. Using the FU-MK1 antibody, similar results were obtained [27].

Songun and colleagues [28]. studied whether Ep-CAM expression in primary tumor specimens from primary gastric adenocarcinoma was indicative for the presence of lymph node metastases, but it was not. However, loss of Ep-CAM expression is an independent prognostic value for poor survival prognosis. This can be explained by the fact that loss of Ep-CAM expression, as an epithelial adhesion molecule, may reflect a loss of epithelial differentiation. Furthermore, low expression levels of Ecadherin in carcinoma, increases the role for Ep-CAM adhesions in interconnecting cells. The loss of Ep-CAM expression probably results in loss of cell-cell adhesion, which promotes metastasis [29]

## Colon

Ep-CAM is widely expressed in the highly proliferative cells of the intestinal epithelium. Ep-CAM is expressed from cells in the basal cells throughout the crypts at





TABLE 2 Expression pattern of Ep-CAM in normal tissue, dysplasia and carcinoma (ca).

| EPITHELIUM (species) | AB | EXPRESSION PATTERN EP-CAM | | |
|---|---|---|---|---|
| | | normal tissue | premalignant | carcinoma |
| oral mucosa, head&neck region | KS1/4<br>323/A3 | negative<br>positive | dysplasia:<br>positive | positive<br>positive |
| esophagus | KS-1 | negative (columnar cells)<br>negative (squamous) | metaplasia:<br>positive (BE) | |
| gastric | 323/A3 | positive only in crypts<br>negative (mucosa) | | positive constitutive from crypts till villae |
| colon | KS1/4<br>323/A3 | positive | adenomas:<br>enhanced | enhanced<br>(colorectal ca) |
| liver | 323/A3<br>17-1A<br>MOC31<br>Ber-EP4 | negative<br>positive (regenerating/ proliferating hepatocytes) | positive | positive (CHC)<br>negative (HCC) |
| pancreas | KS1/4 | positive | | enhanced |
| kidney | Ber-EP4<br>FU-MK-1 | positive (*) | | heterogeneous (clear cell ca) |
| bladder | AUA1<br>FU-MK-1 | positive | | enhanced |
| testes | HEA125 | positive (*) | | positive (*) |
| prostate | 323/A3<br>FU-MK-1 | positive | | enhanced |
| mammary | 2G8<br>ESA<br>323/A3<br>17-1A | positive | | enhanced |
| ovary | AUA-1<br>Ber-EP4 | low positive (oocyte) | | enhanced (serous)<br>enhanced (mucinous) |
| uterine cervix | 323/A3 | negative | dysplasia:<br>positive | positive |
| lung | Ber-EP4 | low positive | | positive (SCC)<br>negative (mesothelioma)<br>lymph node metastasis from NSCLC: positive |
| skin | Ber-EP4<br>MH99 | negative (squamous)<br>positive (sweat ducts/ proliferating squamous cells) | | positive (BCC)<br>positive (basosquamous)<br>negative (SCC) |

(*) for details on cell types and tumor types: see text.

the basolateral membranes, and only the apical membrane facing the lumen is negative [30]. The development of adenomas is accompanied by an increased Ep-CAM expression and Ep-CAM overexpression (mAb GA733) has been frequently demonstrated in colorectal carcinomas [31,32].

In clinical trials, colorectal cancer has been targeted with the monoclonal antibody CO17-1A and anti-idiotypic antibodies mimicking the CO17-1A or GA733-2 epitope. An improved survival was accompanied by a prolonged systemic immune reaction to the antibody [33]. Presently, its anti-tumor effect is being studied as monotherapy after resection of stage II colon cancer, and in combination with chemotherapy in patients with stage II or III rectal cancer [34]. Patients with resected Dukes' C colorectal cancer were randomly allocated to infusions of CO17-1A antibody [20]. The follow-up study shows that 17-1A antibody administered after surgery prevents the development of distant metastasis in approximately one-third of patients. The therapeutic effect is maintained after 7 years of follow-up.20 Various mechanisms can be responsible for the clinical observed effects of Ep-CAM immunotherapy. According to Haller [34], the murine IgG2a mAb against Ep-CAM mediates an antibody-dependent cellular cytotoxicity, complement-mediated cytolysis, and anti-idiotypic network.

## Liver

Ep-CAM (mAb 323/A3) is expressed on hepatocytes in embryonic liver and maturing liver cells, but is absent in adult hepatocytes [35]. Ep-CAM does mark a pluripotent stem cell, the progenitor for both bile duct cells and hepatocytes. The de novo expression of Ep-CAM in regenerating/proliferating hepatocytes is explained by the fact that these stem cells replace the damaged cells, and decreased intercellular adhesion by E-cadherin is required for proliferation. On maturation of the new cells, ie, on differentiation, Ep-CAM expression is lost again. The down-regulation of Ep-CAM and thereby the signal for proliferation precedes the restoration of cadherin-mediated cellular adhesion.

Diseased liver tissue displayed a strong Ep-CAM expression (mAb 17-1A) in the epithelium of typical and atypical bile ducts [36]. In addition, periportal or periseptal hepatocytes revealed variable staining of Ep-CAM, which is directly related to acute and chronic inflammatory changes. The Ep-CAM expression in hepatocytes was most pronounced in acute and chronic active hepatitis, with Ep-CAM expression levels that are common to bile ductular cells. This suggests that the hepatocytes in diseased liver represent transformed hepatocytes.

It was demonstrated that all hepatocellular carcinomas (HCCs), including the pseudoalveolar type, were uniformly negative for Ep-CAM [37,38]. In the mixed HCC-cholangiocarcinoma cases, Ep-CAm (mAb MOC31) highlighted the glandular component, but did not stain the HCC portion of the neoplasm.

Furthermore, Ep-CAM differentiated between HCC and metastatic adenocarcinoma from the colon, lung, breast, pancreas, small intestine, kidney, or ovary [37,38]. However, according to Sansonno and Dammacco [36], neoplastic bile duct epithelium did not react for Ep-CAM (mAb17-1A) in cholangiocarcinoma, whereas neoplastic liver cells acquired cytoplasmic-positive staining in clustered areas in HCC. The intensity of staining and Ep-CAM distribution were inversely related to the grade of tumor differentiation.

## Pancreas

In the mature pancreas, the ductal compartment strongly stained for Ep-CAM exhibited the highest proliferation index [39]. The authors established a correlation between frequency of proliferating cells and increased expression of Ep-CAM (mAb KS1/4) in each cell compartment. The highest Ep-CAM expression was recorded at the cell-cell boundaries of intercalar ductal cells, in interlobular ducts, and in main ducts. Islets of Langerhans, identified by the insulin- and glucagon-specific antibodies, exhibit a significantly less intense Ep-CAM expression. The authors suggest that Ep-CAM expression negatively regulates the endocrine differentiation in pancreatic islet cells.

In cell lysates, increased expression levels of Ep-CAM were detected in human islet β-cell tumors (insulinoma) [39]. This increase is most likely also detectable with immunohistochemistry on tissue sections, but this has not yet been performed.

## Kidney

Few studies have described Ep-CAM in normal and neoplastic kidney. Normal renal tubules are in general strongly positive, while clear cell carcinomas show a more heterogeneous pattern. Five of twelve cases were positive for Ep-CAM (mAb Ber-EP4), whereas only one of five cases of renal carcinoma was weakly positive with the FU-MK1 antibody [40,41]. Concluding from the stained sections presented in the study, the use of the FU-MK1 antibody may not be the best suitable antibody to use for diagnostic purposes.

## Urothelium

Transitional epithelium of the bladder is only slightly positive for Ep-CAM (mAb AUA1/FU-MK1). In dysplastic lesions of urothelium and transitional cell carcinoma, enhanced expression of Ep-CAM was observed, although antigenic heterogeneity exists between tumors of the same grade and within the same tumor [42]. Using the FU-MK-1 antibody, only two of five bladder carcinomas were positive [41].

## Testes

In tissues of the male genital tract, some of the cells in testis (spermatogonia, low Ep-CAM expression), epididymis (ciliated, basal and cuboidal cells, intermediate expression), and seminal vesicle (positive expression) reveal Ep-CAM expression when using the HEA125 antibody [25].

Kommoss and colleagues [43]. concluded that among other antibodies, immunohistochemical staining for Ep-CAM in testicular neoplasms are helpful in the differential diagnosis when distinction on morphological grounds is difficult. Using

HEA125, he demonstrated Ep-CAM reactivity in cases of seminoma (3 of 12, 25%), embryonal carcinoma (3 of 12, 25%), yolk sac tumor (6 of 8, 75%), teratoma (1 of 2, 50%), whereas juvenile granulosa cell tumor, Sertoli cell tumor, primary and metastatic Leydig cell tumor, choriocarcinoma, and sex cord tumor all were negative [43]

## Prostate

Secretary, basal, and ductal cells of the prostate revea an intermediate Ep-CAM expression when using the HEA125 antibody [25].

Positive Ep-CAM staining (mAb FU-MK-1) was detected in normal prostate and in adenocarcinoma, although a small number of cases was studied.41 However, no clear staining pattern was observed with this antibody.

A low immunoreactivity was found for Ep-CAM (mAb323/A3) in benign prostatic epithelium, concentrated on the luminal cells [44]. Strong immunopositivity was detected in luminal cells of high-grade prostatic intraepithelial neoplasias, as well as in adenocarcinomas, suggesting that increasing levels of Ep-CAM expression represent early events in the development of prostatic adenocarcinoma. However, Ep-CAM positivity was not correlated to the clinical outcome of patients.

## Mammary Gland

The mammary gland epithelium undergoes several stages of development and dedifferentiation. In normal human mammary glands, Ep-CAM is mainly expressed in luminal epithelium [45]. Ep-CAM expression during the developmental phases was extensively studied in C57BL6 mice by Balzar and colleagues [46]. Using the mAb G8.8, it was clearly demonstrated that endogenous Ep-CAM expression very well correlated with the proliferative state of the mammary gland postnatal development, while the E-cadherin expression was unchanged during this period. With the start of milk production, the epithelium is differentiated and Ep-CAM expression decreases. Mice transfected with human Ep-CAM under the control of the MMTV-LTR promotor showed not only an association of Ep-CAM with regulation of mammary gland morphogenesis, but also direct involvement. The virgin mammary glands of transgenic mice displayed increased budding and secondary branching as compared to their nontransgenic littermates.

The staining pattern of mAb 323/A3 in benign breast disease was analyzed by Courtney and colleagues [47]. Patients who have had both a benign biopsy and a later biopsy for breast carcinoma were screened. In apocrine metaplasia, the cytoplasm of benign tissue did not stain with 323/A3, whereas in the biopsies with associated breast cancer did (five of seven). The authors noted a positive predictive value of 100% for strong cytoplasmic staining to indicate the presence of carcinoma.

An immunohistochemical study on breast cancer biopsies showed that Ep-CAM (mAb 17-1A) was expressed in the majority of the breast carcinomas, especially on paraffin sections [32] Spizzo and colleagues [48]. stated that the overexpression of Ep-CAM, detected with mAb ESA, in 205 cases of localized invasive breast cancer was an independent prognostic marker by multivariate analysis. Ep-CAM overexpression correlated significantly with disease- free and overall survival, independent of tumor size, nodal status, histological grade, and hormone receptor expression. Specific immunotherapy with mAbs against Ep-CAM in minimal residual stages of breast cancer should be considered [32]

## Ovary

In ovaries, the oocytes display a moderate Ep-CAMpositive staining (Ab HEA125), but the follicular epithelial cells are negative. In the oviduct, (non-) ciliated cells show a low reactivity [25].

Ovarian clear cell carcinomas showed Ep-CAM positivity with both AUA1 and Ber-Ep4 [40,49]. Cherchi and coworkers [50]. showed 50% and 79% Ep-CAM positivity (mAb Ber-EP4) in ovarian cancer of serous and mucinous type. Furthermore, Ep-CAM positivity (mAb Ber-EP4) was directly proportional to tumor differentiation; 70% of the well-differentiated tumors were Ep-CAM-positive, compared to 37.5% of the poorly differentiated tumors. No positivity was observed for Ep-CAM (mAb Ber-EP4) in either metastatic ovarian tumors or germ cell tumors.

## Uterine Cervix

Normal, mature squamous epithelium of the uterine cervix does not express any Ep-CAM (323/A3)[21]. Squamous differentiation marker cytokeratin 13-positive staining appears from parabasal cells, and the staining intensity increased toward the lumen in normal squamous epithelium. This is also observed for the squamous terminal differentiation marker involucrin.

Sections of the uterine cervix stained for Ep-CAM and Ki67 have demonstrated that in squamous dysplasia, both low and high grade, Ep-CAM is associated with proliferation [21]. In cervical intraepithelial neoplasia (CIN) grade I, Ep-CAM-positive areas were found in the parabasal layer, where now cytokeratin 13 was absent (figure 2). In progressing grades of CIN, grade II and III, larger layers of Ep-CAM expression were observed, while cytokeratin 13 almost disappeared. Similar staining patterns were found for the terminal differentiation marker involucrin. In progressing CIN lesions, involucrin staining is lost and Ep-CAM expression expanded. The highly proliferative activity in undifferentiated cells of CIN layers is associated with Ep-CAM expression. The Ep-CAM expression is inversely correlated with E-cadherin participating in cell-cell junctions [17]. Ep-CAM can be used as an early marker for disturbed tissue proliferation and differentiation in cervical premalignant stages. In the majority of both squamous and adenocarcinomas of the cervix a strong expression of Ep-CAM was observed, although some decrease in the expression (both the intensity and the number of positive cells), as compared with CIN III lesions, was observed in the areas of squamous differentiation [21]. Because it is unlikely that E-cadherin-mediated adhesion had returned and the tissue was differentiating, the population of cells that are less positive for Ep-CAM may be submitted to genetic imbalance where Ep-CAM transcription was lost.

### Lung

In normal lung tissue, the ciliated bronchial epithelium, alveolar duct, and alveolar epithelial cells type I and II all show a low Ep-CAM expression. In pulmonary fibrosis, Ep-CAM could further be detected on the cell surface of epithelial remnants [25,51].

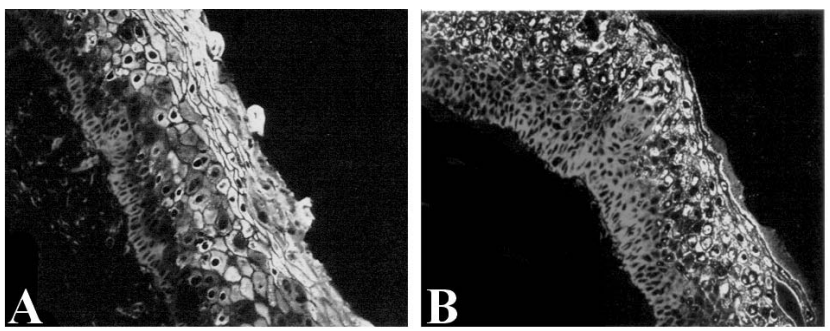
Two studies by Piyathilake and collegues [52,53], reported strong Ep-CAM positivity in 98% of the squamous cell cancers (SCC) and uninvolved bronchial mucosa and in 100% of the hyperplasias and dysplasias. There was increased Ep-CAM expression in luminal cells as compared to basal cells and was more consistent in hyperplasia than in uninvolved mucosa. The authors described a statistically significant stepwise increase in Ep-CAM expression from uninvolved bronchial mucosa to epithelial dysplasia to SCC. A significant association was detected with lower tumor differentiation, advancing nodal status, and advancing clinical stage. Well-differentiated SCCs expressed more Ep-CAM than poorly to moderately differentiated SCCs, and the increase in the Ep-CAM expression tends to correspond with increasing size or local extent of the primary tumor and involvement of regional lymph nodes. In contrast to squamous carcinomas, Ep-CAM is not expressed in mesothelioma. Using MOC31, a distinction between carcinoma and mesothelioma can be made on the basis of Ep-CAM expression [54].

The Ber-EP4 antibody was used to discover small tumor cell deposits in regional lymph nodes in patients with resected non-small cell lung cancer. In a prospective study of 125 patients, the detection of single Ep-CAM positive (mAb Ber-EP4) cells proved to be an independent prognostic factor for the overall survival [55]

### Skin

In the skin, the keratinocytes and melanocytes are Ep-CAM-negative, while the sweat ducts (eccrine and apocrine coils, apocrine ducts) and the proliferative zone of the hair follicle are Ep-CAM-positive.56,57 However, within the basal layers of the

FIGURE 2 Immunofluorescent double staining for Ep-CAM (red) and squamous differentiation marker cytokeratin 13 (green) in uterine cervix, stage CIN 1 (A), and stage 2 (B). The area where Ep-CAM is expressed is larger in CIN 2 as compared to CIN 1. For details see text and Litvinov and colleagues [21]








epidermis, some Ep-CAM reactivity can be observed in the reserve cells with mAb MH99 [58].

Cutaneous neoplasms reported to stain for Ep-CAM (mAb Ber-EP4) include basal cell carcinoma (BCC), Merkel cell carcinoma, and mixed tumor of skin (chondroid syringoma). In BCC, Ep-CAM (mAb Ber-EP4) is constantly and diffusely expressed, while SCC, squamous intraepithelial neoplasia, and actinic keratosis are Ep-CAM-negative [57,59,60]. This pattern was observed in nodular, cystic, superficial, and infiltrative BCC, but not in SCC, irrespective of the degree of differentiation. Using Ber-EP4, the identification of basosquamous carcinoma is also possible, because the studied tumors all showed at least some areas of Ep-CAM positivity [61]

## Discussion and Conclusions

This report has presented an overview of the expression patterns for Ep-CAM in several epithelial tissues, and the pathology thereof. Ep-CAM can be detected in all simple, columnar, and pseudostratified epithelia, but is absent in adult squamous epithelium. In vitro, a clear association was demonstrated between Ep-CAM and cell proliferation. Overexpression of Ep-CAM, as well as a *de novo* expression was observed in colon carcinoma and in squamous carcinoma of the uterine cervix [15,16]. Simultaneously, Ep-CAM expression abrogates the cadherinmediated adhesions, which has serious implications for differentiation of epithelial tissues, and may by itself be the reason behind increased cell proliferation. We believe that the enhanced expression and *de novo* expression is an early step in the malignant transformation of epithelium, and can be used as a marker for diagnostic purposes.

The discussed studies here all used antibodies that have their epitope in the EGF-like domain I. Other antibodies are known to have their epitope in the EGF-like domain II or in the cysteine-poor region (table 1) but were apparently not suited for immunohistochemistry because we retrieved no published reports in which reliable results on histological slides with these antibodies were described. To our knowledge, no antibodies have been developed that have their epitope in the intracellular domain of Ep-CAM.

The most frequently used antibodies are the monoclonal antibodies 323/A3, KS1/4, and Ber-EP4. They are known to have a high affinity and specificity for human Ep-CAM. Observed reported heterogeneity in reactivity of Ep-CAM-specific antibodies with subpopulations of Ep-CAM with cell or tissue suggests that intracellular and cell surface Ep-CAM differ in the conformational state of the protein, and that some epitopes may be masked on the molecules participating in intercellular adhesions [62]. One study described the favor of MOC31 to Ber-EP4: one case of HCC (Ep-CAM-negative) was detected with Ber-EP4, but not with MOC31 [37]. This was independently confirmed, and the authors concluded that the MOC31 staining was readily interpretable with rare exceptions [38].

For our research, the 323/A3 antibody is routinely usedon both frozen and paraffin-embedded tissue samples. After standard xylene and graded alcohol series, formalin-fixed and paraffin-embedded tissues are fixed in methanol and incubated in 0.3% H2O2 in methanol. For 323/A3 staining, tissues are pretreated with 0.1% trypsin (w/v) in 0.1% $CaCl_2$. Standard two-step biotin/streptavidin labeling is often used for detection. Fresh tissue samples can be stained with standard methods, without any pretreatments (see table 3). Immunofluorescent labeling for co-localization studies has been reported frequently by Balzar and colleagues [12,30], Litvinov and colleagues [18], Cirulli and colleagues [39], and Winter and colleagues [9]. The staining can be scored easily; for normal squamous tissues, staining is negative, whereas premalignant lesions display positive cellmembranes. For other epithelial cell types, aberrant cells show a more intense Ep-CAM positivity than normal tissue at the basolateral membranes. Furthermore, the cytoplasm and apical membranes can be positive as well in case of Ep-CAM overexpression.

To summarize the discussed studies, Ep-CAM expression can be detected at membranes of proliferating (epithelial) cells of colon, pancreas, mammary gland, lung, and regenerating liver, and is absent in normal liver, oral mucosa, gastric mucosa, skin, and uterine cervix. In those tissues with pre-existing Ep-CAM expression, Ep-CAM positivity is enhanced during neoplastic development. In tissues where Ep-CAM is absent in the normal situation, *de novo* expression of Ep-CAM indicates dysplasia or malignancy. The dysplastic squamous epithelia start to express Ep-CAM *de novo* at the basal layer. Tumors of epithelial origin virtually all express Ep-CAM at a high level, often the apical membrane is also Ep-CAMpositive. In some tumors, intracellular Ep-CAM-positive vesicles can be detected. Cervical dysplasia is correlated with Ep-CAM positivity; in low-grade dysplasia Ep-CAM is confined to the basal and parabasal cell layers, whereas in severe dysplasia the luminar cells are positive as well.

The association of Ep-CAM with metastases is less clear. One would expect to find higher Ep-CAM expression in metastasized cells, because these cells are more likely to escape the epithelium than well-differentiated cells anchored by E-cadherin-mediated junctions. Momburg and co-workers [25]. demonstrated that micrometastases originating from carcinomas could be detected with for instance the HEA125 antibody. However, in nodal metastasis originating from head and neck squamous carcinomas, Ep-CAM expression was found to be reduced compared to the primary tumor [23]. In contrast, Chaubal and co-workers [24]. concluded that Ep-CAM gene expression could be used as a useful tool to identify disseminated tumor cells. In both SCC and non-small cell cancer of the lung, Ep-CAM-positive cells were detected in the regional lymph nodes. In metastases from primary tumors in the ovary, Ep-CAM expression is decreased. Although loss of Ep-CAM expression is associated with the progression of intestinal metaplasia, it is not an indicative marker for the presence of lymph node metastases in patients with adenocarcinoma of the stomach.

Ep-CAM expression in immunohistological diagnostics may have additional value over the use of Ki-67 in suspected neoplasias. While using Ki-67, proliferative cells





can always be detected in the basal layers of squamous tissues, Ep-CAM positivity is only found in aberrant tissue. To discriminate between (hyper-) proliferative squamous tissue and premalignant squamous tissue, Ep-CAM is only expressed in the latter. In simple epithelia, Ep-CAM is always detectable on the basolateral sides of the cell. Premalignancies display overexpression of Ep-CAM and the apical membrane becomes Ep-CAM-positive as well, for instance in colon.

A useful application of Ep-CAM immunohistochemistry is to discriminate tumors of epithelial and nonepithelial origin. In human tissue, Ep-CAM is only expressed in epithelium or neoplasias from epithelial origin. Most squamous carcinomas are positive for Ep-CAM, except for squamous carcinoma of the skin. BCC can therefore be distinguished from SCC of the skin, squamous intraepithelial neoplasia, and actinic

TABLE 3 Staining for the three most frequently used antibodies directed against Ep-CAM. Tr: room temperature;

| ANTIBODY | STAINING METHOD | |
|---|---|---|
| | **Paraffin** | **Cryostat** |
| 323/A3 | *Fixation*: methanol (5 min; Tr) and 0.3% H2O2 (20 min; Tr)<br>*Pretreatment*: 0.1% trypsin (w/v) in 0.1% CaCl2 (w/v) (20 min; Tr)<br>*Incubation 1st Ab:* 1-5 μg/ml (overnight; Tr)<br>*Detection*: 2-step biotin/ streptavidin system | *Fixation*: acetone (10 minute; Tr)<br>*Pretreatment*: -<br>*Incubation 1st Ab*: 5 μg/ml in PBS/1.0% BSA (1 hour; Tr)<br>*Detection*: two-step biotin/ streptavidin system or envision |
| Ber-EP4 | *Fixation*: formalin<br>*Pretreatment*: 0.1% trypsin (w/v) in 0.1% CaCl2 (w/v) (20 min; Tr)<br>*Incubation 1st Ab:* 6 μg/ml (overnight; Tr)<br>*Detection*: 2-step biotin/ streptavidin system | *Fixation*: formalin<br>*Pretreatment*: -<br>*Incubation 1st Ab:* 5 μg/ml (45 min; 40 °C)<br>*Detection*: Vectorstain Elite kit |
| KS1/4 | *Fixation*: formalin<br>*Pretreatment*: 0.1% trypsin (w/v) in 0.1% CaCl2 (w/v) (20 minute; Tr)<br>*Incubation 1st Ab*: 5-10 μg/ml (overnight; Tr)<br>*Detection*: two-step biotin/ streptavidin system | *Fixation*: 4.0% formaldehyde (20 min; 4 °C)<br>*Permabilization*: 0.1% saponin (5 min; Tr), 50 mM glycine in PBS<br>*Pretreatment:* -<br>*Incubation 1st Ab:* 5 μg/ml (1h; Tr)<br>*Detection*: immunofluorescent labelled 2nd Ab |

keratosis [57,60]. The positive staining pattern in BCC is a useful tool to locate latent BCC in inflammatory Mohs margins [63]. In liver, surprisingly, not all liver neoplasias are positive for Ep-CAM. Almost all analyzed cholangiocellular carcinomas were Ep-CAM-positive, whereas the majority of HCCs were not [27,35,37,38]. One of the two Ep-CAM-positive HCC cases in our own study was diagnosed as fibrolamellar carcinoma, a rare variant of primary liver carcinoma [35]. In combined type tumors consisting of a mixture of HCC and cholangiocellular carcinoma, only the cholangiocellular carcinoma areas react Ep-CAM-positive [27]. Sheibani and co-workers [64]. used the Ber-EP4 mAb, which may have great use in the differential diagnosis of mesothelioma versus adenocarcinoma, particularly when only formalin-fixed tissue is available.

According to Friedman and co-workers [65], using the combination of mAbs Ber-EP4, carcinoembryonic antigen, and vimentin are useful immunohistochemical markers in differentiating malignant mesotheliomas from adenocarcinomas, whereas immunohistochemistry does not reliably distinguish malignant from benign hyperplastic mesothelial cells. The addition of DNA ploidy studies is useful for differentiating the latter two groups. Roberts and colleagues [66]. postulated that mesotheliomas, adenocarcinomas, and reactive pleura could only be accurately diagnosed with a panel of antibodies, in which the Ber-EP4 is only positive in adenocarcinomas. To distinguish peritoneal mesothelioma in women from serous papillary ovarian and peritoneal carcinoma, the use of Ber-EP4 is discriminative in contrast to other mesothelial markers thrombomodulin, cytokeratin 5/6, and CD44H and carcinoma markers polyclonal and monoclonal CEA, and Leu- M1 [67]. Also, the AUA1 antibody was demonstrated to distinguish between carcinoma cells and mesothelial cells in serous effusion [68]

Taken together the above-described findings, it is clear that expression of (epithelial) adhesion molecules may represent different stages in tissue development. Extending the definition of adhesion molecules to morphoregulating molecules is nowadays accepted. To diagnose disturbed or suspected lesions in epithelium, the expression pattern of epithelial adhesion molecule Ep- CAM can be of help. Normal, Ep-CAM-negative epithelia (squamous tissue) show a *de novo* expression in metaplasia, whereas an enhanced Ep-CAM expression can be found in other preneoplastic epithelia. The advantage of Ep-CAM staining over Ki-67 staining is described above. Also, Ep-CAM can serve in determining the tissueorigin of tumors. Therefore, we conclude that Ep-CAM immunohistology proves to be a useful tool in the diagnostics of epithelial lesions.

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Chapter 8

# Summary and future perspectives

Tissues are dynamic groups of cells with specific functions for each cell type. Healthy, mature tissues are in perfect balance concerning growth, differentiation, repair or adaptation. In order to respond to external and internal stimuli, cells are in constant communication with the environment and each other for orchestrated responses of various cell types. A disturbed communication between the cells of the same or different type can lead to a variety of diseases, for example diabetes mellitus or cancer. A number of signal transduction pathways of varying complexity enable cells to adequately respond to the (dynamic) conditions of the microenvironment. Cell adhesion Molecules (CAMs) and adhesion receptors are modalities that mediate direct contact and signaling between various cell types.

The General Introduction in chapter 1 describes the fading border between adhesion molecules and adhesion receptors. Adhesion molecules were formerly considered to act solely as mechanical connections between cells whereas only the adhesion receptors were assigned a role in signal transduction. Nowadays, the signaling and regulatory properties of adhesion molecules are widely acknowledged, and the term 'morphoregulating molecule' seems more appropiate. The characteristics of a relatively novel epithelial morphoregulating molecule, Ep-CAM, are presented, together with plausible signaling pathways that may affect the epithelial morphoregulating molecule E-cadherin, a key protein in epithelial cell biology. Since little is known of Ep-CAM and its morphoregulatory properties, this thesis focuses on the question if and how Ep-CAM mediated adhesions affect cell functions.

## Biology of Ep-CAM, (chapter 2)

The properties and putative function of Ep-CAM as well as its role in tumor biology are reviewed on the basis of the literature . Ep-CAM was formerly known as the 17-1A antigen, named after the monoclonal antibody 17-1A. Ep-CAM expression was detected in most carcinomas, and later studies have demonstrated the association between (epithelial) expression of Ep-CAM and cell proliferation. Ep-CAM is capable of mediating $Ca^{2+}$-independent homotypic cell adhesions. Co-expression with E-cadherin results in abrogation of cadherin-mediated junctions, blocking cellular differentiation. A role for Ep-CAM in normal tissue development and tumor development and progression is discussed.

## Introduction of active Ep-CAM molecules into cells interconnected by E-cadherin leads to abrogation of E-cadherin mediated adhesions (chapter 3)

In this chapter we described a negative effect of Ep-CAM expression in cells on E-cadherin-mediated cellular adhesions. E-cadherin transfected fibroblasts (L-cells), with

and without Ep-CAM supertransfection, were subjected to segregation assays, which resulted in E-cadherin transfected cells in the core of the aggregate, while E-cadherin/Ep-CAM transfected cells formed the outer layer, with decreased strength of the intercellular adhesions. Cell morphology had changed upon Ep-CAM introduction in fibroblasts transfected with both E-cadherin/Ep-CAM, as well as in Ep-CAM transfected epithelial cells. In these transfectants, biochemical analysis showed a decrease of insoluble β- and α-catenin. This demonstrates that the E-cadherin mediated adhesion complex is abrogated by introduction of Ep-CAM .

## Expression of Ep-CAM shifts the state of cadherin adhesions from strong to weak without involvement of β-catenin, (chapter 4)

We investigated which mechanisms may underly the regulation of E-cadherin mediated adhesions by EpCAM, The chimerized E-cadherin/α-catenin, Eα, or the chimerized E-cadherin/ c-terminal half of α-catenin, EαC, were transfected into L-cells and mediated cell-cell juncions. Introduction of Ep-CAM weakened the strength of cell-cell interactions, and decreased the cytoskeleton-bound fraction of the cadherin/catenin chimeras of both types of established junctional complexes. By seemingly disrupting the link between α-catenin and F-actin, Ep-CAM affects E-cadherin-mediated adhesions. Since the binding site for β-catenin was not available in the chimerized molecules, and no β-catenin was expressed, this showed that β–catenin, the acknowledged key regulator of cadherin mediated adhesions, was not involved.

## Downregulation of Ep-CAM leads to augmentation of cadherin mediated junctions in tumor cells, (chapter 5)

Overexpression of Ep-CAM introduction results in abrogation of the cadherin junctional complex, but is this effect reversible? Immortalized UMSCC-2 and -22B cells (U2 and U22B) are poorly differentiated squamous carcinoma cells that overexpress Ep-CAM to high (U2) or moderate levels (U22B), while E-cadherin expression is virtually gone. Stable and transient transfection of antisense DNA of Ep-CAM resulted in decreased cell proliferation and to morphological changes that are related to squamous differentiation. These observations are easily explained by the marked downregulation of Ep-CAM expression and the restauration of E-cadherin mediated adhesions, and the re-appearence of the squamous differentiation markers involucrin and cytokeratin-13. The restauration of cadherin mediated adhesions by suppressing the Ep-CAM expression, is of great importance in the quest for new modalities for curing cancer .

## Cadherins are regulated by Ep-CAM via activation of Pi3K, (chapter 6)

It was demonstrated that Ep-CAM can affect cadherin-mediated adhesions without direct physical interaction, so other molecules have to be involved. Ep-CAM transfected epithelial cells were capable of abrogating N-cadherin mediated cell-cell adhesion, which was inhibited by Pi3K inhibitor LY. Ep-CAM from cell lysates precipitated a protein complex, including the Pi3K subunit p85, and this complex showed Pi3K activity. Similar experiments with Ep-CAM deletion mutants suggest a regulation of Ep-CAM by Pi3K. Abrogation of the N-cadherin adhesion complexes in epithelial cells by cross signaling Ep-CAM involves Pi3K.





## Ep-CAM as a tool in surgical pathology (chapter 7)

After reviewing the literature on immunohistochemical studies of EpCAM expression in tumors, we conclude that Ep-CAM is a useful diagnostic marker for the identification of neoplastic epithelial changes and tumors of epithelial origin.

## Clinical implications

Aberrant expression of cell adhesion molecules is usually characteristic for [pre-]neoplastic cells. As discussed in chapter 7, immunohistochemistry can be a useful tool for the diagnosis of carcinomas, and the level of differentiation of the carcinomas. Recently, Xie and collegues concluded that Ep-CAM was distributed differently in various colon cancers compared to normal colon gland epithelium [Xie et al., 2005]. Staining patterns in tissue arrays showed that in adenocarcinoma and papillary adenocarcinoma the expression of Ep-CAM increased from grade I to grade III. These observations may have a diagnostic value in the correct recognition of carcinoma type.

Overexpression of Ep-CAM appears to correlate with impaired survival of patients with different types of carcinomas [Trzpis et al, 2007]. Exceptions to this rough-and-ready-rule are renal cell carcinoma and gastric carcinoma [Songun et al., 1997; Trzpis et al, 2007].

Over the past years, immunohistochemical studies of large series of cases have shown an increased or de novo expression of Ep-CAM in premalignant lesions. These data form a compelling argument/reason for developing immunotherapy or other ways of treatment targeting Ep-CAM.Theoretically, anti-Ep-CAM antibodies may be the easiest way to therapeutic implementation of this knowledge. Ep-CAM is mainly expressed at the baso-lateral cell membrane and the majority is complexed in cell-cell adhesions. Only aberrant cells express Ep-CAM at the apical cell site due to the overexpression, so for example local administration of anti Ep-CAM antibodies in the lumen of the colon in case of a colorectal carcinoma might be able to reduce tumor size and growth. A systemic approach was tested for edrecolomab/ Panorex®. Although earlier studies had shown promising results, the adjuvant treatment of patients with resected stage III colon cancer or postoperative adjuvant treatment with edrecolomab in patients with resected stage II colon cancer did not improve overall or disease-free survival [Punt et al., 2002; Hartung et al, 2005]. Short half-life and rapid neutralisation may have contributed to the low clinical benefit. This may be overcome by modifying the murine antibody edrecolomab to a subcutaneously administered vaccine, IGN101. This molecule IGN101 was only succesful in prolonging disease free survival of patients with stage IV rectal cancer, but not in other carcinomas [poster at ASCO, 2005].

Other antibodies, including human anti-Ep-CAM antibody adecatumumab (MT201) [Neundorf etal. 2002], and tri-specific Ep-CAM/CD3/APC hybrid antibody catumaxomab, have entered the phase I and phase II clinical trials [Kiman and Whelan, 2007]. and Ep-CAM/CD3 bispecific antibody MT110 [Brischwein et al., 2006]. showed promising results in immunodeficient mice and human cancer cell lines.

An immune response against tumour cells can aslo be elicited by Ep-CAM specific immunotoxins. Local presentation of the immunotoxin might elicit an immune response that may eradicate the tumour cells. For head and neck carcinoman, Ep-CAM specific immunotoxin Proxinium® is being tested in phase II/III clinical trials and other immunotoxins are under development [Di Paolo et al., 2003; Baeuerle and Gires, 2007]. Focuss in this process is on the new routes of local delivery of the toxin [Baeuerle and Gires, 2007].

Alternatively, (local) downregulation of Ep-CAM expression and signalling may halt tumor growth. Osta and collegues tested the hypothesis if silencing EpCAM gene expression with EpCAM short interfering RNA (siRNA) could have any clinically relevant effect [Osta et al., 2004]. They found that Ep-CAM siRNA decreased the rate of cell proliferation in four different breast cancer cell lines, as well as decreased cell migration and cell invasion in vitro. Consistent with results discussed in this thesis, EpCAM silencing was also associated with an increase in the detergent-insoluble protein fraction of E-cadherin, α-catenin, and α-catenin. The clinical approach, i.e. local administration, and dose etc., needs to be developed and explored further before the first patient can be enrolled in a phase I clinical trial.

## Future perspectives

In this thesis, we demonstrate that expression of Ep-CAM regulates cadherin-mediated complexes via a Pi3K -linked signaling pathway. However, many open questions remain.

**1.** Ep-CAM is most likely one of the earliest steps in the transformation from normal squamous epithelium to a dysplastic lesion. Only 10% of the cells in the basal layer of squamous tissue are tissue stem cells that supply the tissue with 'new' cells. The stem cells are located in clusters, and differentiation starts as they migrate sidewards along the basal membrane [Watt, 1998]. It will be interesting to study the expression pattern of Ep-CAM in the normal stem cells, transformed/abnormal/neoplastic stem cells and the internal or external signals to which these cells react.

**2.** The role of Ep-CAM in embryogenesis is unknown. Ep-CAM is associated with proliferation, but is it mere a participant in the proliferative signal or does it more? The fact that Ep-CAM regulates insulin and glucagon gene transcription and translation in fetal pancreatic cell clusters [Cirulli et al., 1998]. suggests a morphoregulatory function for Ep-CAM. What signal(s) balance(s) the proliferation and differentiation status in developmental epithelial tissue?

**3.** The signaling through Ep-CAM, soluble or committed in cell-cell adhesion, is facillitated by proteases; cleavage of Ep-CAM results in the release of intracellular peptide EpIC. The role of EpIC needs further investigation, in both normal cells as well as in preneoplastic and malignant cells.

**4.** During mitosis in normal epithelium, Ep-CAM mediated cell-cell adhesions may provide the integrity while the E-cadherin mediated adhesions are abrogated. With the destruction of the cadherin/catenin complex, β-catenin is set free from this complex and pooled/ stored in other compartments. The β-catenin bound in the Adenomatous Poliposis Coli (APC) /axin complex is phosphorylated by kinase GSK3β and phosphorylated β-catenin is subjected to degradation. However, Wnt signaling antagonizes the GSK3β, resulting in the accumulation of cytoplasmic β-catenin, inducing gene transcription via Tcf4/LEF1. Interestingly, Pi3K was demonstrated to block GSK3β. Is there a correlation between Ep-CAM expression, Pi3K activity and induction of Tcf4/LEF1?

**5.** The role of Ep-CAM in cell motility needs further attention. Our preliminary data from wound-healing experiments suggest that Ep-CAM expression decreases cell migration in experimentally wounded monolayers only where cadherin mediated adhesion complexes are intact. Ep-CAM increased the motility of fibroblasts when deletion mutants of E-cadherin were expressed, or no E-cadherin was expressed. The role of Ep-CAM in regulation of motility may involve accumulation of cytoplasmic Pi3K and β-catenin. Ep-CAM signaling involves Pi3K and abrogates E-cadherin mediated adhesions, leading to 'free' β-catenin. Pi3K was demonstrated to block the GSK3β leaving β-catenin available for motility/ intergin signaling.

**6.** Ep-CAM is not the initial step in the transformation to a malignant phenotype. Therefore it will be interesting to find out at which stage of (malignant) transformation the Ep-Cam/Pi3K complex is formed. Both Ep-CAM and Pi3K are capable of binding α-actinin [Balzar et al, 1998]. and the role of α-actinin is still unclear.

**7.** Furthermore, amplification of PIK3CA, the gene coding for Pi3K, may provide an answer to the question what the signal is for the de novo or over-expression of Ep-CAM. PIK3CA acts as an oncogene in cervical- and ovarian carcinomas [Ma et al., 2000; Shayesteh et al, 1999]. In these tumours, elevated Pi3K activity may induce de novo expression of Ep-CAM. Kobayashi concluded that PIK3CA is not an oncogene, but merely a gene that drives progression towards a more malignant type. The authors describe two poorly differentiated tumor cell lines, KATO-III and MKN45, that express Ep-CAM to very high levels [Kobayashi et al, 1999]. Wennström and Downward demonstrated that the activation of Ras and ERK by physiological relevant concentrations of EGF can be reduced by LY or Wortmannin. A basal activity of Pi3K is present in (epithelial) cells, and may be provided by signal transduction by Ep-CAM.

**8.** GTPases are key players in epithelial cytoskeletal rearrangements. It was demonstrated that Ep-CAM affects the E-cadherin complex in its juxtamembrane domain, where it can bind pp120. Pp120 regulates and is regulated by Rho activity. Changes in Ep-CAM and E-cadherin affect the balance of Rho, Rac and Cdc42 activation. The GTPase activity is also extensively studied by other groups in relation to the regulatory signaling of integrins. The integrins, involved in migration, are of impor-

tance in the transition of a carcinoma in situ to an invasive carcinoma. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through Pi3K [Keely et al, 1997]. It will be of interest to study the effect of Ep-CAM on integrins in vitro, as well as in tissue samples.

**9.** The demonstration of Ep-CAM complexing and interacting with both a tumor metastasis-promoting cell adhesion molecule CD44v4-v7 and a tight junction protein claudin-7 is intriguing. Although CD44v4-v7 is found to promote metastasis, the other partner in the complex, claudin-7, is underexpressed during invasion and metastatis of breast and esophageal carcinoma [Usami et al., 2006]. Therefore, studying the role of Ep-CAM in a complex may explain the contradicting observations.

It is fair to conclude that Ep-CAM is a major player in the development and maintenance of epithelial tissues that deserves further attention in future cell biology research.

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Chapter 9

# Samenvatting

In de algemene inleiding, *hoofdstuk 1*, is een aantal karakeristieken beschreven van signaal transductie tussesn adhesie moleculen. Bijzondere aandacht is besteed aan de biologische kenmerken van E-cadherine en Ep-CAM. Bij aanvang van de studies zoals beschreven in dit proefschrift, was er voldoende over Ep-CAM bekend om te suggereren dat Ep-CAM dusdanig kon interfereren in de E-cadherine gemedieerde cell-cell contacten, dat er wellicht een verandering in het epitheel kon optreden met een kans op kwaadaardige celgroei. Vanwege deze veronderstelling is het onderzoek voortgezet met de experimenten zoals beschreven in dit proefschrift. De experimenten waren gericht op de morforegulerende eigenschappen van Ep-CAM en de betekenis ervan in epitheliale weefsels.

In *hoofdstuk 2* is de kennis over Ep-CAM samengevat. Het is een relatief klein adhesie molecuul, dat niet behoort tot de vier bekende families adhesiemoleculen en dat $Ca^{2+}$-onafhankelijke cell-cell juncties medieert. Het komt tot expressie in alle eenlagige, kubische epithelia, maar niet in plaveisel epitheel. Ep-CAM komt tot overexpressie of de novo expressie in tumoren van epitheliale oorsprong.

## Ep-CAM moduleert de E-cadherine gemedieerde adhesies, hoofdstuk 3

Ep-CAM komt tot expressie in de meeste epitheliale weefsels, in goedaardige en kwaadaardige proliferatieve laesies of tijdens embryogenese. De rol van het adhesiemolecuul Ep-CAM in deze weefsels was echter nog onduidelijk. Introductie van Ep-CAM in murine fibroblasten die zijn getransfecteerd met E-cadherine, of introductie in humane epitheliale cellen induceerde seggregatie van Ep-CAM transfectanten en cellen die Ep-CAM niet tot expressie brachten. Daarnaast veranderde Ep-CAM introductie de celmorfologie, reduceerde de sterkte van aggregatie, en detergent -niet oplosbare fractie van cadherines en cateninen. Al deze veranderingen werden traden niet op wanneer een deletie mutant van het Ep-CAM molecuul in de cellen werd geïntroduceerd. Deze mutant, waarvan het cytoplasmatisch domein van Ep-CAM is gedeleteerd, werd weliswaar correct op de celmembraan van murine fibroblasten en humane epitheliale cellen tot expressie gebracht, maar deze was niet in staat homotypische cel-cel adhesies te vormen. Met andere woorden, het cytoplasmatisch domein van Ep-CAM is in staat de cadherine gemedieerde adhesies negatief te beïnvloeden.

## Ep-CAM expressie verschuift de cadherine adhesies van sterk naar zwak zonder dat β-catenine hierbij is betrokken, hoofdstuk 4

Als Ep-CAM (over-)expressie de E-cadherine gemediëerde adhesies ontmantelt, is het van belang te weten hoe dit gebeurt. Om te onderzoeken welke factor in het E-cadherine/catenine complex door Ep-CAM aangezet wordt tot afbraak, is gebruik gemaakt van twee fusie eiwitten: E-cadherine/α-catenine, Eα, en E-cadherine/ c-terminale helft van α-catenin, EαC. Deze fusie eiwitten zijn ieder in L-cel fibroblasten getransfecteerd en konden cel-cel contacten mediëren. Na introductie van Ep-CAM was de sterkte van de cel-cel contacten afgenomen in zowel de Eα als de EαC getransfecteerde cellen. Tevens was de cytoskelet gebonden fractie van cadherine/catenine chimeren toegenomen. Deze resulaten zijn opvallend, aangezien β-catenin niet tot expressie kwam en dus geen regulerende rol kon vervullen. Het aangrijpingspunt in het cadherine/catenine complex om de binding met het cytoskelet te verzwakken is dus gelegen in het juxta-membraan deel van E-cadherine of in de COOH-helft van α-catenine.

## Cross signaling tussen E-cadherine en Ep-CAM is reversibel, hoofdstuk 5

Uitgaande van het feit dat introductie van Ep-CAM in cellen kan leiden tot het afbreken van cadherine gemediëerde adhesies, zijn experimenten uitgevoerd om het omgekeerde te bestuderen. UMSCC-2 en -22B cellen (U2 en U22B) zijn twee plaveiselcellijnen die veel Ep-CAM (U2) of redelijk veel Ep-CAM tot expressie brengen. De E-cadherine expressie is laag (U2) en redelijk laag (U22B). Wanneer de cellijnen stabiel of voorbijgaand worden getransfecteerd met antisense Ep-CAM DNA, vermindert deze Ep-CAM expressie, is de celproliferatie verlaagd en veranderen de cellen voor wat betreft de morfologie die nu squameuze differentiatie toont. Deze observaties kunnen eenvoudig verklaard worden door de enorme afname van Ep-CAM expressie als gevolg van de antisense DNA en de vorming van E-cadherine gemediëerde adhesies.

## Cadherinen worden gereguleerd door Ep-CAM via de activatie van Pi3K, hoofdstuk 6

Het is aan te nemen dat Ep-CAM geen fysieke, directe interactie met E-cadherine aangaat. Derhalve is het aannemelijk dat andere moleculen betrokken zijn bij de signaaloverdracht tussen Ep-CAM en cadherines. Ep-CAM getransfecteerde epitheliale cellen toonden een verstoorde N-cadherine gemedieerde cel-cel adhesie, en dit effect van Ep-CAM op N-cadherine kon worden voorkomen door toevoeging van Pi3K inhibitor LY. In Ep-CAM precipitaten uit celextracten kon Pi3K subunit p85 worden aangetoond in het eiwit complex. Het geprecipiteerde eiwit complex was in staat tot kinase activiteit.

## De betekenis van Ep-CAM expressie in epitheliale tumoren (review), hoofdstuk 7

De afgelopen twintig jaar zijn door diverse onderzoekers diverse antilichamen tegen Ep-CAM gegenereerd en is met deze antilichamen de aankleuring van verschillende epitheliale weefsels onderzocht. In dit hoofdstuk zijn deze bevindingen samengevat. Op basis van de beschreven onderzoeken blijkt dat Ep-CAM goed als hulpmiddel kan dienen bij het vaststellen van de juiste diagnose van het afwijkend gedrag van epitheliale weefsels.