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EpCAM expression dynamics in cancer progression

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1. INTRODUCTION

In 2012, approximately 8 million cancer-related deaths were recorded with males being more affected (5 millions) than females (3 millions) (Ferlay, Soerjomataram et al. 2015). The world age-standardized statistics reported over 100 deaths per 100,000 men and around 80 in 100,000 women according to United Kingdom cancer research. Cancer causes are numerous and may vary in different areas of the world. They include unhealthy lifestyle, obesity, physical inactivity, infection with microorganisms such as *Helicobacter pylori*, tobacco and alcohol consumption, genetic factors or exposure to extreme environmental conditions, *e.g.* radiation or poisonous chemicals. There are around 200 different types of cancer based on reports of the National Institute of Health (http://www.cancer.gov/types/by-body-location), mostly named after organ location or the types of affected cells. Hence, the term cancer comprises a collection of diseases, all driven by aberrant regulations of cellular pathways. Throughout disease progression, normal somatic cells transform into malignant cells upon mutagenesis to generate a primary tumor. Subsequently, distinct tumor cells locally invade and spread into surrounding tissues, gain access to blood and lymph circulation and metastasize to distant organs. Eventually, overt metastases impair the function of major organs such as liver, lungs, and brain, and lead to multi-organ failures, which are the major reason of cancer deaths (Chaffer and Weinberg 2011).

Cells are normally communicating with their neighbors in order to complete an orchestra of physiological tasks. A plethora of proteins like hormones and growth factors participate in this orchestration of cell homeostasis, including proliferation, differentiation, metabolism, apoptosis, and motility, amongst others. Activating or inactivating mutations of central proteins involved in the regulation of signaling pathways thus have disastrous consequences

and support tumor development. Hence, either overshooting or lack of signal affects proper homeostasis and can promote cancer. Alterations of genomic sequence, namely mutation, uncontrolled transcriptional programs, and non-physiological protein expression profiles are responsible for these changes.

Two major types of mutations can be discriminated: Loss- and gain-of-function mutations. As the names state, mutation in genomic DNA can either result in the destruction or an up-regulation of gene function. In most cases, mutations of genes cause loss-of function (Griffiths., Miller. et al. 2000). However, some mutations generate new functions and/or expression patterns of central genes. This type of mutation is named gain-of function mutation (Griffiths., Miller. et al. 2000). In the human body, the errors during the process of DNA replication is in the range of 10^{-7} to 10^{-8} per nucleotide (Schaaper 1993). Often, these mutations can be corrected through sophisticated repair mechanisms. For instance, DNA polymerase can recognize and replace 99% of mismatches during DNA duplication, a function that is termed proof-reading (Brutlag and Kornberg 1972). Additionally, the mismatches generated can be also repaired after DNA replication through a process called mismatch repair (Pierce 2005). When the unrepaired genomic mutation occurs on a set of genes that are termed proto-oncogenes, it may lead to cancerous transformation of normal cells. Activating mutations of proto-oncogenes, thus, generate oncogenes (Weinstein and Joe 2006). Oncogenes encode protein products, including growth factors, growth factor receptors, transcription factors, signal transducers that are involved in proliferation or/and apoptosis (Croce 2008). In early studies of breast cancer, mutation of the BRCA1 gene was shown to be highly associated with breast cancer incidence (Easton, Ford et al. 1995). Later on, mutation of the BRCA2 gene was reported to be involved in breast carcinogenesis (Wooster, Neuhausen et al. 1994). Another gene first reported as an oncogene in an animal model of simian virus 40 (SV40)-induced tumors is p53. Antibodies against p53 were found in human

breast cancer patients (Mudenda, Green et al. 1994). Although a function of p53 in carcinogenesis was reported (Tan, Wallis et al. 1986), the tumor suppressive role of p53 emerged as the essential function of p53 in hampering tumor formation. p53 mediates cell apoptosis in both, a transcription-dependent and -independent manner (Chi 2014). Another gene termed *RB1* is involved in the formation of retinoblastoma and was first described by Knudson (Knudson 1971). The retinoblastoma protein (Rb) belongs to the group of genes, which repress cell proliferation and induce apoptosis, termed tumor suppression genes, is found dysfunctional in many cancers. Inactivation of both alleles of *RB1* causes the formation of retinoblastoma during development of the retina (Knudson 1971). Later on, the role of pRb as tumor-suppressor was confirmed by Dunn and colleagues in other human cancers showing mutations and deletions of Rb gene (Dunn, Phillips et al. 1989). Beyond the selected examples described above, there are actually hundreds of genes shown to associate with cancer formation. In addition, infectious organisms have been recognized as a cause of cancer for long time. Viruses such as human papillomaviruses (HPV), Hepatis B, Hepatitis C, Epstein-Barr, and others could induce 20% of the world's cancer by affecting cell mitosis, mutating DNA, and inhibiting apoptosis (Aaron J. Smith, John Oertle et al. 2014). It has been well documented that HPV can promote malignancies including cervix and head and neck cancer (Adams, Wise-Draper et al. 2014). It has been studied in depth that the two HPV-encoded oncogenes E6 and E7 can alter cell signaling mechanisms that play important roles in the control of cell proliferation (Chen 2015). E6 and p53 together with an ubiquitination enzyme E6-AP can form a trimeric complex to cause the degradation of tumor suppressor p53, hence, promoting the proliferation of tumor cells (Crook, Tidy et al. 1991). The E7 oncoprotein is encoded by high-risk HPV subclones, such as HPV 16 and HPV 18. In HPV caused cancers, the E7 oncoprotein binds to the ‘pocket domain’ of pRb, which is a central domain of its tumor suppression function (Jones and Munger 1996). Moreover, the tumor

suppressor p53 can suppress the expression of Nanog, which leads to the differentiation and apotosis of normal cells. E6 protein of HPV can induce the degradation of p53 and, thus, leads to overexpression of Nanog and inhibition of differentiation and apotosis of cells (Lin, Chao et al. 2005). This differentiation inhibition and anti-apototic mechanism implies that HPV contributes to carcinogenesis of infected cells.

1.1 Oncogenes and tumor suppressor genes

One hallmark of cancer is the genetic instability of cells. Abnormal alterations of DNA through mutation on proto-oncogenes and tumor suppressor genes are crucial in cell growth and programmed cell death (Weinberg 1994). These two functionally defined groups of genes regulate fundamental activities of somatic cells including proliferation, apoptosis, and differentiation, amongst others. When balanced expression of oncogenes and tumor suppressor genes is disrupted by extracellular or intracellular detrimental impacts, normal cells undergo tumorigenic changes and become malignant (Bishop 1991).

1.1.1 Proto-oncogenes and oncogenes

The term proto-oncogenes refer to a family of genes that, when they become activated, can transform normal cells to cancerous progeny (Adamson 1987, Weinstein 2006). Mutated, activated proto-oncogenes are then termed oncogenes. Normally, proto-oncogenes are involved in central, physiological cellular activities including embryogenesis, wound healing, cell division. Proto-oncogenes are highly conserved and can be detected in diverse species, such as yeast, human and *Drosophila* (Anderson, Reynolds et al. 1992*)*. The protein products they encode comprise growth factors, transcription factors, regulatory proteins in signal transduction, amongst others (Weinberg 1989, Cantley, Auger et al. 1991, Hunter 1991).

These proteins can play a role in the control of cell proliferation and programmed cell death. For instance, RAS proteins represent a group of small GTPases that control cellular activities including growth, adhesion, migration, survival and proliferation, cytoskeletal integrity (Rajalingam, Schreck et al. 2007). The mutation of *ras* proto-oncogene family members was reported in numerous tumor types. Activated *ras* proto-oncogenes have been found in 47% of colon carcinoma, 81% of pancreatic carcinoma, 32% of lung adenocarcinoma, 88% of cholangiocarcinoma, 47% of endometrical adenocarcinomas, 75% of mucinous adenocarcinomas of the ovary, 47% squamous-cell carcinomas (SCC) of sun-exposed skins, 35% of oral carcinoma (Marshall W. Anderson 1992). Actually, *ras* gene mutations with gain-of-functions were the first reported 30 years ago (Der, Krontiris et al. 1982). H-ras, N-ras, and K-ras oncogenes are the 3 members of Ras superfamily (Rajalingam, Schreck et al. 2007). Mutations of either one of the Ras genes are frequent cellular events in cancer (Fernandez-Medarde and Santos 2011). One of the first ever described somatic mutation associated with cancer related to the *HRAS* gene, which was reported to associate with bladder cancer by Reddy *et al. HRAS* encodes a protein that is crucial in cell mitotic signaling (Reddy, Reynolds et al. 1982). Since then, more and more genes that are involved in tumor formation, namely, tumor-associated genes, have been described from the RAS gene family. RAS proteins transmit signals from the cell surface and couple with different intracellular cell signaling networks, ultimately delivering signals to the nucleus, leading to switches of different cellular processes such as DNA synthesis, mitosis and intracellular signaling pathway (Goodsell 1999). Permanent activation of signaling pathways of the K-ras is strongly associated with more than 90% of pancreatic cancer and around half of colon carcinoma (Goodsell 1999). Two hot spots of mutations have been reported for *ras* genes that localized to codons 12 and 61. For example, pancreatic adenocarcinomas harbour K-ras mutations with a frequency as high as 95% according to literature (Almoguera, Shibata et al. 1988). The

mutations located on K-ras codon 12, mutating glycine to either aspartic acid, arginine, or valine, result in constitutively activated Ras protein variants (Forbes, Bindal et al. 2011). H-ras is frequently mutated in bladder carcinoma and is especially found in low-gerade tumors (Castillo-Martin, Domingo-Domenech et al. 2010, Goebell and Knowles 2010). N-ras activating mutations occur in 20-30% of melanomas (Omholt, Karsberg et al. 2002, Hocker and Tsao 2007). One of the downstream effector of Ras is the BRAF oncogene, which was described in melanomagenesis together with N-ras, indicating that Ras and its downstream altered signaling pathway are an important trigger of melanoma formation (Brose, Volpe et al. 2002, Davies, Bignell et al. 2002, Singer, Oldt et al. 2003).

There is increasing evidence showing that oncogenes are generated from their precursors, proto-oncogenes, through three major pathways: (a) Point mutations of proto-oncogenes can lead to permanently active oncogenes, (b) chromosomal rearrangement, deletions, insertions and translocation of genes can cause abnormal expression of proto-oncogenes, (c) Extra chromosomal copies of a proto-oncogene is generated during gene replication events by mistake (Krishna, Singh et al. 2015).

1.1.2 Tumor suppressor genes

Tumor suppressor genes are genes that protect normal cells from transforming into cancer cells. Loss-of-function of these genes or/and gain-of-function of oncogenes due to mutation leads to cancer. The possible mechanism is the repression of tumor suppressor genes inducing tumor growth by inhibiting proteins that normally act to control cell proliferation (Hinds and Weinberg 1994). Among many identified tumor suppressor genes, p53 is the best and most intensely investigated.

The *TP53/P53* (tumor protein) was described firstly as an oncogene in the year 1979, however, later a role as a tumor suppressor was determined too (Lane 1992). Understanding the role(s) and function(s) of p53 was a difficult task. The first evidence was reported by David Wolf and Varda Rotter in the year of 1984, demonstrating that p53 was suppressed in Abelson murine leukemia-transformed mouse cells (Wolf and Rotter 1984). Moreover, results originating from murine leukemias caused by the Friend erythroleukemia virus pointed to a similar direction. These assays lead to the same conclusion that sustained p53 expression in cells is crucial to prevent cancer. However, in that time the major knowledge of p53 was restrained to its cancer promotion function, and thus it was hard to imagine p53 being endorsed with an opposite role in cancer.

In fact, wild type p53 is a tumor suppressor gene, which is crucial for cells when they encounter stress. Abnormal p53 expression is a very common event in human cancer, which can cause deregulation of cell division, instability of genome, resistance to stress, and eventually formation of cancer (Vogelstein, Lane et al. 2000, Petitjean, Mathe et al. 2007). The activation of p53 can be triggered by many types of stress, such as telomere attrition, hypoxia, oncogene activation, DNA damage, loss of normal survival and growth signals (Ryan, Phillips et al. 2001). Cells with these abnormalities are highly prone to malignant transformation. Loss of function of p53 fosters such malignant alterations and, therefore, p53 is recognized as a guardian gene preventing development of cancer. The activation of p53 can lead to the differentiation of cell, senescence and DNA repair, and anti-angiogenesis. However, the best-studied function of p53 relates to the induction of cell cycle arrest and apoptosis (Bates and Vousden 1999). These functions of p53 can either prohibit the formation of oncogenic cells or/and lead to programmed death of abnormal cells. It was reported that p53 could regulate the expression of the cyclin-dependent kinase inhibitor p21 directly through two specific binding sites on the promoter of its gene (el-Deiry, Tokino et al. 1993,

el-Deiry, Tokino et al. 1995). Once p21 becomes activated, it binds to the cyclin-CDK complex and prohibits the kinase activity of this complex (el-Deiry 1998). Since CDK kinase is a key factor of cell cycle transition, p21 is a potent cell cycle suppressor. p21 could also inhibit the function of proliferating cell nuclear antigen PCNA, thereafter arresting DNA replication (el-Deiry 1998).

Apoptosis refers to programmed cell death via intrinsic and extrinsic cues (Fridman and Lowe 2003). p53 regulates the apoptotic program through both, intrinsic and extrinsic pathways (Fridman and Lowe 2003). The intrinsic pathway becomes initiated by DNA damage, hypoxia, loss of adhesion, inhibition of growth factors, cytoskeleton damage, endoplasmic reticulum stress, macromolecular synthesis inhibition and others (Chipuk, Bouchier-Hayes et al. 2006). The intrinsic pathway is controlled by Bcl-2 family members via mitochondrial outer-membrane permeabilization (MOMP), which governs the release of cytochrome *c* from mitochondrial intermembrane space into the cytosol (Haupt, Berger et al. 2003). The most direct link between p53 and the Bcl-2 family in the intrinsic apoptotic pathway relies in its role in the transcriptional regulation of Bcl-2 family members (Fridman and Lowe 2003). These include Bcl-2 antagonist/killer (*Bak*), Bcl-2-associated X protein (*Bax*) (Miyashita, Krajewski et al. 1994), 'BH3-only' members *Puma* (Nakano and Vousden 2001), *Noxa* (Oda, Ohki et al. 2000) and *Bid* (Sax, Fei et al. 2002). Once cells encounter stress such as DNA damage and other pressure as mentioned above, p53 proteins become stabilized upon phosphorylation and additional modifications (Vogelstein, Lane et al. 2000, Xu 2003). Stabilized p53 accumulates in nucleus and regulates the expression of *Bax, Noxa, Puma, Bid* (Miyashita, Krajewski et al. 1994, Oda, Ohki et al. 2000, Nakano and Vousden 2001, Sax, Fei et al. 2002). The extrinsic apoptosis pathway gets activated by p53 through the induction of several transmembrane proteins including the cell-surface receptor Fas (CD95/Apo-1), death-domain-containing receptor DR5 and apoptotic gene PERP (Haupt, Berger et al. 2003). In

response to γ-irradiation, p53 can induce the expression of Fas transcriptionally via binding to its promoter and first intron of the *Fas* gene (Nagata and Golstein 1995). In line with this finding, DR5 can be also induced by p53 when cells undergo DNA damage (Wu, Burns et al. 1997).

Approximately 50% of tumors display p53 mutations (Béroud and Soussi 1998, Hollstein, Moeckel et al. 1998, Hainaut and Hollstein 2000), and p53 deregulation is crucial in many types of malignancies. In head and neck cancers the mutation rate is even higher with up to 90% of cases of mutations (Kropveld, Rozemuller et al. 1999). Mutations of p53 predispose cells for malignant transformation not only through loss-of-function mechanism, but also via gain-of-function (van Oijen and Slootweg 2000). The main mutations occur in the core domain (120–292 bp) (van Oijen and Slootweg 2000), which is critical for its DNA-specific binding function. For instance, mutated p53 is not able to regulate the CDK inhibitor p21 and therefore p21 will not be activated when cells undergo DNA damage. Subsequently, cell cycle arrest will not occur.

Taken together, cancer cells appear as a result of an imbalance of oncogenes and tumor suppressor genes, such as activation of *ras* or/and inactivating mutations of p53. In fact, cancer formation is often a much more complicated process comprising multiple aberrations in numerous different genes. In 1983, a single oncogene was introduced into normal cells by Land and colleagues, which resulted in a lack of transformation of normal cells into a transformed status. In fact, the activation of leastwise two oncogenes in a cooperative manner was required for full transformation of fibroblasts (Land, Parada et al. 1983). Actually, cancer is caused by accumulation of multiple gene mutations in aging process of human beings (w.Ruddon 2007), each mutation providing surviving advantage to transformed cells.

Overtime, the most aggressive phenotype becomes the major population of tumor entity. This complex process is referred to as multistep clonal evolutionary theory (Nowell 1976).

1.2 Cancer clonal evolution

Evolution is a process that involves successive genetic and phenotypic changes of creatures as an adaptation in response to natural selection (Hall 2008). This process was first postulated by Darwin in his book '*On the Origin Of Species'*. Heritable alteration of biological populations imply natural selection and are responsible for the emergence of new species (Murugaesu, Chew et al. 2013). Dobzhansky's molecular definition of evolution as the alteration in an allele's frequency within a population associated the evolution concept to population heterogeneity at the genotypic level (Dobzhansky 1937). Although this concept was initially used in biology to explain that evolution across generations occur over time, this notion was adapted to describe its role in cancer formation by several scientific groups (Cairns 1975, Nowell 1976, Merlo, Pepper et al. 2006). Of note, Nowell cited the Darwinian evolutionary concept, claiming that originally transformed somatic cells with high genetic instability could generate variability, become sequentially selected, resulting in extremely malignant subtypes of tumor cells with highly heterogeneous karyotypes and biology (Nowell 1976). According to this concept, it is not difficult to imagine that once mutations take place at the single cell level, they might provide proliferative advantage to the cell carrying them. As tumor progresses, a large population of uncontrolled, mutated cells are growing, and, due to their genomic instability, more and more tumor cells with additional mutations can be generated. Thus, over time, a huge tumor population with great genetic heterogeneity is formed. Additionally, subpopulations with invasive, metastatic, drug resistant potential among the heterogeneous tumor population can come up (Campbell and Polyak 2007).

The cancer clonal evolution was not solely proposed by Nowell, as described previously, but was further supported by other observations in cancer. For instance, genotoxic agents such as chemicals can lead to the development of epithelial cancers, and are termed carcinogens. Carcinogens contribute two major forces to evolutionary change: increased genetic diversity in a population and alterations of selective pressures (Casas-Selves and Degregori 2011). One of the most toxic agents is Aflatoxin B1, which is a product of fungus *Aspergillus flavus.* Chronic exposure to this toxin has been demonstrated to bear high potential of hepatocellular carcinoma induction (Farazi and DePinho 2006). Aflatoxin B1 can be metabolized by cytochrome-P450 enzymes, the metabolite of this reaction is AFB1-8,9 epoxide (AFBO), which can intercalate into cellular DNA, generating DNA adducts primarily in cells of the liver. DNA adducts that interact with guanine bases can amongst others lead to mutation of p53 at codon 249 hotspot in exon 7, which may cause the formation of hepatocellular carcinoma (Hamid, Tesfamariam et al. 2013). Moreover, infectious organisms were reported to be involved also in cancer evolution. For example, infection of *Helicobacter pylori* is recognized as a cause of stomach cancer (Piazuelo, Epplein et al. 2010). The resulting inflammation may be causative of stomach cancer both by producing DNA damaging agents, as well as by establishing an abnormal tissue circumstance favoring cancer cells. Hence, extra selective pressure is provided for cancer cells during the cancer formation process over time (Casas-Selves and Degregori 2011). Another evidence supporting the cancer evolution theory is that many subclones of cancer cell populations are resistant to chemotherapeutics after treatment, such as temozolomide and the tyrosine kinase inhibitor imatinib (Swords, Quinn et al. 2005, Hunter, Smith et al. 2006). In other words, treatment removes cancer cells that are sensitive therapeutics, thus maintaining resistant subpopulations. Therefore, therapeutics play a role as an artificial selective pressure on cancer evolution over time.

To sum up, it is very important to understand cancer from an evolutionary perspective. As mentioned above, cancer progression follows the route of evolution; with acquisition of mutations cancer cells can proliferate faster, and once under pressure of lacking resources, more aggressive phenotypes, including invasive and metastatic cancer cells, can survive and become dominant populations. Subsequently, multiple organs are invaded and their functions largely devastated by metastatic cancer cells.

1.3 Cancer stem cell

Generally, the term "stem cell" refers to cells that retained unlimited potential to differentiate into diverse types of cells with characteristic phenotypes and are able to self-renew. In mammals, two types of stem cells are distinguished: adult stem cells and embryonic stem cell. Both types of stem cells can act as progenitors of differentiated cells.

The cancer stem cell (CSC) theory postulates that only a small subpopulation of cancer cells within the primary tumor can initiate tumors. This small group of cancer cells are termed 'CSCs' because they harbor traits resembling features of normal stem cells, including self-renewal capacity, differentiation, but, additionally, tumorigenicity (Ffrench, Gasch et al. 2014). Hence, CSCs are responsible for the maintenance of whole tumor cell populations (Tao Wang and Li 2015). In a strict CSC model, only cancer stem cells are capable of generating tumors, whereas non-CSCs originate from CSC through aberrant differentiation, but lack significant tumorigenic capacity (Tirino, Desiderio et al. 2013). Since CSCs are essential for the maintenance of a cancer cell population, they became a major therapeutic target in oncology. However, treatment opportunities are scarce due to the fact that CSCs display characteristics of resistance to chemo- and radio-therapy owing to their low cell division frequency (Kaiser 2015), elevated DNA repair activity with lower apoptotic ratio (Skvortsov,

Debbage et al. 2015), and efficient drug efflux mechanisms (Lou and Dean 2007, Chen, Huang et al. 2013).

The CSCs concept has been studied for years, but additional insight in this perspective is necessary. As mentioned above, CSCs are considered as the precursors of tumors. Hence, numerous strategies are being developed to counteract these cells. Currently, methods to study CSCs include the analysis of cell surface proteins, sphere formation assays and in vivo tumor initiation upon xenotransplantations. The later assay, i.e. the xenotransplantation of selected, marker-positive cancer cells into immunodeficient mice to monitor tumor formation in serial dilutions remains the ‘gold standard’ of CSC analysis (Tsuyada and Wang 2013).

CSC markers are generally cell surface proteins intimately linked with phenotypes and physiological activities of CSCs. Enrichment of marker-positive CSCs has demonstrated their greater tumorigenic potential over marker-negative cells in numerous entities including breast (Al-Hajj, Wicha et al. 2003) and colon cancer (O'Brien, Pollett et al. 2007, Ricci-Vitiani, Lombardi et al. 2007) amongst others (Visvader and Lindeman 2008). For example, CD44, CD133, CD24, EpCAM, CD166, Lgr5, CD47, and ALDH have been recognized as CSC markers that allow selective enrichment of CSCs (Gires 2011). Based on these findings, CSC markers might represent potential treatment targets. However, until today a fully CSC-specific marker could not be defined. All reported CSC markers are expressed not only on CSCs, but also in a varying extent on non-CSCs or even healthy somatic cells (Karsten and Goletz 2013, Liu, Nenutil et al. 2014). This implies that it is difficult to strictly distinguish CSCs from non-CSCs using CSC markers only. When considering the high heterogeneity in different types of cancer, it is even harder to find universal markers for CSCs of all types of cancer. However, if one marker that is only expressed in tumor cell and is negative or at an extreme low level in healthy tissues, this marker might be applicable as a cancer therapy target (Karsten and Goletz

2013). The epithelial cell adhesion molecule EpCAM is a recurrent marker of numerous carcinoma entities (Visvader and Lindeman 2008, Gires, Klein et al. 2009). Furthermore, it was found by Went and colleagues that EpCAM overexpression was detected frequently in colon, pancreas and prostate adenocarcinomas (Went, Lugli et al. 2004). We will discuss EpCAM molecule in detail in next section.

1.4 Epithelial cell adhesion molecule

Epithelial cell adhesion molecule, in abbreviation EpCAM, was first reported as a tumor-specific antigen in colon carcinoma cells (Herlyn, Steplewski et al. 1979). Later, the cell-cell adhesion function of EpCAM was demonstrated by the group of Litvinov (Litvinov, Velders et al. 1994). It was described by the same group that E-Cadherin-mediated cell interconnections were weakened following the expression of EpCAM (Litvinov, Balzar et al. 1997). EpCAM was studied by different research groups and termed in numerous ways according to its function and primarily after the monoclonal antibodies used for its isolation. Ultimately, an agreement was reached to name the protein EpCAM (Baeuerle and Gires 2007).

In carcinoma patients' cohorts, it was reported that the overexpression of EpCAM was strongly related with poor overall survival of breast cancer patients (Spizzo, Obrist et al. 2002, Brunner, Schaefer et al. 2008). In normal tissue, EpCAM is located at basolateral membranes of cells and weak expression of EpCAM in pseudo-stratified, simple and transitional epithelia can be detected (Balzar, Winter et al. 1999). Later on, it was reported by Munz et al. and Osta et al. that EpCAM contributed to cell proliferation in different cell lines (Munz, Kieu et al. 2004, Osta, Chen et al. 2004). In 2009, the mode of action of EpCAM in the regulation of proliferation was disclosed as a 'membrane-to-nucleus missile' (Carpenter and Red Brewer

2009) that transmits proliferative signals via a regulated intramembrane proteolysis (RIP)-dependent mechanism (Maetzel, Denzel et al. 2009). In this process, EpCAM becomes sequentially catalyzed by TACE and a presenilin-2-containing gamma-secretase complex, leading to the generation of an extracellular domain EpEx and an intracellular domain EpICD (Maetzel, Denzel et al. 2009, Hachmeister, Bobowski et al. 2013, Tsaktanis, Kremling et al. 2015). EpICD can bind to FHL2, beta-catenin and Lef-1 to form a nuclear complex that can interact with DNA at Lef-1 consensus sites including the Cyclin D1 promoter, fostering the proliferation of tumor cells (Maetzel, Denzel et al. 2009, Chaves-Perez, Mack et al. 2013).

Moreover, EpCAM expression was shown in progenitors of hepatocytes during developmental processes of the liver, whereas EpCAM expression is lacking in adult hepatocytes, except bile ducts (de Boer, van Krieken et al. 1999). Furthermore, it was reported that EpCAM is critical in the maintenance of pluripotency of mouse and human embryonic stem cells (ESCs) (González B 2009, Ng, Ang et al. 2010). In human embryonic stem cells (hESCs), it was reported that the EpCAM nuclear complex directly regulates several pluripotency genes including OCT4, NANOG, SOX2, and KLF4 (Lu, Lu et al. 2010). Recently, it was shown that EpCAM, together with its associated protein Cldn7, is essential in reprogramming of mouse embryonic fibroblasts (MEFs) (Huang, Chen et al. 2011). Reprogramming efficiency was enhanced when EpCAM was overexpressed, whereas down-regulation of EpCAM reduced the reprogramming efficiency in MEFs (Huang, Chen et al. 2011).

To sum up, EpCAM was one of the first proteins recognized as a tumor proteins that induces immune responses (Herlyn, Steplewski et al. 1979). Thereafter, it was shown to function as a cell-cell adhesion molecule, before its role in promoting tumor growth was disclosed. The critical role of EpCAM in maintenance of pluripotency of stem cells was also reported.

However, the role of EpCAM in cancer is not fully understood. Given the consistent and strong expression of EpCAM in numerous carcinoma entities (van der Gun, Melchers et al. 2010), knowledge on the precise function of this molecule in cancer generation and progression remains of greatest interest.

1.4.1 EpCAM gene

The human *EPCAM* gene is localized on chromosome 2 (2p21) and is approximately 14 kb in size (Szala, Kasai et al. 1990). The *EPCAM* gene encompasses 9 coding exons: exons 1 to 6 encode the EpCAM extracellular domain, including the signal peptide and EGF-I and EGF-II like domains. The transmembrane domain is encoded by exon 7. The intracellular domain of EpCAM, also named EpCAM intracellular domain (EpICD), is encoded by exon 8 and 9 (Szala, Froehlich et al. 1990, Szala, Kasai et al. 1990).

In 2007, it was demonstrated that the *EPCAM* gene was a target of the Wnt-beta-catenin signaling pathway. In both, normal human hepatocyte and carcinoma cell lines, the accumulation of beta-catenin in the nucleus could induce the expression of EpCAM, whereas inhibition of formation of Tcf/beta-catenin complex or degradation of beta-catenin led to the repression of EpCAM expression (Yamashita, Budhu et al. 2007). Moreover, two Tcf binding DNA elements in the EpCAM promoter were found to be specifically bound by Tcf-4 (Yamashita, Budhu et al. 2007). It was reported by our lab that tumor necrosis factor alpha (TNFalpha) inhibits the expression of EpCAM. The suppression of EpCAM is mediated by TNF receptor 1 via the TNF receptor-associated death domain protein (TRADD) and through the activation of nuclear factor kappaB (NF-kappaB). EpCAM expression might be suppressed by NF-kappaB via competing for the transcriptional coactivator p300/CREB binding protein (p300/CBP) (Gires, Kieu et al. 2001). Another repressor of the transcription

of the *EPCAM* gene is p53. It was found that p53 binds to DNA elements within the *EPCAM* promoter. A significant reduction of EpCAM expression could be detected when p53 was induced in a dose-dependent manner. Inversely, EpCAM expression increase was associated to ablation of p53 (Sankpal, Willman et al. 2009).

Chromatin structure controls the accessibility of transcription factors to the specific binding sites within the *EPCAM* gene, which is affected by epigenetic regulation, including DNA methylation and histone modifications (Esteller 2008). DNA methylation takes place mainly on cytosines within cytosine-guanine dinucleotides (CpGs) (Bernardina T.F.van der Gun 2010). CpGs tend to cluster in islands that are usually located in the 5´-regulatory region of many genes (Bernardina T.F.van der Gun 2010). Methylation of CpG islands in promoters results in transcriptional inactivation of genes.

It was shown that inactivation of p53 led to demethylation of the *EPCAM* gene, therefore, resulting in increased expression of EpCAM (Nasr, Nutini et al. 2003). In line with this finding, upregulation of EpCAM can be induced by downregulation of p53 (Sankpal, Willman et al. 2009). Moreover, endogenous EpCAM could be permanently silenced via methylation of EpCAM promoter, whereas treatment of EpCAM-negative cells with demethylating agents could activate the expression of EpCAM, meanwhile, demethylation of the *EPCAM* gene led to upregulation of EpCAM expression in EpCAM-positive cells (van der Gun, Wasserkort et al. 2008). However, treatment with demethylating agent 5-aza in hypermethylated EpCAM-negative cell lines including K562 leukaemia and liver HepG2 failed to induce *de novo* EpCAM expression. The reason behind this observation might be that the expression of EpCAM does not always depend on demethylation in different cells (Yu, Zhang et al. 2008). In addition, the discrepancy might be caused by different numbers and

localizations of CpGs detected in methylation measurements (Bernardina T.F.van der Gun 2010).

Compared to DNA methylation of *EPCAM*, the histone modification mechanisms at the *EPCAM* promoter are less clear. It was reported that in EpCAM-positive ovarian cancer cells, the *EPCAM* gene was related with acetylated histone 4 (acH4), acetylated histone 3 (acH3) and with trimethylation of lysine 4 of histone 3 (H3K4me3), whereas in EpCAM-negative cells, no histone modifications were detected in most cases (van der Gun, de Groote et al. 2011).

1.4.2 EpCAM protein

1.4.2.1 EpEx domain

The human EpCAM protein consists of 314 amino acid (aa), which can be separated in a long extracellular domain with 242 aa, a single-spanning 23 aa transmembrane domain, and an intracellular domain of 26 aa (Balzar, Winter et al. 1999). The EpCAM protein is composed of a signal peptide, which is recognized during synthesis process for proper ER (endoplasmic reticulum) recognition and becomes removed later (Fig.1.1 arrow 1) (Strnad, Hamilton et al. 1989). In addition, the EpCAM extracellular domain (EpEX) can be cleaved between two arginine residues (Fig.1.1 arrow 2) (Thampoe, Ng et al. 1988, Szala, Kasai et al. 1990, Schon, Schon et al. 1993).

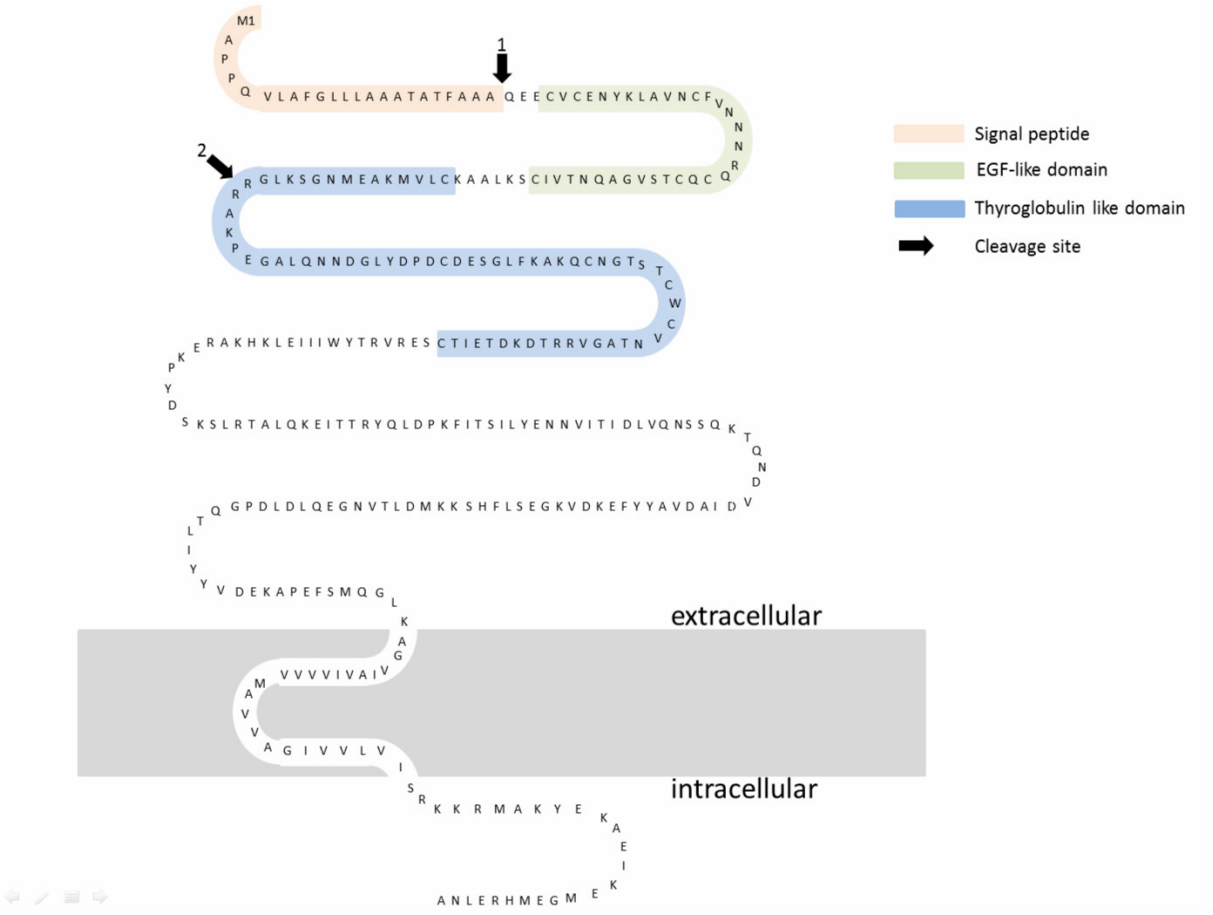


Figure 1.1: Amino acid sequence of EpCAM protein molecular (Schnell, Cirulli et al. 2013).

Arrow 1: signal peptide cleavage site; arrow 2: N-terminal cleavage site between Arg-80/Arg-81. (Modified picture from U. Schnell)

Different analyses were performed to identify the structure of EpCAM molecule. It was shown that EpCAM is composed of three motifs. The extracellular region of EpCAM consists of epidermal growth factor (EGF) like repeats that are located at amino acid position 27-59 and 66-135 (Molina, Bouanani et al. 1996) and are followed by a cysteine-poor region. The epidermal growth factor-like domains can establish a globular structure, which was claimed to be necessary for the homophilic cell-cell contact of EpCAM (Balzar, Winter et al. 1999). In 2014, Pavsic and colleagues reported the crystal structure of the heart-shaped homodimer, which is formed by EpEx. In addition, they also described that cleavage of EpCAM by

cathepsin L within EGF II domain at Gly79-Arg80 and Leu78-Gly79 leads to disruption of the cis-dimeration capacity of EpCAM (Pavsic, Guncar et al. 2014).

1.4.2.2 EpICD interacts with the actin cytoskeleton

The intracellular domain of EpCAM, namely EpICD, is composed of 26 aa. It has been described that two potential alpha-actinin binding sites at aa 289-296 and 304-314, facilitate the binding of EpICD and the actin cytoskeleton, therefore, supporting the hypothesis that EpCAM mediates cell-cell adhesion (M. Balzar and Litvinov 1998).

1.4.3 EpCAM signaling

In 2009, our group demonstrated that EpCAM becomes cleaved by TACE and a presenilin-2-containing gamma-secretase complex through regulated intramembrane proteolysis (RIP) (Maetzel, Denzel et al. 2009). EpCAM cleavage releases the extracellular EpEx ectodomain and the intracellular EpICD domain. EpICD can form a complex together with FHL2 and ß-catenin, shuttle into nucleus, and activate cell division through promoting the expression of c-myc, cyclins and other genes associated with cell proliferation (Munz, Baeuerle et al. 2009, Chaves-Perez, Mack et al. 2013) (Fig.1. 2).

It is also reported that in HCT116 cells, EpICD can activate reprogramming factors including c-Myc, Oct4, Nanog, and Sox2, by binding to their promoter regions (Lin, Liao et al. 2012). When EpCAM-positive cells were treated with the inhibitor of γ-secretase (DAPT), the expression of reprogramming transcription factors such as Nanog, Sox2, c-Myc and Oct was impaired as well as some EMT transcription factors. Moreover, elevated EpEx production led to enhanced EpICD release and subsequently increased expression of reprogramming factors (Lin, Liao et al. 2012).

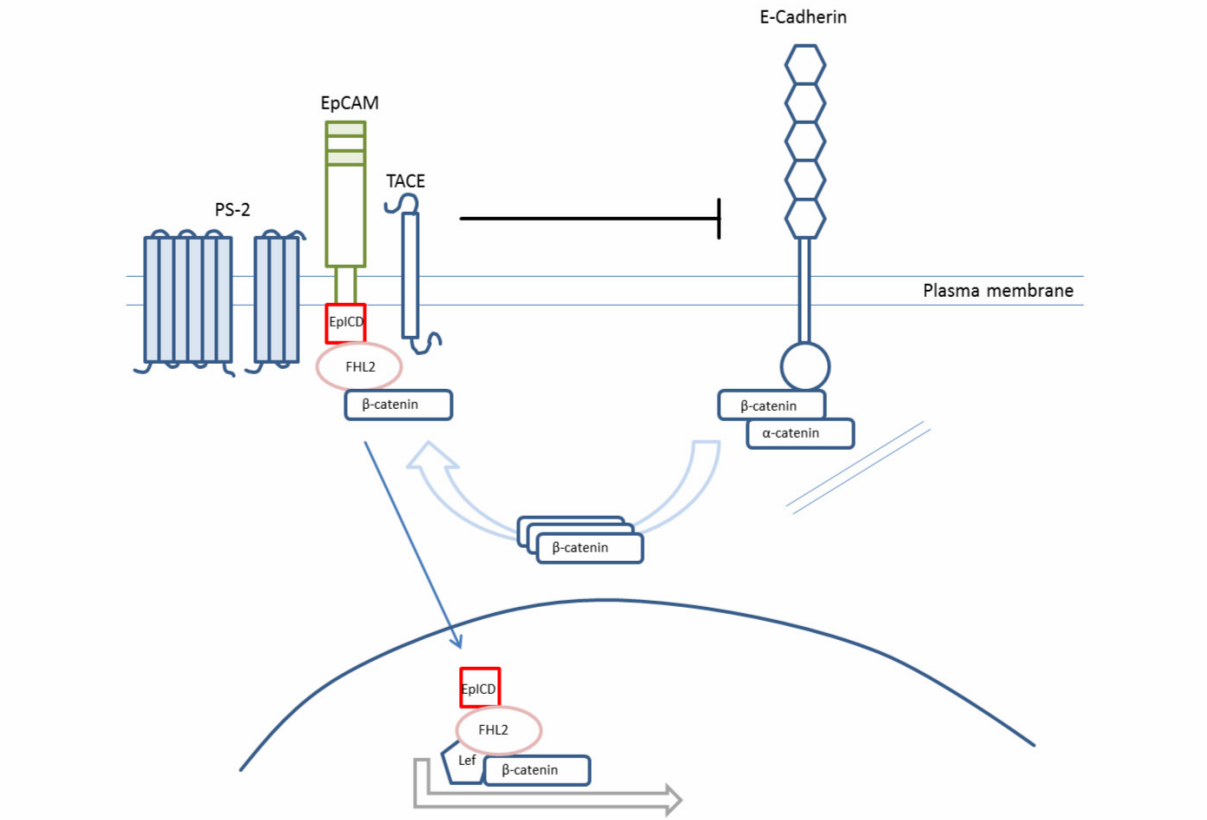


Figure 1.2:Schematic of signaling pathways of EpCAM. Upon cleavage by TACE/PS-2, EpICD shuttles into the nucleus in a protein complex. Together with FHL2, β-catenin, and Lef-1, EpICD binds to DNA at Lef-1 consensus sites. Owing to its capability to interrupt E-cadherin-mediated adhesion, EpCAM provides itself with β-catenin as an essential interacting protein. Modified picture from Munz et al (Munz, Baeuerle et al. 2009).

1.5 EpCAM in Epithelial-to-mesenchymal transition (EMT)

Most tumor cells within primary carcinomas display epithelial traits. In order to loosen from the primary tumor bulk and invade into surrounding tissue, disseminate to distant localization, and subsequently form metastases, subsets of primary tumor cells shift at least transiently from an epithelial into a more mesenchymal status. This change is conducted through a systematic cellular biological program named epithelial-to-mesenchymal transition (EMT). Since it is a transient alteration between epithelial and mesenchymal status, it is reported that the whole mechanism is regulated by epigenetic regulation rather than permanent genetic

mutations (Huangyang and Shang 2013). During the EMT process, epithelial neoplastic cells lose their typical traits, for instance, cell-cell adhesion, squamous-like shape, low migration capacity, and gain invasive features, motility, as well as a spindle-shaped phenotype (Polyak and Weinberg 2009, Thiery, Acloque et al. 2009).

Metastasis formation is a multi-step process, that consists of several critical steps: primary tumor cells undergo a phenotypic shift via EMT, tumor cells penetrate into the blood or lymphatic stream (intravasation), circulating tumor cells (CTCs) survive in the circulation, under certain conditions exit from circulation (extravasation), colonize in distant organs and reverse their phenotype through the inversion of EMT: mesenchymal-to-epithelial transition (MET) (Kalluri and Weinberg 2009).

Circulating tumor cells (CTCs), which were detectable in cancer patients´ blood stream, showed their potential prognostic significance for several carcinoma entities (Zhe, Cher et al. 2011, Tsai, Chen et al. 2016). Therefore, these cells have prognostic and functional significance, and have gained great attention with respect to quantification and molecular characterization. Since EpCAM is ubiquitously overexpressed in carcinoma cells, which differs from normal tissue, it was rational to design a platform to capture cancer cells via the EpCAM antigen. In 2004, Cristofanilli and coworkers described the CellSearch system to isolate CTCs via capture of EpCAM-positive cells in the blood, which was later certified by the Food and Drug Administration (FDA) (Cristofanilli, Budd et al. 2004). Genetic and epigenetic events are taking place in CTCs in order to escape immune-surveillance and survive under harsh conditions in a low metabolic status and behaving like cancer stem cells, sticking together to avoid extra damage in the blood stream (Alix-Panabieres and Pantel 2014). A potential dynamic change of CTCs from epithelial to a mesenchymal phenotype would impair the detection of CTCs that relies on the typical epithelial marker EpCAM, since

down-regulation of EpCAM was detected in the EMT program (Gorges, Tinhofer et al. 2012, Liu, Zhang et al. 2015). Indeed, the implication of dynamic changes between epithelial and mesenchymal statuses in CTCs and DTCs was discussed (Gires and Stoecklein 2014).

Taken together, the expression of the typical epithelial marker EpCAM may be suppressed during EMT program during cancer metastatic cascade (Rao, Chianese et al. 2005, Gorges, Tinhofer et al. 2012), which might contribute to the aggressiveness and dormancy of CTCs, hence, provide better chances for CTCs to survive under harsh conditions. By studying these mechanisms, we aim at identifying the function of EpCAM in cancer progression.

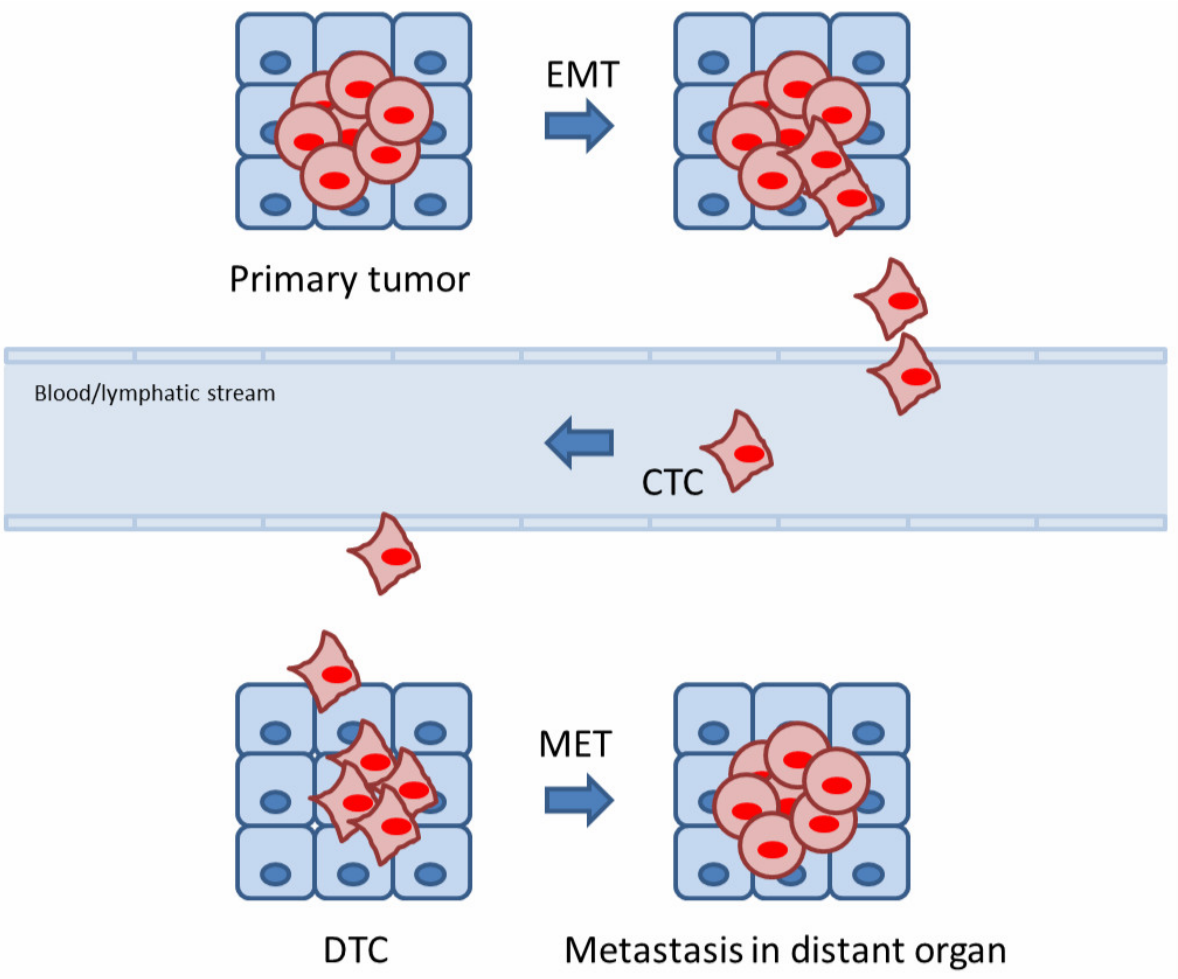


Figure 1.3: Schematic view of cancer progression. Due to the accumulation of mutations, primary tumor is formed. Some tumor cells are able to transit their phenotype from epithelial to mesenchymal (EMT), thereby facilitating them to invade into local tissue, intravasate into the circulation system.Thereafter, they become

circulating tumor cells (CTCs), extravasate from the circulating system and localize in distant organ to form micrometastases. They are termed disseminated tumor cells (DTCs), and can go through a reverse program namely mesenchymal-to-epithelial transition (MET), to propagate and establish macrometastases in secondary organ. Picture adapted from Gires O (Gires O 2014)

1.6 Study aims of the project

Metastasis is a leading cause of death of cancer patients (Gupta and Massague 2006). Although major advances have been made in the treatment of cancer, it is still urgent to disclose mechanisms of cancer cell mobilization from primary tumors to distant organs in order to generate new therapeutic strategies. Many research groups have pointed out that the epithelial-to-mesenchymal transition (EMT) is critical during the cancer metastatic cascade. Down-regulation of cell-cell adhesion proteins is a frequent event. Due to this reason, tumor cells lose contact to their neighboring cells and become motile. Furthermore, the proteins of the cytoskeleton, for example vimentin, might also be affected in parallel. EpCAM is illustrated by many studies as a typical epithelial marker, and is frequently overexpressed in most carcinomas. In vitro, down-regulation of EpCAM facilitates the migration of tumor cells. Since the intracellular portion EpICD accelerates proliferation of tumor cells, it is rational to imagine that EpCAM is dispensable for circulating tumor cells, but required again at later stages of the metastatic process.

However, all the experiments reported to identify potential role(s) of EpCAM during the metastatic cascade were performed in differing systems so far. For instance, data stem from clinical studies, rodent animal models and in vitro assays with human cell lines, which have been implemented into a tentative model of a dynamic expression of EpCAM during the metastatic cascade. Therefore, the aim of present study is to elucidate the EpCAM expression during tumor progression in an all-in-one mouse model that mimics the situation of the

metastatic cascade closely, to provide fundamental data for understanding mechanism of EpCAM expression pattern in cancer metastasis.

2 MATERIAL

2.1 Chemicals

Table 2.1 List of chemicals in present project.

Chemicals	Company
3-amino-9-ethylcarbazol	Sigma-Aldrich GmbH, Taufkirchen
ABC-Kit Vectastain® Elite® PK6100	Vector Laboratories, Burlingame (USA)
Agarose Roche	Mannheim
Acrylamide	Protogel ultra pure Schröder Diagnostics, Stuttgart
Ammonium chloride lysing reagent	PharM Lyse™, BD Biosciences, USA
Anorganic salts	acids and bases Merck KGaA, Darmstadt
Antibody dilution buffer	DCS Innovative Diagnostik-Systeme GmbH & Co. KG, Hamburg
Ammonium persulfate (APS)	BioRad, Hercules (USA)
Aqua dest	Braun, Melsungen
β-Mercaptoethanol	Sigma-Aldrich GmbH, Taufkirchen
Bovine serum albumin (BSA)	Sigma-Aldrich GmbH, Taufkirchen
Dimethylsulfoxide (DMSO)	Sigma-Aldrich GmbH, Taufkirchen
DMEM (4,5g glucose/ with L-glutamine)	Biochrom AG, Berlin
EDTA(Ethylenediaminetetraacetic acid)	Carl Roth GmbH & Co.KG, Karlsruhe
Eosin solution 0,5%	Pharmacy Klinikum Großhadern, Munich
FACSFlow	Becton Dickinson, Heidelberg
FACSSafe	Becton Dickinson, Heidelberg

Chemicals	Company
FACSRinse	Becton Dickinson, Heidelberg
Fetal calf serum (FCS)	Biochrom AG, Berlin
Glycerine	Sigma-Aldrich GmbH, Taufkirchen
Hematoxylin Gill`s Formula H- 3401	Vector Laboratories, Burlingame (USA)
Hydrogen peroxide (H_2O_2)	Merck KGaA, Darmstadt
Mayers Hemalaun solution	Merck KGaA, Darmstadt
Oligonucleotides	Metabion, International AG, Planegg
Organic solvents	Merck, KGaA, Darmstadt
Paraformaldehyde	Carl Roth GmbH & Co.KG, Karlsruhe
PBS tablets	Invitrogen, Karlsruhe
PBS solution	Pharmacy Klinikum Großhadern, Munich
Penicillin Streptomycin (Pen Strep)	Biochrom AG, Berlin
Propidium iodid	Sigma-Aldrich GmbH, Taufkirchen
Protease Inhibitor Cocktail Complete	Roche, Mannheim
Sodiumdodecylsulfat (SDS)	Sigma-Aldrich GmbH, Taufkirchen
Temed BioRad	Hercules (USA)
TissueTek® O.C.T Compound	Sakura Finetek, Staufen
Tris-(hydroxymethyl)-aminomethan (TRIS)	Merck KGaA, Darmstadt
Triton X-100	Sigma-Aldrich GmbH, Taufkirchen
Trypan blue	Biochrom AG, Berlin
Trypsin/ EDTA	Biochrom AG, Berlin
Vectashield® with DAPI	Biozol GmbH, Eching

2.2 Buffer

2.2.1 Cell culture

PBS:	8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4 to 1l H_2O
Cryopreservation medium:	DMEM; 10% DMSO
DMEM/10%FCS:	DMEM; 10% FCS; 1% PenStrep

2.2.2 Flow cytometry

Flow cytometry (FC) buffer:	3% FCS in PBS
Antibody solutions:	1:50 in 50µl FC buffer
Propidium iodide staining solution:	1µg/ml propidium iodide (PI) in FC buffer

2.2.3 SDS-PAGE and western blot

Whole cell lysis buffer (2x):	2 complete protease inhibitor tablets, 1% triton-X100 in 50ml PBS
Laemmli buffer (5x):	62.5mM Tris pH 6.8, 2% SDS; 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue
Stacking gel (4%):	13.3ml 30% acrylamide, 16.6ml 2M Tris pH 6.8, 0.663ml 0.5M EDTA, 69.44ml dd. H_2O
Resolving gel (15%):	50ml 30% acrylamide, 16,6ml 2M Tris pH 8.9, 0.663ml 0.5M EDTA, 32.74 ml dd. H_2O
Running buffer SDS-PAGE:	150g Tris, 720g glycine, 50g SDS to 5l dd.

	H_2O
Blotting buffer (10x):	250mM Tris, 1.26M glycerine in dd. H_2O
Western blot washing buffer (PBST):	8 tablets PBS, 4ml Tween-20 to 4l dd. H_2O

2.3 Commercially available kits

Table 2.2 List of kits in present project.

Product	Manufacturer
BCA Protein Assay	Pierce,Rockford (USA)
Immobilon Western Chemiluminescent HRP substrate	Millipore, Bedford (USA)
LightCycler 480 SYBR Green I Master	Mannheim Roche,
MATra transfection reagent	Iba GmbH, Göttingen
Prestained protein marker V	Peqlab,Erlangen
QiaShredder	Qiagen, Hilden
QuantiTect Reverse Transcription Kit	Qiagen, Hilden
RNeasy Mini Kit	Qiagen, Hilden

2.4 Antibodies

Table 2.3 List of primary antibodies in present project.

Product	Species	Manufacturer
α-EpCAM	Rat IgG2a,κ	Becton Dickinson, Heidelberg

	cloneG8.8	
α-EpCAM	Mouse IgG,clone4A7	Sigma-aldrich
α-Vimentin	Rabbit IgG1	Abcam, Cambridge (USA)
α-hNGFR	Mouse FITC labeled IgG1, κ	BioLegend, San Diego (USA)
α-Cytokeratin	Rabbit polyclonal	Invitrogen, Waltham (USA)
α-E-Cadherin	RabbitIgG,clone24E10	Cell Signaling Technology, Danvers (USA)
α-CD45	Rat (LOU) IgG2b, κ	BD Pharmingen, Heidelberg

Table 2.4 List of secondary antibodies in present project.

Product	Manufacturer
Fluorescein rabbit-α-rat IgG (H&L)	Vector Laboratories, Burlingame (USA)
Biotinylated goat-α-rabbit IgG (H&L)	Vector Laboratories, Burlingame (USA)
PO Goat-α-rabbit IgG (H&L)	Jackson Immuno Research, Newmarket (UK)
Isotype control α-rat IgG	Santa Cruz Biotechnology, Heidelberg
ABC-Kit Vectastain® Elite® PK6100	Vector Laboratories, Burlingame (USA)

2.5 Oligonucleotids

2.5.1 qRT-PCR primer

Table 2.5 List of primers in present project.

Primer	Sequence
Fw m Vimentin	5'-CGG AAA GTG GAA TCC TTG CA-3'

Bw m Vimentin	5'-CAC ATC GAT CTG GAC ATG CTG T-3'
Fw m N-cadherin	5'-AGG GTG GAC GTC ATT GTA GC-3'
Bw m N-cadherin	5'-CTG TTG GGG TCT GTC-3'
Fw m E-cadherin	5'-CAG GTC TCC TCA TGG CTT TGC-3'
Bw m E-cadherin	5'-CTT CCG AAA AGA AGG CTG TCC-3'
Fw m Snail	5'-GCG GAA GAT CTT CAA CTG CAA ATA TTG TAA C-3'
Bw m Snail	5'-GCA GTG GGA GCA GGA GAA TGG CTT CTC AC-3'
Fw m Slug	5'-TCC CAT TAG TGA CGA AGA-3'
Bw m Slug	5'-CCC AGG CTC ACA TAT TCC-3'
Fw m Twist	5'-CGG GTC ATG GCT AAC GTG-3'
Bw m Twist	5'-CAG CTT GCC ATC TTG GAG TC-3'
Fw m Zeb1	5'-CCA TAC GAA TGC CCG AAC T-3'
Bw m Zeb1	5'-ACA ACG GCT TGC ACC ACA-3'
Fw m Zeb 2	5'-CCG TTG GAC CTG TCA TTA CC-3'
Bw m Zeb2	5'-GAC GAT GAA GAA ACA CTG TTG TG-3'
Fw m EpCAM	5'-CAG TGT ACT TCC TAT GGT ACA CAG AAT ACT-3'
Bw m EpCAM	5'-CTA GGC ATT AAG CTC TCT GTG GAT CTC ACC-3'

2.6 Plasmid

pMXs-Puro Retroviral Vector, SV40, puromycin resistance

2.7 Cell lines

Table 2.6 List of cell lines in present project.

Cell line	Description
4T1	From Dr. Sebastian Kobold lab
4T1CTC#1	CTC from blood of 4T1 injected Balb/c mice
4T1DTC#1	DTC from bone marrow of 4T1 injected Balb/c mice
4T1 ex vivo #4	Primary tumor from 4T1 injected Balb/c mice
4T1 ex vivo #9	Primary tumor from 4T1 injected Balb/c mice
4T1 ex vivo #10	Primary tumor from 4T1 injected Balb/c mice
4T1human NGF-Rtrunc	4T1 transduced with pMXs puro retrovirus vector containing truncated human NGF-R

2.8 Consumables

Table 2.7 List of consumables in present project.

Product	Company

3 MM Whatman paper	Bender & Hobein, Munich
6-well cell culture plate, flat bottom Nunc,	Wiesbaden
96-well cell culture plate, flat bottom	Nunc, Wiesbaden
96-well cell culture plate, round bottom	Nunc, Wiesbaden
96 magnet bar plate	Iba GmbH, Göttingen
Cell culture flasks and dishes	Nunc, Wiesbaden
Centrifugation tube 15ml/ 50ml	Becton Dickinson, Heidelberg
Centrifugation tube 1,5ml (nuclease-free)	Costar, New York (USA)
Centrifugation tube 1,5ml/ 2ml	Eppendorf AG, Hamburg
Corning® Costar® stripettes	Sigma-Aldrich GmbH, Taufkirchen
Cryomold Tissue-Tek®, Biopsy (10x10x5mm)	Sakura Finetek, Staufen
Cryo tubes	Becton Dickinson, Heidelberg
FACS-tubes	Becton Dickinson, Heidelberg
Gauge needle Microlance™ 3	Millipore, Schwalbach
Glass flasks	Schott AG, Jena
Glass pipettes	Costar, New York (USA)
Glass plates	Amersham Bioscience, Glattbrugg (Switzerland)
Gloves sempercare latex	Sempermed, Vienna (Austria)
Gloves sempercare nitril	Sempermed, Vienna (Austria)
Microlance 3 / 23G 1.25”	Becton Dickinson, Heidelberg
Microlance 3/ 24G 1” - Nr. 17, 0.55x25mm	Becton Dickinson, Heidelberg
Neubauer chamber	Assistent, Sondheim/Rhön
Object slides „Super Frost”	Nunc, Wiesbaden

Parafilm	American National Can, Menasha (USA)
Pipette tips	Starlab, Hamburg
Quadriperm	Sarstedt, Nümbrecht
Reagent reservoir	Costar, New York (USA)
Safe Seal Tips Professional	Biozym Scientific GmbH, Hessisch Oldendorf
Scalpel	Feather/ PFM, Cologne
Syringe	Braun, Melsungen
Sterile filters	Millipore, Wiesbaden

2.9 Equipment

Table 2.8 List of equipments in present project.

Equipment	Company
Autoclave Systec 95	Systec GmbH, Wettenberg
Blotting System Mini trans Blot	BioRad, Hercules (USA)
DSC-W290 camera	SONY(Japan)
Centifuge Mikro 20	Hettich Lab Technology, Tuttlingen
Centifuge Mikro 22R	Hettich Lab Technology, Tuttlingen
Centrifuge Rotanta 46 R	Hettich Lab Technology, Tuttlingen
ChemiDoc XRS+ imaging system	BioRad, Hercules (USA)
Flow cytometer „FACS-Calibur“	Becton Dickinson, Heidelberg
Fluorescence microscope „Axiovert 200“	Carl Zeiss AG, Jena
Freezer (-20°C, -80°C)	Liebherr, Ochsenhausen

Fridge (4°C)	Liebherr, Ochsenhausen
Light Cycler 480 System	Roche, Mannheim
Magnet stirrer with heat block	Janke & Kunkel, Staufen
Microliter pipettes	Gilson Inc., Middleton (USA)
Microwave	Sharp Electronics GmbH, Hamburg
Phase contrast microscope “Axiovert 25”	Carl Zeiss AG, Jena
pH-meter	WTW, Weilheim
Pipetboy® Comfort	Integra Biosciences, Fernwald
Power supply E835	Consort bvba, Turnhout (Belgium)
Power supply E865	Consort bvba, Turnhout (Belgium)
Precision scales	Mettler, Gießen
Safety cabinet HLB 2448 GS	Heraeus Holding GmbH, Hanau
Scales CP 4202 S	Sartorius, Göttingen
Scales Mettler PM 4600	Mettler, Gießen
Spectrophotometer „GeneQuantPro“	GE Healthcare, Solingen
Thermocycler Comfort	Eppendorf AG, Hamburg
Water bath Exotherm U3e1	Julabo, Seelbach

2.10 Software

Table 2.9 List of softwares in present project.

Software	Company
ApE	Wayne Davis (University of Utah), Salt Lake City (USA)

BD Cell Quest Pro Version 5.2.1	Becton Dickinson, Heidelberg
Endnote	Thomson Reuters Corporation, New York (USA)
Image Lab	BioRad, Hercules (USA)
Image J	Wayne Rasband (National Institutes of Health), Bethesda (USA)
LightCycler® 480 SW 1.5	Roche, Mannheim
MS Office 2010	Microsoft, Redmond (USA)
Photoshop CS3	Adobe Systems Inc., San Jose (USA)
Revelation 4.2.5	DYNEX Technologies Inc., Chantilly (USA)

3 METHODS

3.1 Cell culture

3.1.1 Passaging of cells

Required reagents:

- Dulbecco`s Modified Eagle Medium (DMEM)
- PBS
- Trypsin

All the cell lines applied in the current project were cultured in DMEM with 10% FCS and 1% penicillin-streptomycin at 37°C at the atmosphere of 5% CO_2. Selection of stably transfected cell lines was by the adding of 1µg/ml puromycin into the culture medium. Cells were passaged following their individual proliferation speed. For performance of cell lines passaging, cells in 80% confluence were rinsed with sterile PBS three times, 3ml each time, gently. Trypsin solution was innoculated into culture vessels to cover all the cells on culture surface and cells were incubated in 37°C subsequently. Until adherent cells were detached from culture surface, trypsin reaction was stopped by adding 3 fold more DMEM containing 10% FBS and cell suspension was divided with a ratio of 1:6or 1:8, only one portion of cell suspension was inoculated and cultivated in flasks.

3.1.2 Counting of cells

Cell numbers were determined via a Neubauer chamber by using 20µl of the cell suspension mixed 1:1 with trypan blue. Dead cells incorporated with the dark blue dye and were excluded from counting. Cells concentration was calculated by using the equation demonstrated following:

Cells/ml = (cells counted/ number of counted large squares) * 10^4

3.1.3 Freezing and thawing of cells

Required reagents:

- DMEM
- PBS
- Trypsin
- Cryopreservation medium (DMEM containing 10% DMSO)

For cryopreservation, adherent cells were rinsed gently with PBS three times and harvested from culture flasks using trypsin and centrifugation at 280g for 5 minutes. Supernatants were discarded, and cell pellets were resuspended in cryopreservation medium then kept in cryotubes stored at -80 °C for short-term and in liquid N_2 for long-term.

For thawing, cells in cryotubes were warmed up in a 37 °C water bath quickly and centrifuged at 280g for 5 minutes. Supernatants were discarded and cells were then resuspended in fresh DMEM with FBS and antibiotic kept in flasks for cultivation at 37 °C.

3.1.4 Flow cytometry

Flow cytometry is a biological method that characterizes cells via the fluorescence of the antibodies bind to cells. In present study, antigen specific primary and secondary antibodies were used for staining of living cells, fluorescence intensity was proportional to the expression level of tested antigens of cells. Besides, propidium iodide was applied as a dye to exclude dead cells from whole detected cell population.

3.1.4.1 Flow cytometry analysis of membrane proteins

Required reagents:

- PBS
- FC buffer
- Specific primary and secondary antibodies
- Propidium iodide (PI) (1mg/ml)

For flow cytometry analysis, cells were collected from culture flasks using trypsin solution as described above. Cells were pelleted by centrifuge force of 280g for 5 minutes. The supernatant was discarded and cells were washed with PBS gently, half million cells were then incubated with first antibody at a 1:50 dilution ratio in FC buffer at room temperature for 15 minutes. Then, cells were centrifuged and washed, secondary antibody incubation was performed as same as first antibody. Afterwards, cells were collected and washed, resuspended into 500µl FC buffer with 0.5µl PI. Cells were detected with a BD FACS-Calibur work station and analyzed with Cell Quest Pro (BD) software.

3.1.5 Cytospin

Cytospin is a biomedical method that is innovated for detection of cells in body fluids on glass slides. Cells were forced to attach on the glass slides surface by a special centrifuge platform and immunohistochemistry staining and later on analyses can be performed on the cells.

Required reagents:

- PBS

For cytospin preparation and staining, cells were harvested and washed once with PBS, then resuspended in 100µl PBS and transferred into a device containing a cytofunnel, filter paper,

and a glass slide. Cells were concentrated onto the glass slides surface upon centrifugation, while the PBS was drained through the filter paper. Cytofunnel and filter paper were carefully removed from glass slides, which were dried over night at room temperature. On the next day cells were fixed and stained according to standard IHC staining protocol.

3.1.6 Scratch assay

Scratch assay is applied to measure the migration velocity of cells on 2D surface. Cells were cultured on 2D surface until confluent before a scratch was generated on monolayer of adherent cells. The scratch area was documented and calculated throughout time, and migration velocity was determined.

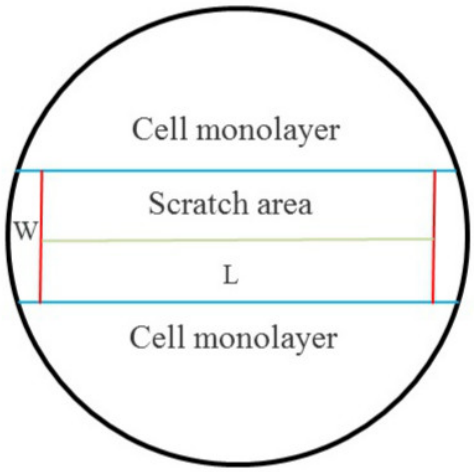


Figure 2.1: Calculation of scratch width. Average width was calculated with the area in the box formed by blue and red lines dividing the length of the green line.w=width, l=length.

3.1.6.1 4T1 and 4T1CTC#1 scratch assay

Reagents required:

- DMEM w/FCS

- DMEM w/o FCS
- PBS

Cells were cultured in flasks and pelleted, $2*10^5$ cells of 4T1 and 4T1CTC#1 were plated and cultivated with 2 ml DMEM with 10% FCS, 1% pen/strep in 6-well plate. $0,5*10^5$ cells per well were seeded in 6-well plate as a proliferation control. Cells were cultivated until approximately 90% confluent, medium was replaced with DMEM without FBS and cells were starved for 24 hours. Afterwards, a scratch was generated on cell monolayer through the center of well using a 200µl sterile tip, in parallel a photo was taken by using SONY DSC-W290 under Axiovert 25 microscope (Zeiss Q5) at initiate time point. The position on 6-well plate of initiate time point photo was marked to ensure that at following photos at different time points were taken at same position. Later on, all the photos were analyzed by Image J software. All the calculations were performed by using Microsoft Excel.

Equation of calculations are following:

W=Scratch area/L

$V=(W_{t0}-W_{tx})/\Delta t$

$P_r=N_f/N_i$

$V_{abs}=V/P_r$

W= width of the scratch

L= length of the scratch

V= migration velocity of tumor cells

W_{t0}= width of the scratch at time point 0

W_{tx}= width of the scratch at time point x

Δt= time difference between time point 0 and x

P_r= proliferative ratio

N_i= seeding cell number of proliferation control

N_f= final cell number of proliferation control

V_{abs}= absolute migration velocity of tumor cells

3.2 Molecular methods

3.2.1 Isolation of mRNA

For the extraction of mRNA from cells, the RNeasy Mini Kit (Qiagen) with QiaShredder columns (Qiagen) was applied following the manufacturer's protocol. Extracted mRNA was stored at -80°C until further use.

3.2.2 Reverse transcription polymerase chain reaction (RT-PCR)

Isolated mRNA was reverse transcribed into cDNA and used as template for PCR or qRT-PCR amplification assays.

When mRNA was isolated from cells, the concentration was measured by „GeneQuantPro" spectrophotometer (GE Healthcare). Then, 1µg of the mRNA was added to 2µl of gDNA wipeout buffer and the mixture was filled up to 14µl with RNAse-free H_2O. The mixture was warmed up to 42°C for 2 minutes to eliminate genomic DNA in the probes and then promptly placed on ice. For cDNA generation, 1µl reverse transcriptase, 1µl primer mix and 4µl Quantiscript RT-buffer were added to the previous solution and the mixed solution was incubated for 30min at 42°C. Ultimately, the mixture was heated up to 95°C for 3 minutes to stop the reverse transcription reaction.

Standard reaction procedure:

Mix 1:	Mix 2:
mRNA 1µg	Quantiscript RT 1µl
gDNA wipeout buffer 2µl	Quantiscript RT-buffer (5x) 4µl
RNAse free H_2O add to 14µl	Primer mix 1µl
Mix well at the beginning	Mix with Mix1 for reaction

Standard temperature settings:

Genomic DNA elimination	2 min	42°C
Pause	1 min	on ice then add mix 2
RT-PCR reaction	30 min	42°C
RT reaction break down	3 min	95°C

After reverse transcription, cDNA samples were stored at -20°C until further use.

3.2.3 Quantitative Real-Time PCR (qRT-PCR)

Quantitive RT-PCR is a method that can facilitate the comparison of the expression of a gene of interest transcriptionally across cell lines or within the same cell line with different treatments.

In our qRT-PCR assays, the LightCycler 480 SYBR Green I Master kit (Qiagen) was applied. A master-mix was mixed beforehand according to the amount of templates and samples to be detected. Each probe was repeated in triplicates.

Standard master-mix (per reaction):

cDNA template	1µl
Primer mix	2µl
SYBR Green master-mix (2x)	5µl
ddH_2O	2µl
Total	10µl

Primer mix: containing forward and backward primers (each 10µl of a 100µM stock), 1:10 diluted with 180µl ddH_2O.

2x SYBR Green master-mix (Roche): encompasses DNA-polymerase, SYBR-Green and reaction buffer.

Standard reaction setup:

Initial denaturation	10 min,95°C
denaturation	30 sec, 95°C
Annealing and elongation	60 sec, 72°C back to denaturation step, 45 repeats
Cooling/Storage	∞ 4°C

Data was acquired using a Light Cycler 480 (Roche) and calculated with LightCycler 480 SW 1.5 (Roche) and Microsoft Excel.

Calculation of different mRNA levels was based on crossing points (Cp)-values, which showed fluorescence signal of a probe emited above the threshold (Roche 2014).

Calculations were the following:

1. Average of Cp-values: $Cp = \sum Cp/3$

2. Standardisation to a housekeeping gene (HG):

$\Delta Cp = Cp\ (gene) - Cp\ (HG)$

3.3 Biochemical methods

3.3.1 Preparation of whole cell lysates

Required reagents:

- Whole cell lysis buffer (2x)
- PBS
- Laemmli buffer (5x)

For generation of whole cell lysates, cells were harvested and washed gently with PBS. Lysis buffer (2 fold of pellet volume) 2x concentrated was added to cell pellets. The probes were maintained on a rotation wheel at 4°C and mixed thoroughly for 10 minutes. Afterwards, probes were centrifuged at 16000rpm for another 10 minutes to clean cell debris. Supernatants containing proteins were kept at -20°C or directly utilized for protein concentration measurement via BSA-assay. Ultimately, Laemmli buffer was added to the lysates and samples were denatured at 95°C for 5 minutes (Laemmli 1970). Then, protein probes were stored at -20°C until further use.

3.3.2 Measurement of protein concentration (BCA-assay)

Required reagents:

- BCA-assay kit

Protein concentrations were measured by using the BCA-assay kit following the manufacturer`s protocol. 1µl of the protein samples was mixed with 99µl BCA solution and absorbance at 595nm wavelength was measured with a spectrophotometer („GeneQuantPro“, GE Healthcare). All measurements were performed in triplicates. For calculation of protein concentrations, a probe containing bovine serum albumin (BSA) with standard concentration was used as a standard curve and background (BG) value of BCA was subtracted.

Calculation was performed with Microsoft Excel via the equation below:

$$C_{(probe)} = ((A\lambda_{(probe)} - A\lambda_{(BG)})/(A\lambda_{(BSA)} - A\lambda_{(BG)})) * C_{(BSA)}$$

$C\lambda$ = protein concentration/ml

$A\lambda$ = absorbance

3.3.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Required reagents:

- 10x SDS running buffer
- Resolving gel
- Stacking gel
- APS
- TEMED
- ddH_2O

Resolving gel (15%)	Stacking gel (4%)
30% acrylamide 50ml	30% acrylamide 13.3ml
2M tris pH 8.9 16.6ml	2M tris pH 6.8 16.6ml
0.5µ EDTA 663µl	0.5µ EDTA 663µl
ddH_2O 32.74ml	ddH_2O 69.44ml

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) is commonly used to separate proteins based on their molecular weight. The migration capacity of proteins is proportional to molecular weight, given they do not differ substantially in charge. Accordingly, smaller proteins migrate faster than those with high molecular weight.

Standard SDS-PAGE consists of two different types of matrix, i.e. a stacking gel, which collects all proteins at the boundary between the two gels, and the resolving gel, in which the proteins are actually separated. 10ml resolving gel (15%) was generated using a mixture of 50µl APS and 30µl TEMED, transferred into the gel chamber and covered with ddH_2O to form a level surface. After polymerization the water was removed and 2ml of stacking gel solution was generated with a mixture of 30µl APS and 15µl TEMED, inoculated and solidified on top of the running gel. Subsequently, same amounts of protein probes were loaded on gels. Gel electrophoresis was conducted for 15min at 15mA and 2h at 30mA per gel saturated with SDS running buffer. Thereafter, gels were used for immunoblotting.

3.3.4 Immunoblotting (western blot)

Required reagents:

- Methanol
- 1x blotting buffer

- Blocking solution (5% milk in washing buffer)
- Washing buffer (PBST)
- Specific primary and secondary antibodies
- Primary antibody solution (3% BSA in washing buffer)
- Secondary antibody solution (5% milk in washing buffer)
- Chemiluminescent HRP substrate

A wet blotting system (Blotting System Mini trans Blot, BioRad) was used for immunoblotting. With this system, proteins separated in a polyacrylamide gel can be blotted onto a polyvinylidene fluoride (PVDF) membrane. Firstly, membranes were first merged into methanol for one minute and then moved into blotting buffer. After assembling the blotting device, blotting was performed for 50 minutes at 100V at room temperature.

After blotting, PVDF membranes were first merged in blocking solution for minimally 30 minutes at room temperature to minimize unspecific antibody binding on membranes. Then membranes were washed in PBST for 15 minutes and incubated with first antibody (diluted in 5ml primary antibody solution) for 1 hour at room temperature or overnight at 4°C. Subsequently, membranes were washed in PBST for 15 minutes and incubated with the appropriate secondary antibody for 45 minutes at room temperature (diluted in 5ml secondary antibody solution). After washing in PBST for 15 minutes, antigen-antibody signals were amplified upon chemiluminescent HRP substrate (Millipore). Protein bands were detected with a ChemiDoc XRS+ imaging system (Biorad) and analyzed using ImageLab (Biorad) and Photoshop (Adobe) software.

3.4 Immunohistochemistry

Required reagents:

- Methanol (-20°C)
- PBS
- Paraformaldehyde (PFA)
- Horse serum
- Tris buffer (0.05M, pH 7.4)
- Brij solution (50% Brij in PBS)
- Specific primary and secondary antibodies

Tumor probes were preserved in cryomolds, embedded with Tissue Tek gel and snap frozen in liquid nitrogen. Frozen tumor samples were sectioned to serial slices with 4µm thickness by a Cryostat model CM 1900 (Leica) and fished with glass slides.

In immunohistochemical staining assays, probes were fixed in acetone for 5 minutes at room temperature, afterwards fixation with 3.5% PFA for 10 minutes in dark at 4°C and 5 minutes in dark at room temperature. Endogenous peroxidase activity was diminished by incubating the samples in 0.03% H_2O_2 in PBS for 10min at room temperature. Probes were washed twice in PBS each time for 5 minutes at room temperature and blocked with horse serum (1:200 in 200µl tris buffer) for 20 minutes at room temperature to prevent unspecific antibody reaction. First antibody (1:1000 in 200µl tris buffer) was incubated for 1 hour at room temperature or overnight at 4°C. After washing probes with PBS and Brij-solution, probes were incubated with a biotinylated anti-mouse antibody (1:200 in 200µl tris buffer) for 30 minutes at room temperature, and washed again using PBS and Brij-solution, then incubation with a peroxidase-labeled avidin–biotin complex was proceeded. Finally, tissues were stained with amino-ethylcarbazole (AEC) as a peroxidase substrate, generating a red signal of the antigen/antibody complexes. Counterstaining was generated by using hematoxylin (blue).

Probes were covered with Kaisers glycerine gelatine and photos were taken using a Olympus BX43F fluorescence microscope and CellEntry software (Olympus).

3.5 Mouse experiments

Required reagents:

- DMEM w/o FCS
- Growth Factor Reduced BD Matrigel Matrix
- TissueTek® O.C.T Compound
- Liquid nitrogen

Note: All assays were conducted with the approval of the Ethics Commission of the Ludwig's Maximilian University Munich (Az 55.2.1.54-2532-90/12).

To study in vivo tumor formation ability of 4T1 and 4T1CTC#1, cells were prepared and harvested then transplanted in 6-8 week old, Balb/c mice. To do so, $1.25*10^5$ cells in 100µl PBS were mixed with 100µl Growth Factor Reduced BD Matrigel Matrix and the mixture was injected intraperitoneally in the flanks of mice using a BD Microlance 3/24G 1". After cells were injected, mice were continuously observed for signs of tumor growth. Objective quantitative endpoints for the experiment were a tumor size larger than 20mm, a tumor weight superior to 4g and an animal weight loss superior to 20% of the initial body weight. Following these endpoints but no later than 28 days after injection, mice were sacrificed. In vivo generated tumors were explanted, tumor weights were assessed using a precision scale, and tumor tissues were embedded in Tissue Tek and snap frozen and sectioned for immunohistochemical analyses.

3.5.1 Ex vivo cell culture

Required reagents:

- DMEM
- Ammonium chloride lysing reagent (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM EDTA, or a commercial preparation e.g. PharM Lyse™, BD Biosciences #555899)
- 2% agarose
- Wash buffer (0.1% sodium azide, 0.1% BSA in PBS)
- Airway Epithelial Cell Growth medium supplemented with SupplementMix C39165

3.5.1.1 Generation of ex vivo primary tumor cells

4T1 has been generated several decades ago in a biomedical lab and applied as a common breast cancer research model. In our present study, 4T1 cells were transplanted into Balb/c mice and EpCAM expression level was determined before transplantation and immunohistochemically analyzed on tumor tissue samples after mice were taken down. However, in mice tumor explants a mixture of non-tumor and tumor cells existed.Ex vivo cultivation of primary tumor cells from mice were conducted. Cells were cultured and selected with DMEM containing 6-thioguanine with a concentration of 30μM.

Primary tumor probes were excised from mice and cut into 1 mm^3 cube-shaped pieces using sterile Feather disposable scalpels N°11. Several grooves were made using scalpels on top of the small tumor explants that were a 1 mm^3 big. These small grooves generated by scalpels can loosen the structure of the tumor samples and facilitate the migration of tumor cells from tumor samples. In order to keep tumor tissue moisture, a drop of PBS was pipetted on top of the tissues. Small tumor tissue pieces were placed in 24-well plates. And the plates were

coated on one side with a crescent-like structure using 2% agarose. 250 µl of agarose solution was used for a crescent-like structure coverage each well in 24-well format plates. Tumor tissue samples were cultivated using Airway Epithelial Cell Growth medium supplemented with SupplementMix C39165 (Promocell, Heidelberg, Germany) for one to seven days (Mack, Eggert et al. 2013). Once sufficient amount of tumor cells adhered to the agarose free area, cells were harvested and transferred to bigger plastic culture flasks for expansion. Later DMEM containing 6-thioguanine with a concentration of 30µM was used to select the cells. When permanent cell lines were generated, cryopreservation was performed and further analyses were conducted in parallel.

3.5.1.2 Generation of CTC and DTC cell from mice blood and bone marrow

In our present study, in order to define the EpCAM status on CTCs and DTCs, we tried to isolate and culture those rare cells from mice whole blood and bone marrow.

After tumor bearing mice were sacrificed, whole blood of animals was collected with heparin covered sterile tubes. For lysis of red blood cells in the samples, BD lysis buffer (#555899) was applied. The 10x lysis buffer was warmed up at room temperature before utilization, and diluted to 1x with destilled water. Per 100 µl fresh blood sample 1ml diluted lysis solution was added. The samples and solution were mixed gently and subsequently incubated at room temperature in the dark. All the living cells were collected at 280g centrifugal force for 5 minutes. Then, the supernatant was carefully aspirated and 10 ml 1x PBS containing 1% heat-inactivated fetal bovine serum (PBS-FBS) was added. The cells were again centrifuged using same program mentioned above, supernatant was carefully removed and the cells were inoculated with culture medium into flasks.

In order to isolate and cultivate DTCs, femur and tibia of mice were collected. The two ends of the bones were cut with sterile surgical scissors. The bone marrow was washed out with sterile PBS and red blood cell lysis and culture process was conducted in the same manner as described above.

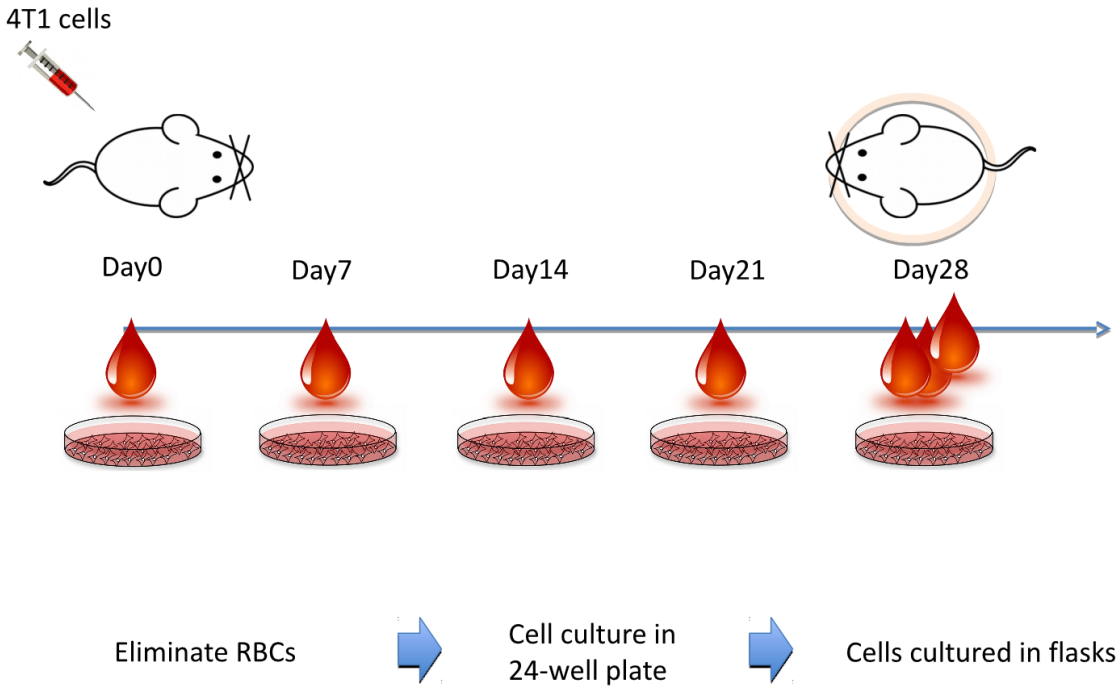


Figure 2.2: Workflow of CTCs isolation from blood samples of tumor bearing mice. 4T1 tumor cells were injected into Balb/c mouse at day 0, the blood of mice was sampled at different time points, such as day 4, day 14, day 21 and all the blood was collected at the time of mice sacrifice. All the blood samples were processed with BD blood lysis buffer and then inoculate into 24-well plate to expand cells. Once the cells in 24-well is nearly confluent, they were transfer into T-75 flasks for passaging.

3.6 Metaphase preparation

Chromosome preparations were executed for Spectral karyotyping (SKY) (Bauer, Hieber et al. 2010). 2.5×10^5 cells were cultivated in 4 mL medium on top of a sterile glass slide placed in a slide tray chamber with addition of 0.05 µg/mL Colcemid overnight to arrest cells in metaphase. Thereafter, the medium was discarded and the slide was incubated with 4 mL

hypotonic KCl-solution (0.075 M) for 20 min at 37 °C. Then, 4 mL ice-cold fixation solution (methanol/glacial acetic acid, 3:1) was added and incubated for 20 min at 4 °C. After this incubation step, incubated solution was discarded and another new 4 mL ice-cold fixation solution was added and exact same incubation step was performed. Finally, the slide was air dried under a laminar flow.

3.7 Spectral karyotyping (SKY)

SKY was performed as published previously by Zitzelsberger *et al* (Zitzelsberger, Lehmann et al. 1999, Bauer, Hieber et al. 2010). Chromosome preparations were treated with RNase A (0.1 mg/mL in 2 × SSC) before hybridization. Chromosomes were denatured by positioning the slides in 70% formamide in 2 × SSC at 72 °C. Thereafter, the slides were dehydrated gradually with 70%, 90% and 100% ethanol and hybridized with a denatured SKY-probe mixture (SKYPaintTM DNA Kit, Applied Spectral Imaging, Edingen-Neckarhausen, Germany) for 1–2 min. After hybridization for 24 hours, slides were washed at room temperature following a rapid washing protocol: 0.5 × SSC for 5 min at 75 °C, 4 × SSC/0.1% Tween for 2 min and aqua dest for 2 min. Probe was detected using anti-digoxigenin (1:250; Roche, Penzberg, Germany), avidin-Cy-5 and avidin-Cy-5.5 antibodies (both 1:100; Biomol, Hamburg, Germany) following the manufacturers' instructions. Prepared metaphase chromosomes were counterstained with 0.1% DAPI (4',6-diamidino-2-phenylindole) in anti-fade buffer (Vectashield mounting medium; Vector Laboratories, Burlingame, CA). Image was captured via a SpectraCube system and analyses were conducted using the SKY-View imaging software (both Applied Spectral Imaging, Edingen-Neckarhausen, Germany).

3.8 Statistical analysis

Statisticatical analyses were performed with Microsoft Excel. The Student's t-Test was performed to assess the statistical significancesbetween experimental groups. P-values smaller or equal to 0.05 were recognized with significance. Bars and error bars in charts represent mean values ± standard deviation (s.d.) of at least three independent experiments.

4 RESULTS

4.1 EpCAM expression in 4T1 murine breast carcinoma cell

4T1 is a Balb/c-derived breast carcinoma cell line that generates primary tumors and metastases upon syngeneic transplantation (Pulaski and Ostrand-Rosenberg 2001). In order to study expression pattern and function of EpCAM in the 4T1 murine breast carcinoma metastasis model, cell surface expression level of EpCAM was detected on 4T1 cells via flow cytometry. In parallel, the morphology of 4T1 cells was assessed in two-dimensional cell culture. For this, 4T1 cells were cultured at low and high density (Fig 3.1A). 4T1 cells display a degree of heterogeneity in cell morphology with the majority of cells are retaining typical epithelial traits with a cobblestone-like morphology and formation of cell-cell contacts (Figure 3.1A). However, at low confluence a minor fraction of cells with mesenchymal appearance, protrusions and spindle-shape with reduced cell-cell contact was observed (Figure 3.1Ac). Flow cytometry analysis revealed a strong and heterogeneous expression of EpCAM on the cell surface. A small fraction of cells did not express EpCAM, whereas the great majority strongly expressed EpCAM (mean fluorescence intensity=105±15) (Figure 3.1B). Hence, 4T1 cells are heterogeneous with respect to morphology and EpCAM expression. However, the majority of cells are epithelial and EpCAM-positive.

Expression status of EpCAM on 4T1 was further assessed via immunocytochemistry using cytospin preparations. 4T1 cells were cultured and harvested, then cell concentration was determined by counting in a Neubauer Chamber. One million cells in 100µL medium were spun down on a slide, fixed and permeabilized according to protocol.

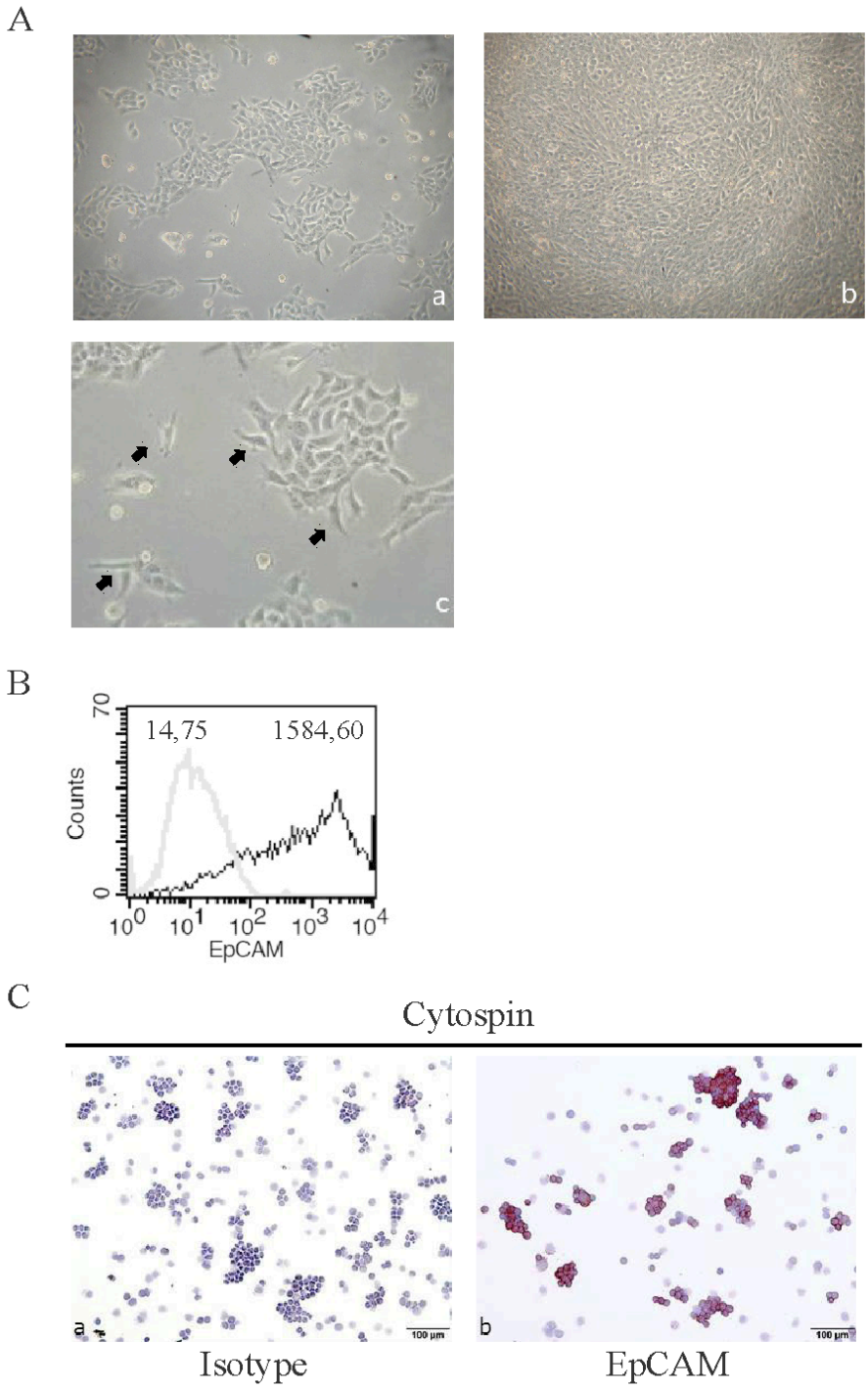


Figure 3.1: Characterisation of EpCAM expression in the 4T1 murine breast carcinoma cell line.

(**A**) Morphology of 4T1 cells at low (a) and high (b) density. 4T1 cells were cultured in T-75 cell culture flask and pictures were taken at different confluence. (c) Magnified picture visualizes a subpopulation of 4T1 cells retain a mesenchymal morphology (closed black arrow). (**B**) Representative histogram of flow cytometry analysis of EpCAM expression on 4T1 cells. 4T1 cells were stained with a primary antibody specific for murine

EpCAM (black line) or an isotype control-specific antibody (grey line). Propidium iodide was used to exclude dead cells during FACS analysis. Mean fluorescence intensities of each staining are given. (**C**) EpCAM expression on 4T1 cells was confirmed via cytospin immunocytochemistry staining. 4T1 cells in cytospins were stained with an isotype control antibody (a) or an EpCAM-specific antibody (b, red chromophore).

Comparably to flow cytometry measurements, EpCAM was strongly expressed in the great majority of 4T1 cells (Figure 3.1C, red color). Consistent with cell surface expression results, the expression of EpCAM was likewise heterogeneous with a majority of cells highly expressing the protein while a subset of cells revealed EpCAM-negative. Control staining with an isotype-matched primary antibody confirmed the specificity of the detection system (Figure 3.1C, left panel).

4.2 4T1 cells generate primary tumors and metastases in the Balb/c mouse model

4T1 cells generate transplantable tumors with high metastatic capacity in various organs. These traits make syngeneic transplantations of 4T1 cells a suitable animal model to study major aspects of breast cancer progression in the presence of an intact immune system.

4T1 cells were cultured and harvested before syngeneic transplantation was performed subcutaneously into the flank of Balb/c mice. Expression of EpCAM in primary tumors was analyzed by immunohistochemistry staining (IHC) with specific antibodies. EpCAM expression in primary 4T1 tumor specimens was heterogeneous with areas of very strong to weak expression and even areas of cells lacking EpCAM expression completely (Figure 3.2a-c), which is consistent with the expression of EpCAM in 4T1 cells cultured in vitro. In swollen lymph nodes, EpCAM positive tumor cell colonies were detected (Figure 3.2d), which represent metastatic growth. 4T1 cells displayed an aggressive growth pattern with spreading into numerous organs including spleen, liver, lung, brain, lymph nodes, amongst others. A nest of disseminated 4T1 in the spleen is depicted in Figure 3.2f.

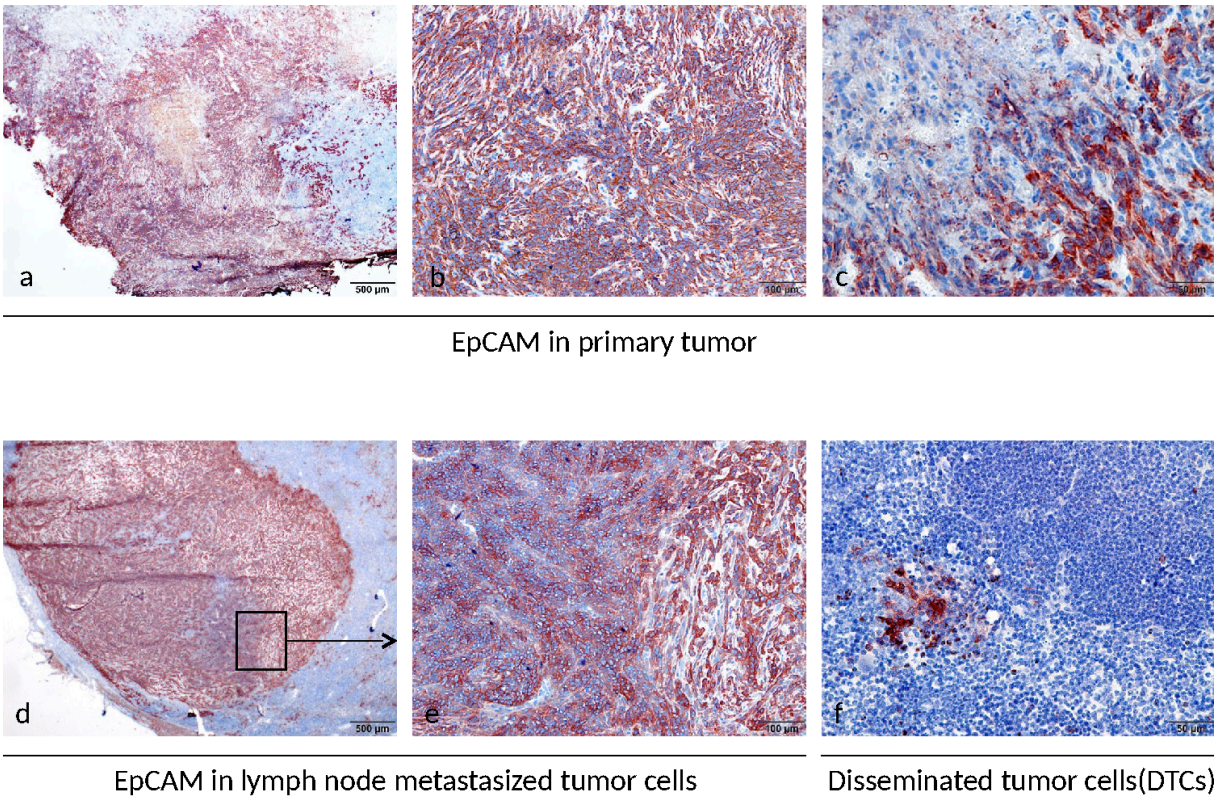


Figure 3.2 EpCAM is expressed in 4T1 primary tumor and disseminated tumor cells (DTCs). 4T1 cells were transplanted subcutaneously into the flank of Balb/c mice. After 28 days, mice were sacrificed and primary tumors and organs were collected, cryopreserved, sectioned and immunohistochemically stained. In primary tumors (a), EpCAM expression was heterogeneous with most areas characterized by high expression of EpCAM (b). However, EpCAM low expression and negative areas were detected too (c). In swollen lymph nodes, macrometastasis with intense expression of EpCAM were visualised (d). In enlarged spleens, EpCAM-positive cells were detected that most probably represent DTCs.

4.3 Circulating 4T1 tumor cells displayed a mesenchymal phenotype

4T1 tumor cells were subcutaneously transplated into the flank of Balb/c mice. Blood of mice was drawn at different time points as illustrated in Fig.2.2. The whole blood of mice (n=5) that had been transplanted subcutaneously with 4T1 cells was collected and processed following a standard protocol to eliminate red blood cells (see materials and methods). Blood collection was performed four weeks after primary inoculation of tumor cells and in the presence of large primary tumors. The remaining cells after red blood cell depletion were

inoculated into 24-well plate and cultured in 1 ml medium. By doing so, one cell line was established and termed 4T1CTC#1. This newly established cell line from mice blood showed a strong fibroblast-like morphology with a pronounced spindle shape and long protrusions (Fig 3.3Ab). In addition, 4T1CTC#1 could not form cell-cell contact and propagated independently from neighbor cells even in a highly dense status, i.e. without forming cell colonies (Fig 3.8b). Measurement of EpCAM expression at the surface of 4T1CTC#1 upon flow cytometry revealed a complete lack of expression (Figure 3.3Ad), which was confirmed as a total lack of EpCAM protein via immunobloting of whole cell lysates in comparison to parental 4T1 cells (Figure 3.3B).

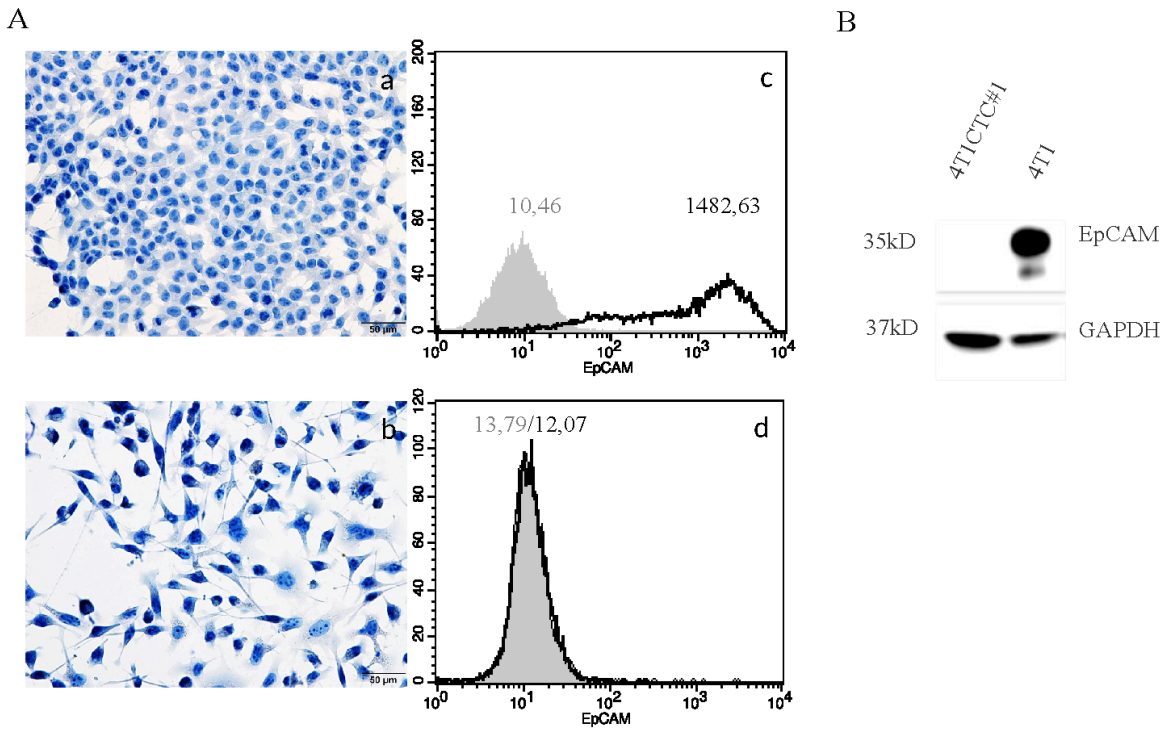


Figure 3.3 4T1CTC#1 cells do not express EpCAM and have a mesenchymal phenotype. 4T1CTC#1 cells show a strong mesenchymal phenotype, do not form cell-cell contact, and display an elongated fibroblast-like shape (Ab), while 4T1 cells display epithelial morphology (Aa). In flow cytometry analysis, 4T1CTC#1 revealed negative for the cell surface expression of EpCAM (Ad).However, 4T1 intensively express EpCAM (Ac). Immunoblot analyses in whole cell lysates of 4T1CTC#1 confirmed the total lack of EpCAM protein (B).

4.4 4T1CTC#1 originates from parental 4T1 cells

4.4.1 CD45 and p53 expression

4T1CTC#1 cells were further characterized in order to substantiates their origin. To this end, cells were analyzed via flow cytometry for the expression of CD45, a marker for hematopoietic cells. As shown in Fig 3.4 right panel, 4T1CTC#1 did not express CD45, whereas control murine B lymphoma 291 cells expressed high amounts of CD45 at the membrane. Parental 4T1 cells served as a further control and revealed negative for CD45, too. 4T1 cells are further characterized by a lack of p53 expression (Yerlikaya, Okur et al. 2012). Hence, the p53 status of 4T1CTC#1 was analyzed at the transcriptional level using quantitative real-time PCR. Both, parental 4T1 and 4t1CTC#1 cells did not express p53 mRNA, whereas control E14 embryonic stem cells (day 0) did (Figure 3.5). Hence, 4T1 parental cells and 4T1CTC#1 derivative cells do not express the hematopoietic marker CD45 and show no expression of p53 mRNA.

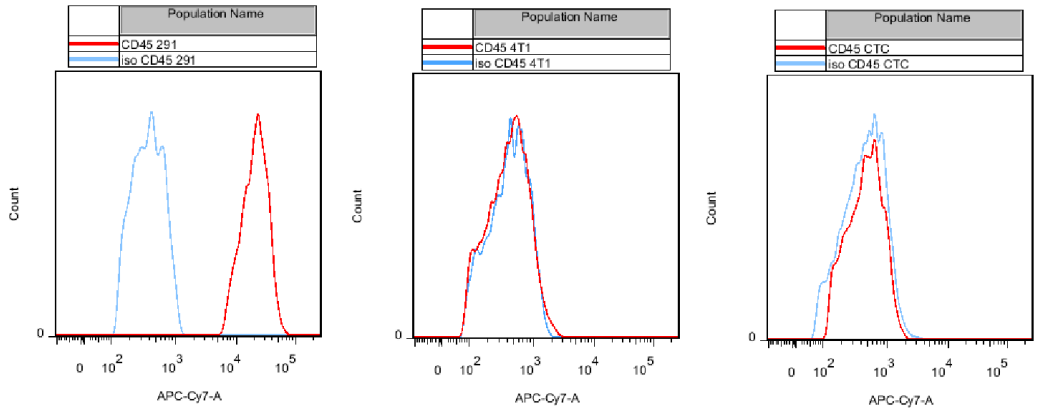


Figure 3.4 4T1CTC#1 is CD45 negative. CD45 expression was determined by flowcytometry analysis on 4T1CTC#1 cells, 4T1 cells were used as a negative control and murine B lymphoma 291 cells as a positive control. CD45 is negative on 4T1CTC#1 cells.

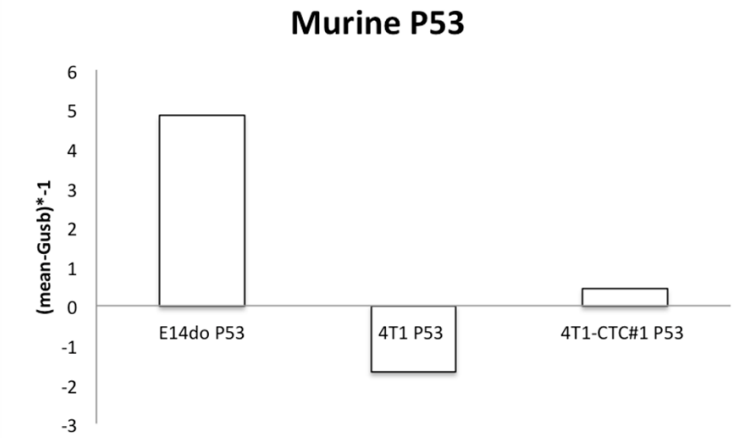


Figure 3.5 4T1CTC#1 is p53 negative. Quantitative reverse transcription PCR results show that p53 is negative in 4T1 and 4T1CTC#1 cells. E14 cells served as a positive control. Total RNAs of 4T1, 4T1CTC#1 and E14TG2a embryonic stem cells (day 0) were extracted, thereafter, cDNA was produced upon reverse transcription. Expression levels of p53 were assessed via qPCR using the generated cDNAs as templates. The house-keeping gene Gusb was utilized as a reference.

4.4.2 4T1CTC#1 cells could survive 6-thioguaine selection

The mouse mammary carcinoma 4T1 cell line originated from a spontaneously arising mammary tumor in BALB/cfC3H mice (Dexter, Kowalski et al. 1978, Heppner, Dexter et al. 1978). A great advantage of 4T1 cell relies on its resistant to 6-thioguanine. 6-Thioguanine is a purine analog of guanine that can be incorporated into the cellular DNA during replication process and, thereby, inhibits the small GTPase Rac1 and possibly hampers translation after incorporation into mRNA (Pulaski and Ostrand-Rosenberg 2001). In order to explore whether 4T1CTC#1 cells originated from 4T1 cells, a 6-thioguanine cytotoxicity assay was performed. Both 4T1 and 4T1CTC#1 were plated in 6-well plate with an initiating cell number of 5000 cells per well. NIH3T3 murine fibroblast cell were seeded under the same condition as a control. All cells were cultivated in 2 ml DMEM with or without 6-thioguanine at a concentration of 60 μM. Cell numbers were determined via counting in a Neubauer chamber

at day 3 and day 7. As displayed in Fig 3.6, 4T1CTC#1 and 4T1 proliferated in both media, with or without selection pressure of 6-thioguanine, while NIH3T3 could only grow in normal medium.

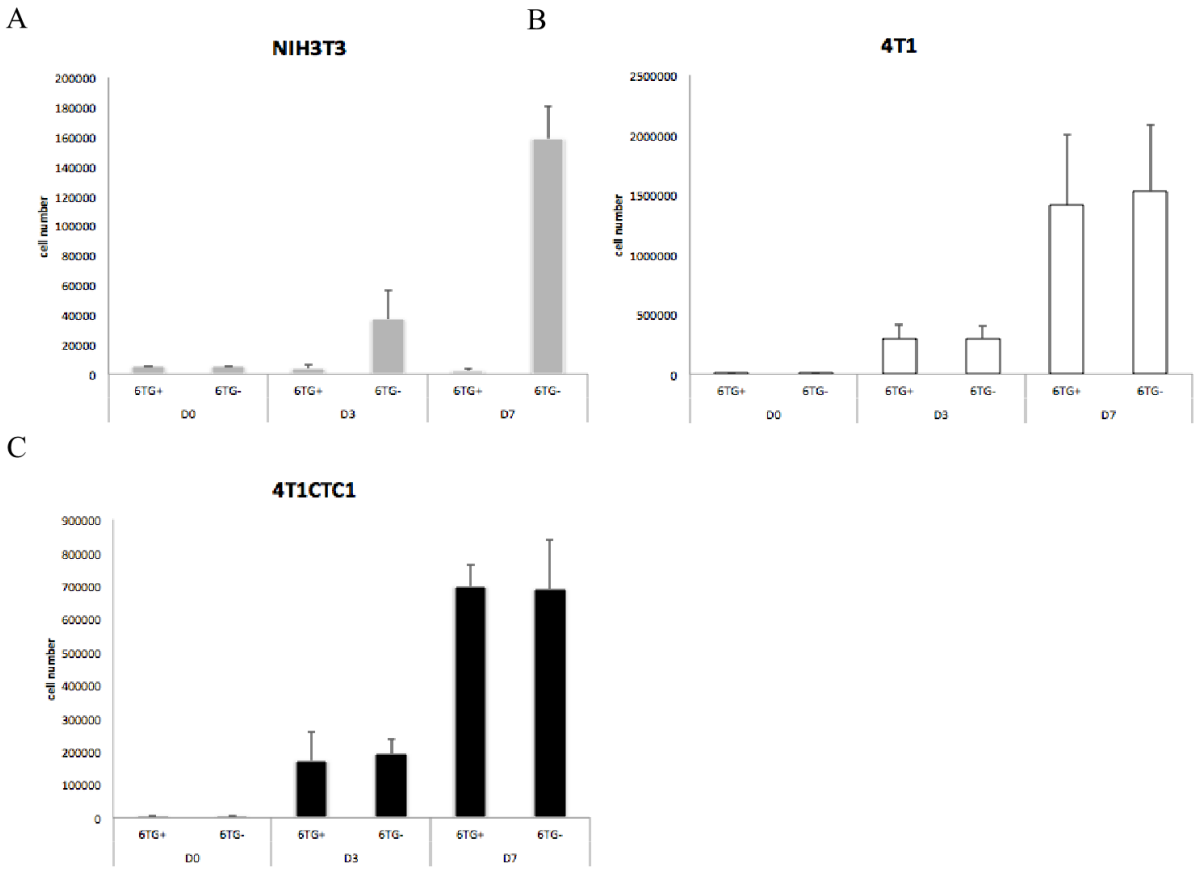


Figure 3.6 4T1CTC#1 cells are resistant to 6-thioguanine. 5000 cells were plated in 6-well plate at day 0, then cultivated with 2 ml of DMEM medium with or without 60 μM 6-Thioguanine. Cell numbers were counted at day 3 and day 7. NIH3T3 cells served as negative control (A). Results show that 4T1(B) and 4T1CTC#1 (C) can both grow in medium with or without 6-thioguanine.

4.4.3 Karyotyping of 4T1CTC#1 confirms their 4T1 origin

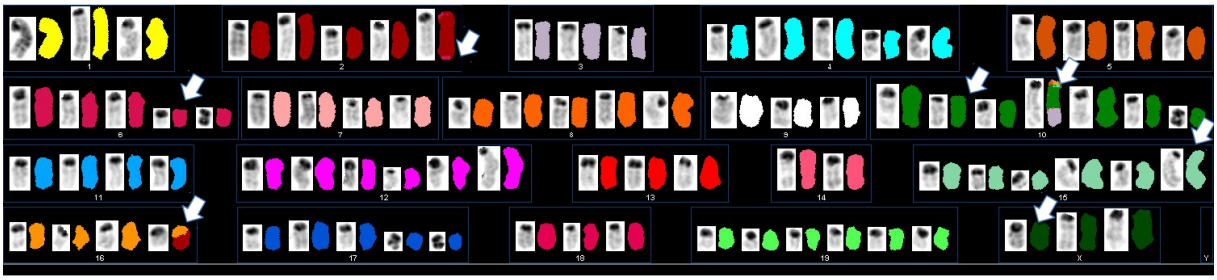
In order to confirm the origin of 4T1CTC#1 as 4T1 cells, spectral karyotyping was performed on both 4T1 and 4T1CTC#1 cell lines. Both cell lines were cultured and blocked in

metaphase through the application of chemical inhibitors of mitotic spindle formation, thus facilitating the detection of individual condensed chromosomes. After fixation, chromosomes of both cell lines were stained with DAPI (4',6-diamidino-2-phenylindole), then black and white pictures of chromosomes with bands were taken (Fig.3.7). The chromosomes were categorized according to banding patterns. Thereafter, the same cells were stained with chromosome-specific DNA probes conjugated with fluorophores. The hybridization of the DNA probes with chromosomes resulted in staining of each chromosome with identifiable color. Based on this process, translocations of chromosomes were effectively detected.

As shown in spectral karyotyping results of Fig.3.7, both 4T1 and 4T1CTC#1 demonstrated intense polyploidy, with a total of 86 and 74 chromosomes identified, respectively. The partial deletion of the X chromosome was identified in both cell lines. On chromosome 2 of both cell lines translocations from chromosome 6 to 2 were detected. Deletions on 2 of 5 copies of chromosome 6 of 4T1 were shown, similar deletion in 1 of 3 copies of chromosome 6 in 4T1CTC#1 could be visualized. On chromosome 15 of 4T1CTC#1, 1 of 5 copy of chromosome showed translocation of chromosome 15. This was also confirmed in 4T1. On the same chromosome of 4T1 3 of 6 displayed partial deletion and 1 of 6 in 4T1CTC#1 showed the same deletion pattern. In 4T1 cells translocation from chromosome 3 to 10 was found and 3 copies of chromosome 10 were partially deleted. On chromosome 16 of 4T1 translocation from 2 was demonstrated. And in most cases, 4T1CTC#1 had similar extra copies of each single chromosome as 4T1 cells, indicating 4T1CTC#1 as a derivative cell line of 4T1. On chromosome 2 of both cell lines translocations from chromosome 6 were detected. Meanwhile, partial deletions of chromosome 6 of both cell lines could be identified. Moreover, in both cell lines similar pattern of translations and deletions of chromosome 15 were demonstrated. Based on these observations, the tumor origin of 4T1CTC#1 was confirmed to 99%. Hence, this represents genetic evidence of the 4T1 origin of 4T1CTC#1

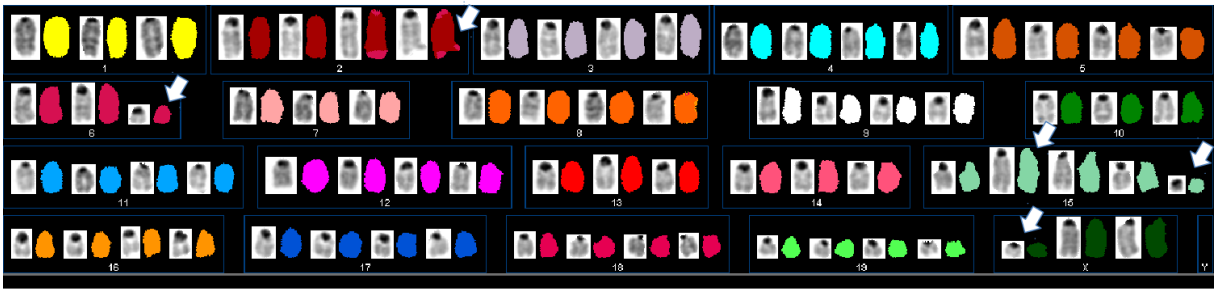
and confirms 4T1CTC#1 cells as circulating tumor cells isolated from mouse blood after the formation of 4T1 primary tumors.

4T1



86,XX,+del(X),mar(2)t(2;6),2xdel(6),mar(10)t(3;10),3xdel(10),mar(15)t(15;15),3xdel(15), mar(16)t(2;16)

4T1CTC



74,XX,+del(X),mar(2)t(2;6),del(6), 2xmar(15)t(15;15),del(15)

Figure 3.7 Karyogram results of 4T1 and 4T1CTC#1. A total of 86 chromosomes were detected in 4T1 cells (upper panel), in 4T1CTC#1 74 chromosomes were detected (lower panel). All the similar features were marked with white arrows.

4.5 Repression of EpCAM expression and partial EMT in 4T1CTC#1 cells

Breast cancer-associated CTC represent a potential source of metastatic cells and are therefore considered as intermediates between primary tumors and overt metastases. For the case of breast cancer, the actual presence of metastatic cells in a subpopulation of CTC was recently demonstrated for the first time. A $EpCAM^{+}$-$CD44^{+}$-$cMet^{+}$-$CD47^{+}$ subpopulation of CTC was

able to generate distant metastases after intrafemural injection into immunocompromised mice (Baccelli, Schneeweiss et al. 2013). Furthermore, CTC are a feature of systemic cancer that can easily be traced in patients´ blood and thus constitute a liquid biopsy for repetitive monitoring of disease progression (Alix-Panabières C 2013). As described above, one cell line, which was termed 4T1CTC#1, was isolated from mice blood and permanently cultured in vitro. In the following, the morphology and antigen expression profile of 4T1CTC#1 were analyzed.

The morphology of both 4T1 and 4T1CTC#1 is shown in Fig 3.3A. 4T1 cells displayed epithelial morphology and formed cell-cell contact (Fig 3.3Aa). However, 4T1CTC#1 showed a fibroblast-like phenotype and no cell-cell contact (Fig.3.3Ab). In flow cytometry analyses, 4T1CTC#1 did not express EpCAM on cell membrane while 4T1 cells expressed EpCAM intensively (Fig.3.3Ac,d). The lack of expression of EpCAM was also confirmed by immunoblotting (Fig.3.3B). In line with the observed loss of EpCAM expression at the plasma membrane and in whole cells lysates (Fig 3.3B), 4T1CTC#1 did not display any expression of EpCAM in immunohistochemistry staining of 2D-cultures (Fig 3.8b). This was true also at the transcriptional level as measured upon quantitative real-time PCR (Figure 3.9). In contrast, the majority of 4T1 cells consistently and strongly expressed EpCAM with only a minor fraction of cells lacking EpCAM (Figure 3.8a). Cytokeratin (CK) are intermediate filament proteins specifically expressed in epithelial cells. CK were highly expressed in both 4T1 and 4T1CTC#1 cells (Figure 3.8c-d), supporting the epithelial origin of both cell lines.

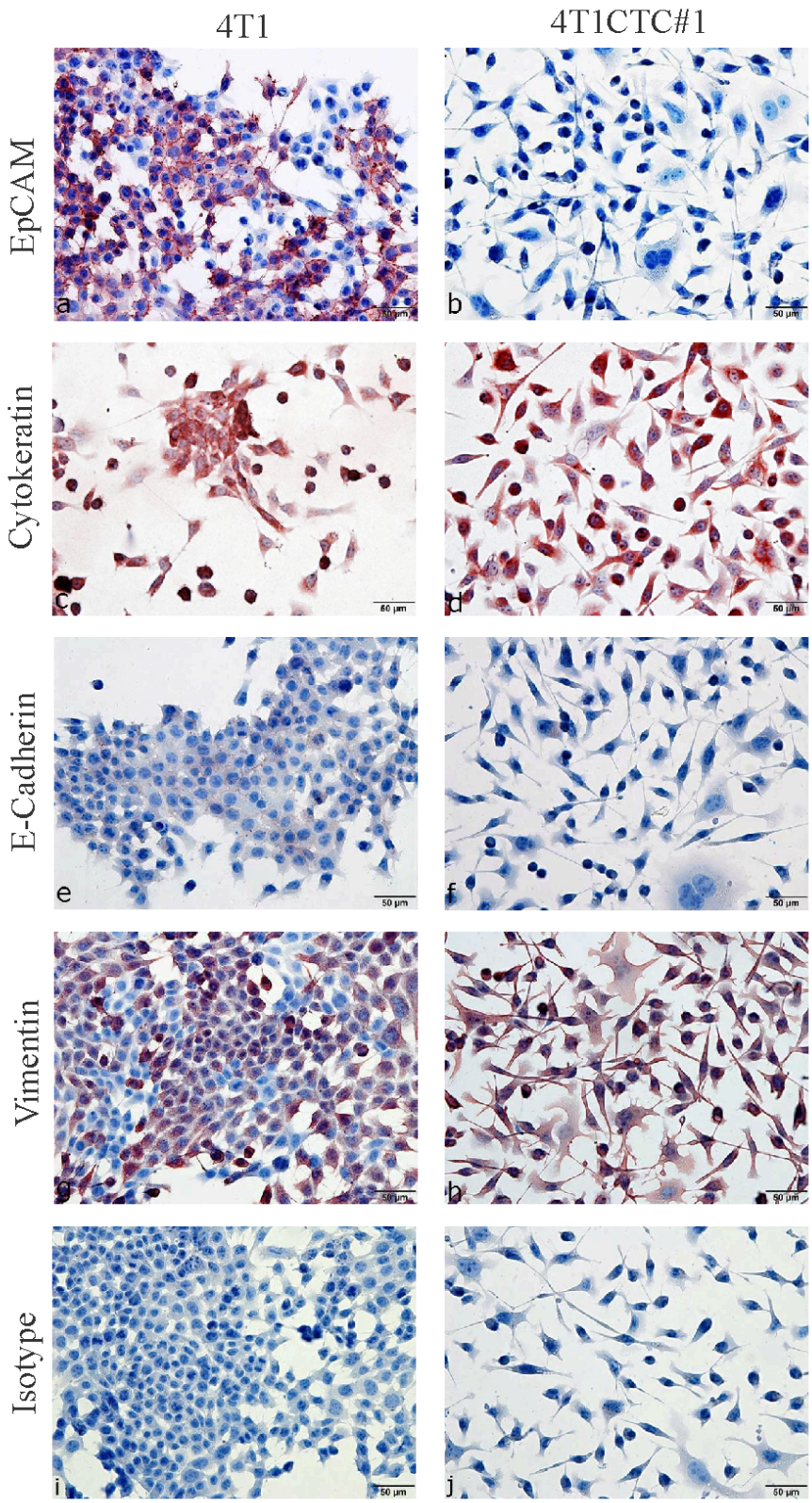


Figure 3.8 Immunohistochemistry staining of 4T1 and 4T1CTC#1. Both 4T1 and 4T1CTC#1 cells were cultivated on glass slides in quadriperm chambers until 50% confluence was achieved. Thereafter, cells were fixed and permeabilized before different antibodies were applied to detect protein expression profiles. In 4T1

cells (a), EpCAM expression is positive (shown in red color), while 4T1CTC#1 cells lack EpCAM expression (b). Both, 4T1 and 4T1CTC#1 cells express cytokeratins (c) (d) (shown in red color). E-cadherin is slightly positive in 4T1 (e) (shown in light red color), while it was absent in 4T1CTC#1 (f). Vimentin is heterogeneously expressed in 4T1 cells (g), whereas vimentin is intensively and homogeneously expressed in 4T1CTC#1 (h) (shown in red color). Isotype controls were always included for all staining, nucleus stained in blue (i) (j).

E-Cadherin is a classical marker and cell adhesion mediator on the plasma membrane of epithelial cells. Although the expression of E-Cadherin was not intensive compared to EpCAM, we observed a complete loss of E-cadherin in 4T1CTC#1 compared to parental 4T1 cells (Figure 3.8e-f).

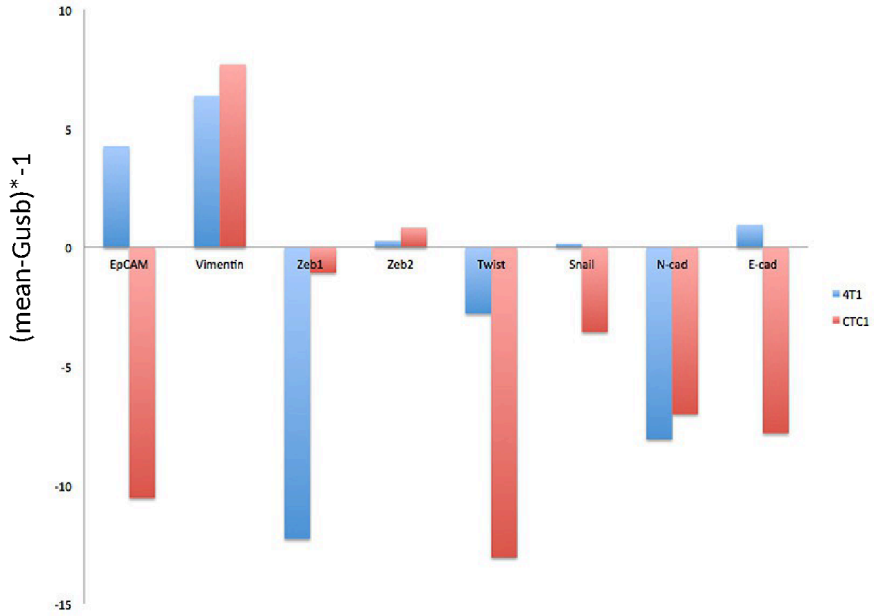


Figure 3.9 Relative mRNA expression level of EMT markers in 4T1 and 4T1CTC#1 cells. EpCAM expression was suppressed in 4T1CTC#1 at the transcriptional level. The expression of vimentin in both cell lines is positive. The expression of Zeb2 in 4T1CTC#1 is higher than 4T1. E-cadherin was expressed in 4T1 but not in 4T1CTC#1. There are no significant differences between 4T1 and 4T1CTC#1 in the expression of Zeb1, Twist, Snail, N-cadherin.

The similar pattern of E-cadherin was detected at the transcriptional level (Fig.3.9). Interestingly, mesenchymal marker vimentin was up-regulated in 4T1CTC#1 and expressed in

100% of cells as compared to 4T1 parental cells (60% positivity) (Figure 3.8g-h). The mRNA expression of vimentin in both 4T1 and 4T1CTC#1 cells were both intensive, however, 4T1CTC#1 showed a slight elevation compared to parental cells (Fig.3.9). Additional EMT markers such as Twist, Zeb1, Zeb2, Snail, N-Cadherin did not show significant differences between 4T1 and CTC derivatives (Fig.3.9).

4.6 4T1CTC#1 have enhanced migration capacity

As described, 4T1CTC#1 cells displayed mesenchymal features with a loss of EpCAM and E-cadherin expression, spindle shape and lack of cell-cell contact. Typically, EMT and the acquisition of a mesenchymal phenotype go along with increased migration. In order to study the migration capacity of 4T1 and 4T1CTC#1 cells, a wound-healing assay was performed in vitro. Both 4T1 and 4T1CTC#1 were cultivated in 6-well plates and a scratch was applied to confluent cultures following the protocol described in materials and methods. For this, similarly sized scratches were generated in 4T1 and 4T1CTC#1 cell monolayers as shown in Figure 3.10A. Over time, cells migrate into the open area, which represents a measure for their migration ability. After 48 hours, 4T1CTC#1 covered significantly more open area of the scratch, as visualized with black lines (Figure 3.10A). Calculation of migration velocity over time disclosed a two-fold increase in migration of 4T1CTC#1 as compared to parental 4T1 cells with comparable proliferation rates of both cell lines (Figure 3.10B-C).

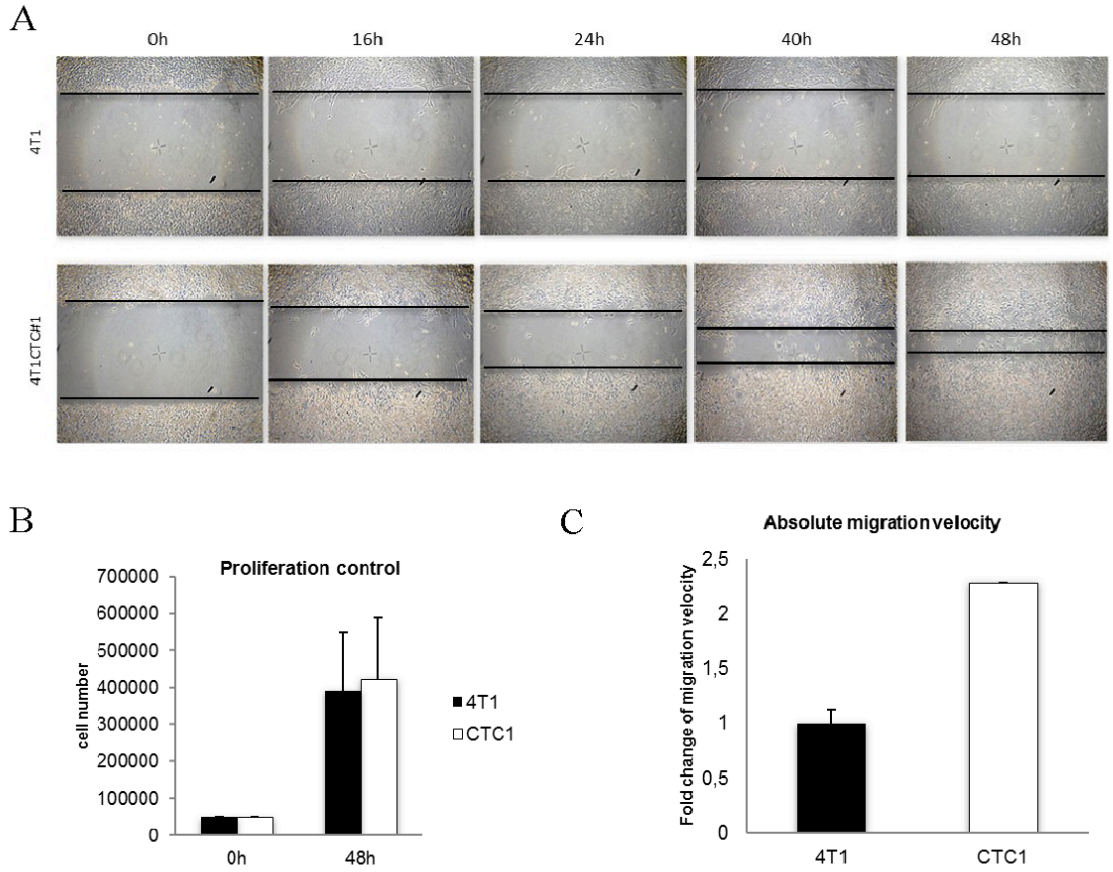


Figure 3.10 4T1CTC#1 cells migrate faster than parental 4T1 cells. A wound-healing assay was performed with 4T1 and 4T1CTC#1 cells. Cells were seeded in 6-well plate and cultured to achieve >80% confluence before starvation with serum free medium for 12-24 hours. Then, a single scratch was applied through the center of the well on the monolayer of cells using 200 µL sterile tips. Pictures were taken under phase contrast microscope at the indicated time points. The scratch areas were measured and calculated according to standard methods using Image J (A). Proliferation control was performed in parallel. No proliferation difference was seen between 4T1 and 4T1CTC#1 in the absence of FCS in the culture medium (B). 4T1CTC#1 is 2 fold more migratory compare to 4T1 cells (C).

4.7 4T1CTC#1 showed reduced tumorigenic capacity *in vivo*

In the following experiments, the tumorigenic potential of 4T1CTC#1 cells was assessed through syngeneic transplantation into Balb/c mice. Twenty-three days after subcutaneous inoculation of 4T1 and 4T1CTC#1 cells into the flank of mice, animals were sacrificed, tumors were removed and weighed. As showed in Fig.3.11, the mean value of tumor weight

in 4T1CTC#1 injected mice group was lower than in the 4T1-injected group. Of note, in 4T1CTC#1 group 2 mice failed to form tumors. However, 4T1 cells could generate tumors in all mice with a median weight of 369,5 mg.

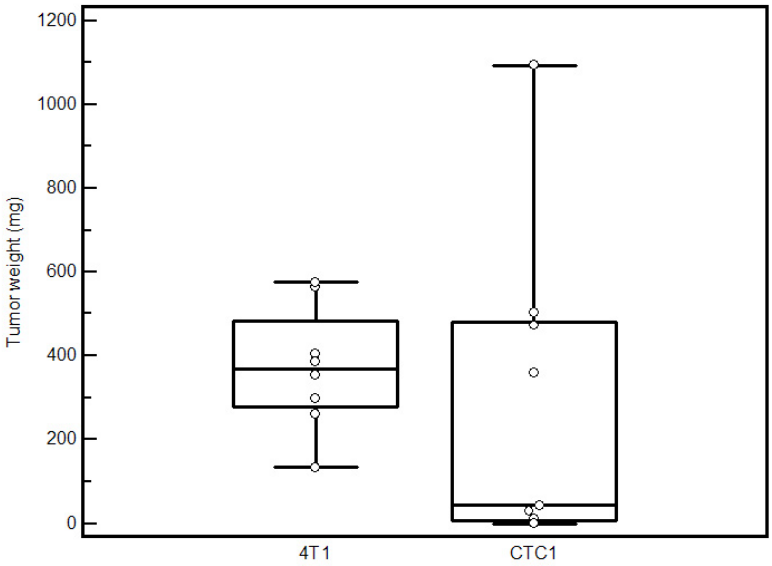


Figure 3.11 4T1 possess stronger tumor formation potential compared to 4T1CTC#1. 4T1 and 4T1CTC#1 cells were syngeneically transplanted into Balb/c in two independent experiments with 5 mice per group and experiment. Tumor cells were allowed to grow at least 23 days in vivo, then mice were sacrificed and primary tumors were excised and weighed. Shown is the tumor weight in box-plot-whiskers graphs from 4T1 and 4T1CTC#1 group. The median tumor weight of 4T1 group was 369,5 mg, while the median value of 4T1CTC#1 group was 42 mg.

4.8 4T1DTC#1 expressed EpCAM comparably to 4T1 primary tumor cells

In order to further investigate the expression of EpCAM in the course of the metastatic cascade, we isolated disseminated tumor cells of mice bone marrows after inoculation of 4T1 subcutaneously in the flank. This way, one permanent cell line 4T1DTC#1 was established in our lab. 4T1DTC#1 displayed epithelial morphology with tight cell-cell contact in low and high density culture conditions (Fig. 3.12 g,h).

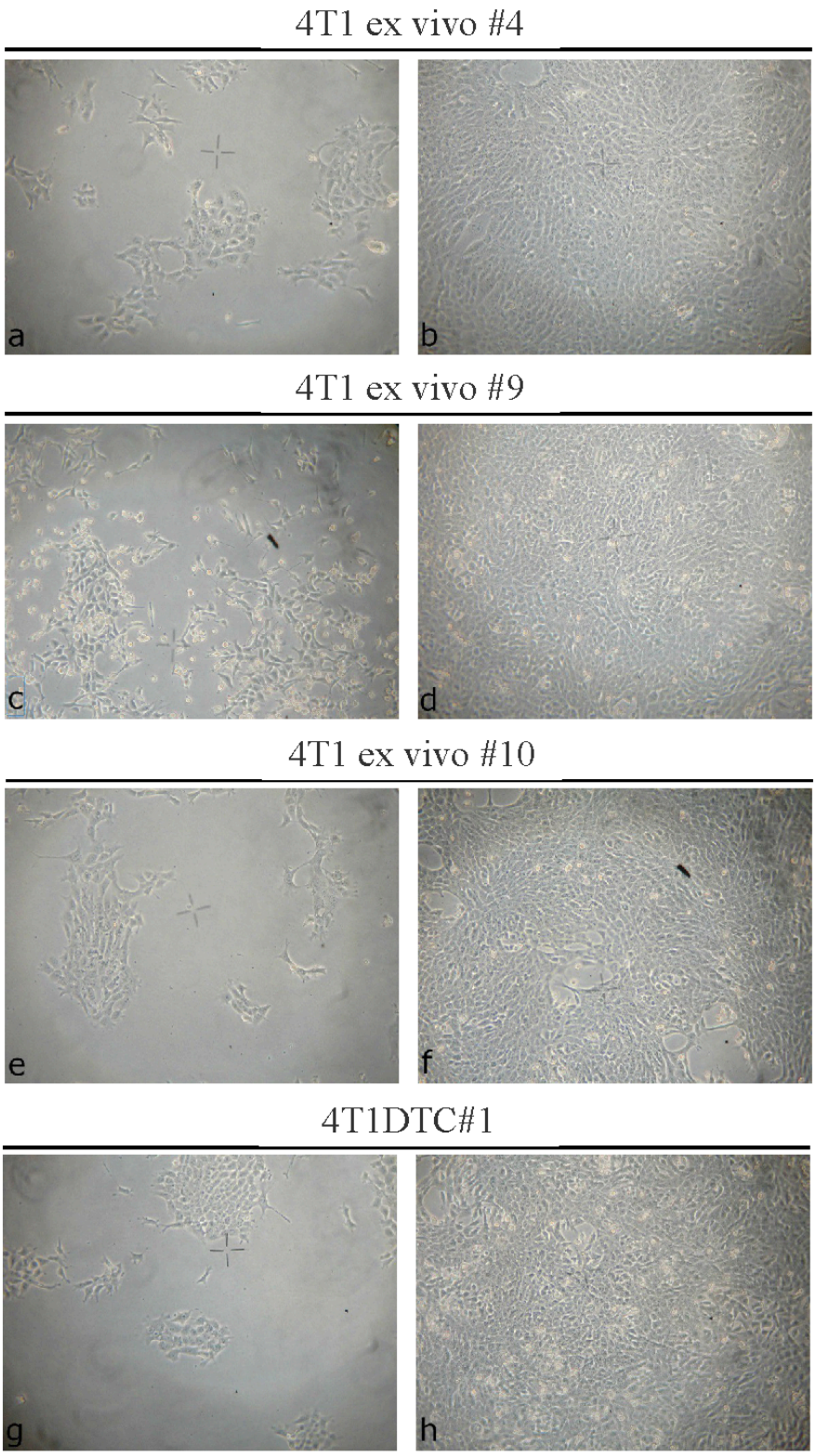


Figure 3.12 *Ex vivo* cultured tumor cells of 4T1 transplantation. 4T1 ex vivo #4, 4T1 ex vivo #9 and 4T1 ex vivo #10 were generated from primary tumors after syngeneic transplantations of 4T1 cells. 4T1 were injected subcutaneously on the flanks of Balb/c mice and tumor formed in vivo gradually. Primary tumors were excised

and ex vivo cell lines were established following the protocols described in materials and methods chapter. Pictures depicting the morphology of ex vivo cell lines at low (left panel a, c, e, g) and high density (right panel b,d f, h) in culture are shown. All cell lines including 4T1DTC#1 showed an epithelial phenotype and could form typical cobblestone-like monolayers in cell culture.

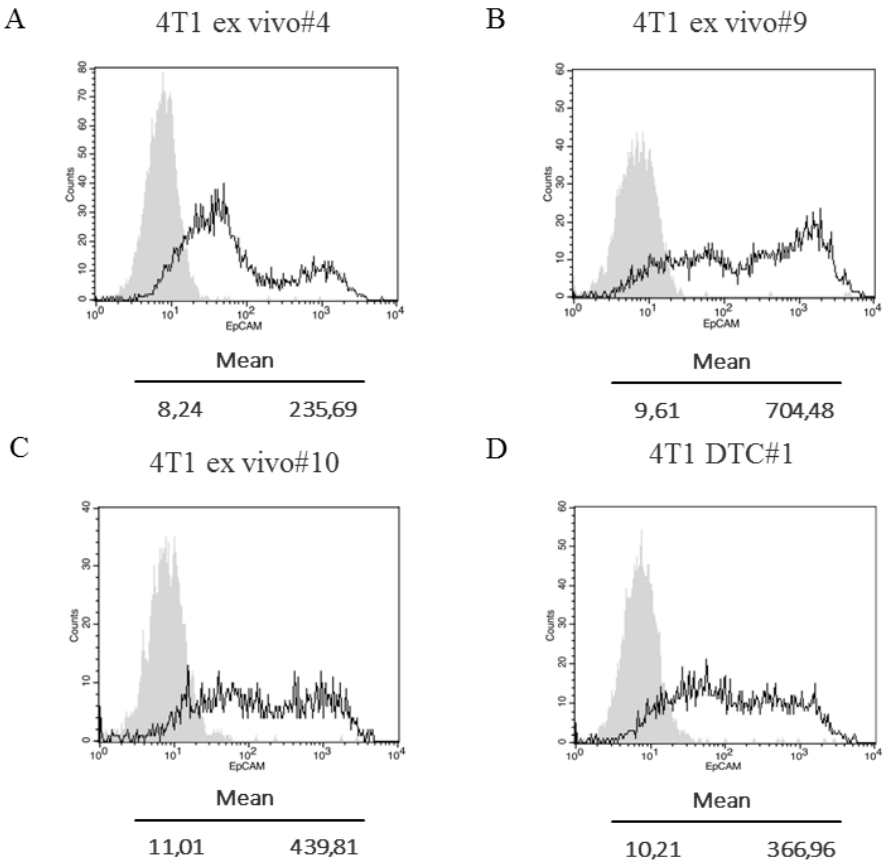


Figure 3.13 Flow cytometry analysis of EpCAM on ex vivo cultured cell lines. After plating tumor cells ex vivo, 4 permanent cell lines were established. In order to investigate the EpCAM expression status, flow cytometry detection of cell surface expression of EpCAM was performed on these cell lines. All 4 cell lines expressed EpCAM comparably to parental 4T1 cells (solid black histogram line)(A-D). The major fraction of the cell population was EpCAM positive and only a small portion revealed negative. 4T1 ex vivo#4 showed two major peaks of EpCAM expression (A), the main peak demonstrated a moderate EpCAM expression. Negative control was always included in all assays (grey closed histogram).

In addition, the EpCAM expression of 4T1DTC#1 on cellular membrane was detected via flow cytometry. EpCAM was moderately expressed by 4T1DTC#1 (MFI=366,96/10,21). Moreover, 3 permanent *ex vivo* cell lines (4T1 ex vivo #4, 4T1 ex vivo #9, 4T1 ex vivo #10) of 4T1 primary tumors were established following Mack *et al* (Mack, Eggert et al. 2013). These 3 primary tumor cell lines displayed the same morphology as parental 4T1 cells (Fig 3.12 a-f). The EpCAM expression of 3 primary tumor cell lines (MFI=459,99/9,62)(Fig.3.13) showed a similar pattern as 4T1DTC#1 (MFI=366,96/10,21).

4.9 Truncated human NGF receptor as a surrogate marker to increase the efficiency of CTC capturing in murine blood

In order to selectively enrich for 4T1 cells in the blood of transplanted mice, a truncated version of the human nerve growth factor NGF-R was stably transduced into parental 4T1 cells. The intracellular domain of NGF-R was shortened from a.a.273 to 427, so that signaling is abbrogated. Syngeneically injected 4T1 tumor cells can be distinguished from haematopoietic and other normal cells using NGF-Rtrunc as a marker using MACS or FACS sorting techniques. After transduction, expression of human NGF-Rtrunc was detected via flow cytometry (Fig. 3.14B). Additionally, the EpCAM expression was also positive on 4T1hNGF-Rtrunc cells (Fig.3.14A).

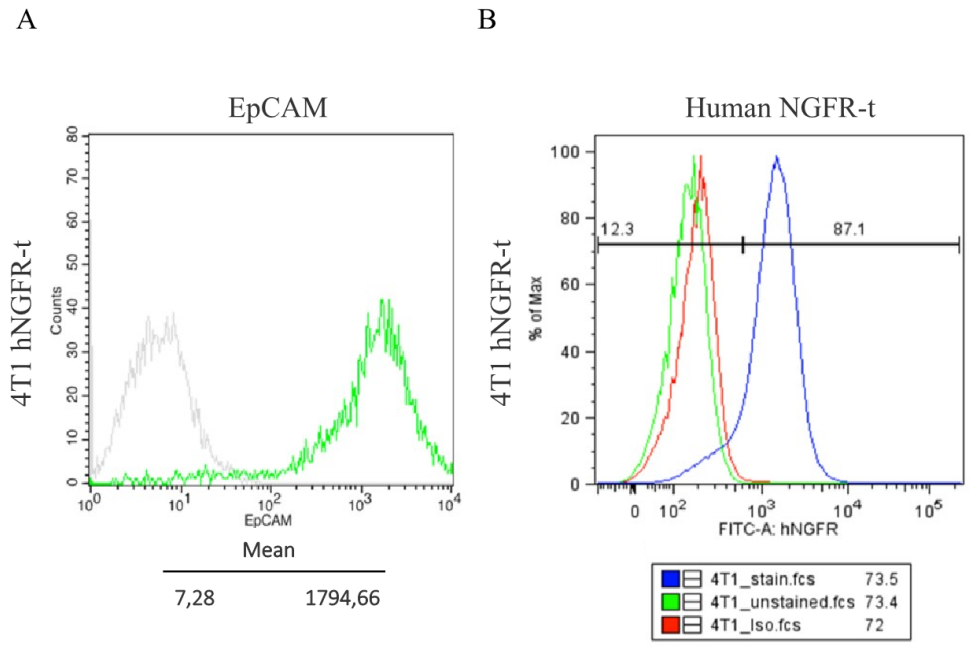


Figure 3.14 Truncated human NGFR as a surrogate marker was expressed in 4T1. The human NGF-Rtrunc was transduced into 4T1 cells. Cells stably expressing human NGF-Rtrunc were selected using puromycin in the culture medium. human NGF-Rtrunc protein expression on the cell surface was detected via flow cytometry. 87.1% of cell population were expressing human NGF-Rtrunc (B),and their EpCAM expression was positive (A).

5 DISCUSSION

Cancer originates from normal somatic cells in the human body and the majority cancer-related deaths are caused by ungovernable metastatic spread of dissociated malignant cells from primary tumor into distant organs. Normally, metastatic cancer cells reach the target organs through the blood stream, in which the circulating tumor cells (CTCs) are best characterized with respect to numbers and phenotype (Allard, Matera et al. 2004). It is believed that in order to form metastases, cancer cells have to detach from the primary tumor, intravasate into the blood stream and possess the ability to survive in an anchorage-independent manner in the circulation (Joosse, Gorges et al. 2015). Upon extravasation, CTCs get access to distant organs including lung lobes, adrenal glands, liver, brain, as well as bone marrow (Langley and Fidler 2011). Tumor cells present in distant organs, for instance in bone marrow, are not termed CTC anymore, but DTC for disseminating tumor cells (Masuda, Hayashi et al. 2016). Owing to their role in the colonization of distant organs, CTCs hold the key to better understand the cancer metastatic cascade. However, since CTCs are rare events in the blood (0 to 4 CTCs in 7,5ml blood) (Lalmahomed, Kraan et al. 2010), it is difficult to isolate them for further characterization. The amount of CTCs was demonstrated to be approximately one to few CTCs in 1 million blood cells of cancer patients (Krebs, Metcalf et al. 2014). In addition, the short half-life (for mammary cancer cells between 1 and 2.4 hour) of CTCs made their detection even more challenging (Meng, Tripathy et al. 2004). In order to enrich rare CTCs from cancer patient blood samples, many platforms based on physical properties, including size, density, electric charges, deformability, and biological properties, such as surface protein expression, mostly EpCAM expression, have been developed (Pecot, Bischoff et al. 2011, Issadore, Chung et al. 2012, Pantel and Alix-Panabieres 2012). Among these CTC isolation technologies, the CellSearch® system (Veridex LLC, Raritan, NJ, USA),

which is the only one cleared by US Food and Drug Administration (FDA), relies on EpCAM as anchor molecule for CTC enrichment, cytokeratins and 4',6-diamidino-2-phenylindole (DAPI) positivity and CD45 negativity. However, the presence of EpCAM-negative CTCs was reported as well (Punnoose, Atwal et al. 2010, Konigsberg, Obermayr et al. 2011, Gorges, Tinhofer et al. 2012). Hence, utilizing EpCAM-based techniques for capturing CTCs is not sufficient to enrich and cover the whole CTC population. Therefore, subsequent analyses may have biases. Since the expression pattern of EpCAM on CTCs is still under investigation, attention must be paid in the case of applying EpCAM-based platforms to isolate CTCs.

The cell surface molecule EpCAM, which is frequently expressed in carcinomas, was previously proposed to be involved in homophilic cell adhesion (Litvinov, Velders et al. 1994). Thereafter, the functions of EpCAM in regulation of proliferation were described by our group and others (Munz, Kieu et al. 2004). Its role in promoting proliferation might answer why frequent overexpression of EpCAM is observed in most carcinomas. Later on, it was demonstrated that EpCAM was downregulated during migration *in vitro* in esophagus carcinoma cell models. Additionally, EpCAM downregulation was observed in most of DTCs from cancer patients in the same study (Driemel, Kremling et al. 2014). This implies that EpCAM may not be persistently expressed on migrating malignant cells. In order to adapt to altered survival circumstances during cancer metastatic cascades, EpCAM downregulation might be necessary for metastatic cancer cells. Since EpCAM might not be persistently expressed during the cancer metastatic cascade, the whole picture of EpCAM expression pattern and function during cancer progression reveals rather unclear. Therefore, we have established cell lines from a murine breast carcinoma model, including 4T1CTC#1, 4T1DTC#1, 4T1 ex vivo #4, 4T1 ex vivo #9, 4T1 ex vivo #10, and described the expression of EpCAM in these cell lines.

5.1 EpCAM on 4T1CTC#1 was suppressed

We used the 4T1 murine carcinoma cell line to investigate expression patterns of EpCAM in a metastasizing breast cancer animal model. From the photographic visualization of cultivated 4T1 cell monolayers, we could see that the major population of 4T1 cells displayed an epithelial phenotype with typical squamous-like morphology. A minority of single fibroblastic-like 4T1 cells could be observed as well. Moreover, EpCAM was heterogeneously expressed as was demonstrated upon flow cytometry measurements and immunohistochemical staining. A subset of 4T1 cells lacked expression of EpCAM or displayed very low levels, however, the main population expressed EpCAM strongly (Fig 3.1). These results were confirming that EpCAM was heterogeneously expressed on the cell membrane of 4T1 cells in Fig 3.8a. Consistent with our results, another group demonstrated the same EpCAM expression profiles in 4T1 cells, with 95% of 4T1 cells being EpCAM positive (Guixin Shi 2013). In contradiction, it was demonstrated by Hiraga and colleagues that the major population of 4T1 cells were EpCAM negative (Hiraga, Ito et al. 2016). Moreover, they published that the EpCAM-positive 4T1 subpopulation, which was recognized as the cancer stem cell subgroup, could change into EpCAM-negative phenotype after 7 passages in culture. However, our colleague Anna found that the EpCAM-negative 4T1 cells slowly switched into EpCAM-positive cells during 2 months cultivation (Data not shown). 4T1 cells were subsequently transplanted into immune-competent Balb/c mice. Expression of EpCAM *in vivo* was detected via IHC on primary tumors, metastases, as well as disseminated tumor cells in different organs (Fig 3.2). However, the expression pattern of EpCAM on CTCs, which are believed to be critical intermediates between primary tumor and metastasis, needs to be addressed. Therefore, CTCs from transplanted mice were isolated and grown permanently in vitro. These cells were termed 4T1CTC#1 and showed a strong

mesenchymal morphology (Fig 3.3Ab), with a loss of EpCAM expression (Fig 3.3Ad). Immuno-blotting of 4T1CTC#1 confirmed that EpCAM expression was suppressed (Fig 3.3B). Based on the suppression of EpCAM and E-Cadherin, and the upregulation of vimentin in 4T1CTC#1, we could conclude that 4T1CTC#1 underwent an EMT program. In line with our finding, it was shown that CTCs captured in colon cancer patients blood displayed EpCAM and E-Cadherin negativity (Satelli, Mitra et al. 2015). Vimentin expression of 4T1CTC#1 was more intensive and uniform compared to 4T1 cells (Fig 3.8h). In accordance with these findings, it was reported by others that CTCs from different types of cancers underwent EMT. For example, Yu and colleague reported that CTCs expressed TGF-ß pathway components and FOXC1 transcription factor, which were evidences of EMT in CTCs. Satelli and colleagues showed that cell-surface vimentin was detected in EMT CTCs with 84-1 monoclonal antibody, which was associated with progressive disease of colon cancer patients (Yu, Bardia et al. 2013, Satelli, Mitra et al. 2015). A similar result was reported with respect to TGFß induced EMT in esophagus carcinoma cells, with an up-regulation of N-Cadherin and vimentin (Driemel, Kremling et al. 2014). Moreover, in the same study, downregulation of EpCAM via siRNA dependent techniques in esophagus carcinoma cells led to trancriptional upregulation of vimentin. In line with our findings, Zhang et al. reported on the generation of three CTC lines from breast cancer patients´ peripheral blood. Interestingly, all three cell lines were EpCAM negative at both translational and transcriptional level (Zhang, Ridgway et al. 2013). Moreover, they have also confirmed vimentin up-regulation at both, the translational and transcriptional level. Indeed, EpCAM negativity on CTCs was reported by other groups as well recently. EpCAM-negative CTCs, which were not enriched upon CellSearch system, could be identified by filtration and fluorescent labeling (de Wit, van Dalum et al. 2015). Moreover, it was found that the $EpCAM^{low}$ subpopulation of squamous carcinoma cell (SCC) exhibited a mesenchymal

morphology and elevated expression of mesenchymal markers such as vimentin and Twist, and suppression of epithelial marker E-Cadherin (Biddle, Liang et al. 2011). Further reports confirming our results in a breast cancer model were published by Santisteban and colleagues (Santisteban, Reiman et al. 2009). They found that in breast cancer cell lines, in which EMT was induced, EpCAM and E-Cadherin were suppressed, whereas N-Cadherin and Snail expression were elevated.

Techniques to differentiate CTCs from hematopoietic cells were based on EpCAM positivity of carcinoma cells. In 2010, a chip with herringbone structure and micro-posts coated with EpCAM antibody on the surface to capture CTCs in cancer patients' blood was published for the first time (Stott, Hsu et al. 2010). However, EpCAM-negative CTCs were reported recently (de Wit, van Dalum et al. 2015). It is conceivable that EpCAM positive and EpCAM negative CTCs co-exist in the circulation system of cancer patients. The rationale behind this might be that not all CTCs undergo EMT before they start to metastasize and that CTCs with varying phenotypes might be generated within the blood through high plasticity of these cells. Another explanation according to Joosse is that there are two ways how CTCs could enter into blood or/ and lymph vessel system, namely, a passive way and an active way (Joosse, Gorges et al. 2015). Active intravasation of tumor cells of into the circulation system requires a 'preparation' before shedding from primary tumor nest. For instance, tumor cells need to lose some features that are inhibitory to migration such as adhesion, and gain some other traits that are supportive of motility. One mechanism believed to facilitate phenotypical changes towards more migratory traits is EMT, which also occurs during embryonic development (Lee, Dedhar et al. 2006). The passive route of intravasation relies on a mechanical push of primary tumor cells into vessels, whereby tumor cells might be passively entering into the blood stream in form of microemboli, maintaining epithelial features of CTCs. At present, it is not possible to discriminate the mode of intravasation of 4T1 cells in the animal model used

herein. However, 4T1 cells retrieved from mouse blood displayed a high degree of EMT-based phenotypic changes, supporting a central role of EMT in the metastatic cascade, along with a loss of EpCAM.

5.2 Loss of EpCAM in 4T1CTC#1 led to enhanced migration ability *in vitro*

In a wound-healing assay, we observed that 4T1CTC#1 migrated faster compared to parental 4T1 cells (Fig. 3.10C). In line with this result, former members of our research group found that down-regulation of EpCAM in esophagus carcinoma cells led to enhanced tumor cell migration activity (Driemel, Kremling et al. 2014). In the same study, one esophagus carcinoma cell population was sorted via FACS for differing EpCAM expression levels into two subpopulation, namely, $EpCAM^{high}$ and $EpCAM^{low}$. Again, the $EpCAM^{low}$ subgroup demonstrated enhanced migration ability with reduced proliferation. More recently, our group reported on the enhanced vimentin expression and loss of EpCAM at the migration front in scratch assays (Tsaktanis, Kremling et al. 2015). Indeed, it was shown by Biddle and colleagues that $EpCAM^{low}$ cancer stem cells in head and neck squamous carcinomas displayed elevated migratory capacity compared to $EpCAM^{high}$ subgroup (Biddle, Liang et al. 2011). Similarly, in human cancer cell lines, when EMT endows the cells with mesenchymal features to locomote, transient EpCAM downregulation enables migration of cancer cells (Jojovic, Adam et al. 1998). One explanation for this observation, including our own data, would be that EpCAM is suppressed via EMT programs, which is hypothesized to endow cancer cells with migratory and invasive properties (Thiery, Acloque et al. 2009). It is imaginable that when tumor cells gradually lose EpCAM on the cell membrane, cell-cell contact between neighbor cells may be disrupted due to this alternation. Indeed, in Figure 3.8 we could see 4T1CTC#1 showing the fibroblastic like morphology with E-Cadherin

repression. Hence, mesenchymal tumor cells could leave from the main tumor population taking advantage of this feature.

Oppositely, it was found that transient down-regulation of EpCAM via siRNA techniques resulted in reduced migration and invasion capacities of breast cancer cells in vitro (Osta, Chen et al. 2004). It was reported that EpCAM could weaken the expression of one of the major adhesion molecule E-cadherin (Litvinov, Balzar et al. 1997). Therefore, it was proposed that EpCAM overexpression may actually promote cancer metastasis through this mechanism (Trzpis, McLaughlin et al. 2007). The cancer metastasis-promoting role of EpCAM was also confirmed by animal experiment by Wuerfel and colleagues. They demonstrated that overexpression of EpCAM in fibrosarcoma led to formation of metastasis in the lung (Wurfel, Rosel et al. 1999). Moreover, down-regulation of EpCAM via siRNA in renal and breast carcinoma cells impeded the migration of tumor cells. (Seligson, Pantuck et al. 2004). The somewhat contradicting finding that EpCAM overexpression promotes tumor cell migration in vitro could be explained by its growth-promoting function. The intracellular domain of EpCAM, EpICD stimulates tumor cells to divide (Maetzel, Denzel et al. 2009), leading to a quick expansion of tumor cells. The proliferation advantage of EpCAM-positive tumor cells might simulate increased migration via cells occupying spare spaces of cell culture surfaces through division. In order to preclude such misleading results, migration assays should preferably be performed in the absence of growth factors and should include growth control curves. By doing so, we did not see differences in growth capacities of 4T1 and 4T1CTC#1 after serum deprivation. Thus, we believe that loss of EpCAM expression rather induces migration.

5.3 Loss of EpCAM in 4T1CTC#1 associates with impaired tumorigenesis *in vivo*

Next, 4T1 and 4T1CTC#1 cells were transplanted into immune-competent Balb/c mice to test the tumor formation capacity *in vivo*. 4T1 generated bigger tumors in vivo compared to 4T1CTC#1 (Fig 3.11). Probably 4T1 tumor cells were benefit from the possible generation of EpICD, the intracellular domain of EpCAM, through regulated intramembrane proteolysis of EpCAM, which initiates an active growing signal (Maetzel, Denzel et al. 2009). Comparably, suppression of EpCAM expression by shRNA in esophageal carcinoma cell lines led to reduced tumor growth *in vivo* compared to EpCAM-positive cells (Driemel, Kremling et al. 2014).

In line with these findings, EpCAM-positive hepatocellular carcinoma stem cells efficiently formed tumours in immune-compromised mice compared to EpCAM-negative cells (Yamashita, Ji et al. 2009). Several studies have shown similar results related to the over-expression of EpCAM that associates with cell proliferation (Munz, Kieu et al. 2004, Maetzel, Denzel et al. 2009, Wenqi, Li et al. 2009, Chaves-Perez, Mack et al. 2013). Induction of EpCAM activates oncogenic transcription factor c-myc, subsequently up-regulates cell cycle related genes such as Cyclin A and E, as well as epidermal fatty acid binding protein (Munz, Kieu et al. 2004, Munz, Zeidler et al. 2005). According to our previous lab data, the EpICD was the key mediator of inducing c-myc upregulation. EpCAM has been also demonstrated its direct effect on cyclin D1 (Chaves-Perez, Mack et al. 2013).

In contrast to our observations, down-regulation of EpCAM via siRNA in ovarian cancer cells did not show an inhibitory effect on cell growth (van der Gun, Huisman et al. 2013). In this cell line probably EpCAM was not a major driver of proliferation, and the inhibition effect of proliferation mediated by EpCAM down-regulation could be rescued by other proliferative signaling pathways. Moreover, it was found that overexpression of EpCAM in CT-26 murine colorectal carcinoma cells induced inhibitory effects on tumor cells proliferation *in vivo*

(Basak, Speicher et al. 1998). Actually, demonstration of a growth-inhibitory role of EpCAM remains rare and potential molecular mechanisms underlying this inhibition are unclear.

5.4 EpCAM was expressed in DTCs

Disseminated tumor cells (DTCs) are released from primary tumors, which can travel through circulation system and localize in bone marrow and other organs. Bone marrow is a frequent homing destination for breast, prostate and lung cancer metastatic cancer cells (Pantel and Alix-Panabieres 2014). Interestingly, it was also reported that patients with small primary tumor already had bone marrow-resident DTCs, which indicated the possibility of a parallel growth of primary tumor and cells of the metastatic cascade (Klein 2009). In some cases, people claimed that DTCs are sources of new micro-metastasis (Massard, Loriot et al. 2011). Recently, it was described that DTCs in bone marrow could be used as a liquid biopsy resource that can guide cancer therapy and provide helpful information of prognosis for cancer patients (Pantel and Alix-Panabieres 2014). Clinical analysis of a large cohort of 4703 breast cancer patients demonstrated that presence of DTCs in bone marrow was associated with poor prognosis (Braun, Vogl et al. 2005). Therefore, DTCs in bone marrow may be another important study target for cancer biologists and oncologists.

In our investigation, we established a permanent DTC cell line from bone marrow of 4T1-injected mice that was termed 4T1DTC#1, which showed an epithelial phenotype and EpCAM positivity in FACS analysis. EpCAM positive DTCs in both lymph nodes and spleen of mice were detected via IHC staining (Fig.3.2). In these findings, the similarities between primary tumor and 4T1DTC#1 were observed, including EpCAM expression and cell morphology as opposed to 4T1CTC#1. In line with our results, it was found that EpCAM-positive DTCs in lymph nodes were detected in 8 (18.2%) of the 44 cancer patients, which

was significantly associated with tumor recurrence (Dhayat, Sorescu et al. 2012). A subset of DTCs with EpCAM expression in bone marrow were found in breast cancer patients (Woelfle, Breit et al. 2005). The presence of EpCAM on DTCs might contribute to their survival, cell-cell communication, and proliferation. Indeed, this point is supported by the fact that $EpCAM^{high}$ DTCs in bone marrow of esophageal carcinoma patients is strongly associated with the presence of locoregional of lymph node metastases and poor overall survival of patients (Driemel, Kremling et al. 2014).

6 CONCLUSION

In the present thesis, we have used an animal model to explore the expression pattern of EpCAM throughout 4T1 breast cancer progression. Primary tumors and metastatic niche could be established in 3 to 4 weeks, which allowed us to perform animal studies in a relative time saving manner. Another advantage relies in the presence of an intact immune system, which allowed us to mimic processes of breast cancer metastatasis formation in patients most closely. Thus, the animal model in use provides valuable data supporting cancer investigation in a relevant system. Using this animal model, we established several cell lines, which originated from the parental 4T1 line, including one circulating tumor cell line (CTC). 4T1CTC#1 cells were lacking the common leucocyte marker CD45, and did not express p53 to relevant levels. Karyotyping of this cell line confirmed 4T1CTC#1 as descendants of 4T1 cells.

Epithelial cell adhesion molecule is a carcinoma-associated tumor marker with a functional role in cell-cell adhesion and mitogenic signaling. EpCAM was strongly expressed in the majority of 4T1 cells, whereas EpCAM expression was persistently suppressed in 4T1CTC#1. Interestingly, 4T1 parental cells displayed an epithelial phenotype with the expression of E-Cadherin. Oppositely, 4T1CTC#1 did not express E-Cadherin, but elevated levels of vimentin along with a mesenchymal morphology. In vitro wound healing analysis showed that 4T1CTC#1 migrate faster compared to parental 4T1 cells. After re-transplantation of 4T1CTC#1 into Balb/c mice, 4T1CTC#1 generated tumors with lower efficiency and decreased tumor weight compared with parental 4T1 cells. Moreover, one disseminated tumor cell line (DTC) was isolated from the bone marrow of 4T1 mice. This cell line termed 4T1DTC#1 demonstrated an epithelial phenotype and EpCAM positivity. This finding

indicated a dynamic expression pattern of EpCAM during cancer progression. Final experiments of my thesis have set the basis for a standardized isolation of CTCs and DTCs in the 4T1 mouse model. To this aim, a truncated version of human NGF-R (neuro-growth factor receptor) was cloned into the PMXs-puro retrovirus vector, which later was transduced into 4T1 cells. The signaling defective NGF-R receptor is expressed at the cell surface of transgenic 4T1 cells and can be used for subsequent enrichment strategies.

Taken together, parental 4T1 and *ex vivo* 4T1 cell lines expressed the tumor antigen EpCAM to high levels. However, EpCAM expression was repressed in circulating tumor cells, which were isolated from the blood of 4T1-injected mice and had undergone a partial EMT program. Disseminated 4T1 tumor cells from the bone marrow of transplanted cells mice displayed a restored expression of EpCAM along with an epithelial phenotype. Hence, EpCAM expression is dynamic in the 4T1 mouse model of breast cancer progression, which might relate to the function of EpCAM throughout the various phases of the metastatic cascade.

ZUSAMMENFASSUNG (German summary)

Tumormetastasen sind die Haupttodesursache bei Patienten mit einer Krebsdiagnose. Dank stetig fortschreitender Technologien gibt es inzwischen eine Vielzahl von Therapiemöglichkeiten, angefangen bei der chirurgischen Resektion von Tumoren, über Chemotherapie und Strahlentherapie bis hin zur antikörperbasierten Immuntherapie. Trotzdem haben viele Tumoren noch immer eine schlechte Prognose und geringe Überlebensraten aufgrund von Metastasen, die häufig inoperable und therapieresistent sind. Um neue Therapiemöglichkeiten zu finden bemühen sich viele Wissenschaftler um ein besseres Verständnis der Pathophysiologie von Tumoren, das heißt zu verstehen, wie sich Tumorzellen aus dem Zellverband lösen, durch den Blutstrom an andere Körperstellen gelangen, die Blutgefäße wieder verlassen und Metastasen bilden.

EpCAM ist ein Typ I Transmembranglykoprotein mit einem Molekulargewicht von ca 40kD. Anfangs wurde EpCAM die Funktion eines Adhäsionsmoleküls zugeschrieben. Dann wurde in verschiedenen Tumoren eine EpCAM-Überexpression beschrieben. Dort konnte es als diagnostischer Marker und therapeutisches Zielmolekül verwendet werden. Da EpCAM ein homophiles Zelladhäsionsmolekül ist, könnte man schlussfolgern, dass eine hohe EpCAM Expression eine Metastasierung verhindert. Das Gegenteil zeigte sich aber bei Patienten mit Tumorerkrankungen. EpCAM-Überexpression in Primärtumoren korrelierte mit einer ungünstiger Prognose. Eine mögliche Erklärung für diesen Widerspruch wäre, dass EpCAM die Expression von E-Cadherin reprimiert, das dadurch als wichtiges Adhäsionsmolekül fehlt und somit die Metastasierungskaskade angestoßen werden kann. Zudem wurde gezeigt, dass EpCAM proteolytisch gespalten wird und daraufhin die intrazelluläre Domäne EpICD in den Kern transloziert wird, wo es Onkogene wie c-myc, Zyklin D1 und A reguliert. Außerdem

wird inzwischen die Funktion der Zell-Zell Adhäsion von EpCAM in Tumorzellen angezweifelt.

Zur Isolierung zirkulierender Tumorzellen (CTC) aus dem Blut wurde die Cellsearch Methode verwendet. Diese Methode macht sich Eisen-Nanopartikel zunutze, die von Biotin-Analoga ummantelt sind und mit Anti-EpCAM Antikörpern konjugiert sind. In letzter Zeit häufen sich kritische Stimmen, die beanstanden, dass diese Methode es nicht ermögliche EpCAM-negative CTCs zu isolieren, die malignes Potential innehaben könnten. Deshalb ist es wichtig das Expressionsmuster von EpCAM in der gesamten Metatstasierungskasakde, CTC und DTCs eingeschlossen zu untersuchen und dadurch Leitlinien für die Thearpie und Diagnostik von Tumoren zu formulieren.

In dieser Arbeit wurde anhand eines murinen Mamma-Karzinommodells gezeigt, dass der Primärtumor EpCAM positiv, die im Blut zirkulierenden Tumorzellen dagegen EpCAM negativ waren. Die aus dem Blut isolierte Tumorzelllinie 4T1CTC#1 zeigte einen eindeutigen mesenchymalen Phänotyp und einen Verlust der EpCAM Expression. Im Vergleich zu parentalen 4T1 Zellen, weisen 4T1CTC#1 Zellen eine erhöhte Migrationsfähigkeit auf. Aus dem Knochenmark isolierten wir eine weitere permanente Tumorzelllinie (4T1DTC#1), die EpCAM exprimiert und, ähnlich den parentalen 4T1 Zellen, einen epithelialen Phänotyp aufweist. Zusammenfassend bestätigen meine Daten eine dynamische Expression von EpCAM im Verlauf der Tumorprogression, insbesondere in zirkulierenden Tumorzellen im Blut, welche als Quelle metastasierender Zellen gelten.

APPENDIX

ABBREVATIONS

°C	Celsius degree
A	adenine
aa	amino acids
APS	ammoniumpersulfate
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CK	cytokeratin
CTCs	circulating tumor cells
ddH_2O	double distilled water
DMEM	Dulbecco`s Modified Eagle Medium
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotidtriphosphate
DTCs	disseminated tumor cells
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EMT	epithelial to mesenchymal transition
EpCAM	epithelial cell adhesion molecule
EpICD	intracellular domain of EpCAM
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
g	acceleration of gravity
G	guanine
h	hour
H_2O	water
IHC	immunohistochemistry
KH_2PO_4	potassium dihydrogen phosphate
KCl	potassium chloride
kDa	kilo Dalton
l	litre
M	molar
mA	milli ampere
max	maximal
mg	milligram
µg	microgram
MET	mesenchymal to epithelial transition
min	minute
ml	millilitre
µl	microlitre
mM	millimolar
µM	micromolar
mRNA	messenger RNA

NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
OD	optical density
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PFA	paraformaldehyde
qRT-PCR	quantitative Real Time PCR
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
T	thymidine
TEMED	N,N,N`,N`-Tetramethylendiamin
V	volt
WB	western blot
Δ	delta

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PUBLICATIONS

During my doctorate study I was able to contribute the following publication.

Tsaktanis T, Kremling H, Pavšič M, von Stackelberg R, Mack B, Fukumori A, Steiner H, Vielmuth F, Spindler V, **Huang Z**, Jakubowski J, Stoecklein NH, Luxenburger E, Lauber K , Lenarčič B, Gires O: Cleavage and cell adhesion properties of human epithelial cell adhesion molecule (HEPCAM). *J Biol Chem* 2015 Oct 2;290(40):24574-91.

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