NOTE TO USERS

A TUMORIGENIC ROLE FOR HUMAN GLYCOPHOSPHATIDYL INOSITOL-ANCHORED CARCINOEMBRYONIC ANTIGEN FAMILY MEMBERS CEA AND CEACAM6 *IN VIVO*

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THESIS ABSTRACT

Human carcinoembryonic antigen (CEA), a well-known clinical tumor marker, belongs to the CEACAM family of cell surface intercellular adhesion molecules that represent a subset of the immunoglobulin superfamily. The glycophosphatidyl inositol-anchored family members, CEA and CEACAM6, are over-expressed in as many as 70% of all human cancers. This correlation suggested their role in tumor progression, which was supported by extensive results obtained with several systems *in vitro* and *ex vivo*. But their contribution and relevance *in vivo* remains uncertain without further validation. Since mice do not possess homologs of the CEA and CEACAM6 genes, transgenic mice harboring a 187 kb portion of the human CEACAM family gene locus contained in a bacterial artificial chromosome (CEABAC) that includes CEA, CEACAM3, CEACAM6, and CEACAM7 genes were constructed in this study. The spatiotemporal expression pattern of these genes is very similar to that in humans. The expression levels of these genes are gene dosage dependent. Moreover, these CEABAC mice are more susceptible to develop carcinogen-induced colon tumors and spontaneous lung tumors. At low to moderate expression levels of CEA/CEACAM6, a partial block in cell differentiation and a mild to moderate hyperproliferation were evident in the transgenic colon; however, these mice develop normally. At higher or tumor-like expression levels, a complete block in cell differentiation, an extreme hyperproliferation and an inhibition of apoptosis were observed. These mice showed reduced survival, growth retardation and chronic diarrhea, and showed massively enlarged colons comprising continuous non-focal cytological and architectural abnormalities, including a dysplastic and serrated adenomatous morphology, by only 3 months of age. These results suggested that, while moderate expression levels of CEA/CEACAM6 cause an imbalance of tissue homeostasis leading to increased tumor susceptibility, tumor-like expression levels alone produce a severe imbalance leading directly to tumor formation. In sum, these results provide direct evidence for the important role of CEA/CEACAM6 in cancer development. These CEABAC mice also provide an *in vivo* model system for studies of other cancers and infectious diseases involving human CEACAM family genes and for pre-clinical trials of CEACAM-based therapy.

RÉSUMÉ DE THÈSE

Le *carcinoembryonic antigen* (CEA) est un marqueur tumoral bien connu et le chef de file d'une famille de molécules de l'adhésion intercellulaire (CEACAM) appartenant à la superfamille des immunoglobulines. Deux de ces membres, CEA et CEACAM6, ancrés à la membrane par un lien *glycophosphatidyl inositol*, sont surexprimés dans presque 70% de tous les cancers humains. Cette corrélation suggère leurs rôles dans le développement du cancer qui est supporté par des résultats obtenus dans les systèmes *in vitro* et *ex vivo*. Cependant, leurs contributions et pertinence dans le système *in vivo* ne sont pas évidentes à ce jour. Puisque les souris ne possèdent pas d'homologue de CEA et de CEACAM6, des souris transgéniques ont été construites dans cette étude avec une portion (187 kb) du locus humain de la famille de CEACAM (CEABAC) contenant les gènes de CEA, CEACAM3, CEACAM6, et CEACAM7. Leurs profils d'expression ressemblent beaucoup à ceux présents chez l'homme. Quant à leurs niveaux d'expression, ils sont directement corrélés avec le nombre de copies du CEABAC intégrées. De plus, les souris CEABAC sont prédisposées plus à développer des tumeurs colorectales provoquées par les carcinogènes ainsi que des tumeurs pulmonaires spontanées. À un niveau d'expression modéré de CEA/CEACAM6, une réduction de la différentiation cellulaire et une augmentation modérée de prolifération cellulaire sont évidentes dans les côlons transgéniques, bien que ces souris se développent normalement. À un niveau d'expression plus élevé et semblable à celui d'une tumeur, on observe une absence de la différentiation cellulaire, une prolifération extrêmement élevée et une inhibition de l'apoptose. Une réduction de la longévité, un retard de croissance et une diarrhée chronique ont également été observés. À l'age de 3 mois, ces souris développent des côlons beaucoup plus larges, composés d'anomalies cytologiques et architecturales, incluant des dysplasies et des structures festonnés. Les résultats suggèrent que l'expression modérée de CEA/CEACAM6 cause un déséquilibre de l'homéostasie menant à la prédisposition à développer des tumeurs. Par contre, l'expression élevée de CEA/CEACAM6 est suffisante pour produire un déséquilibre sévère menant directement à la formation de tumeurs. En conclusion, ces résultats démontrent l'importance du CEA/CEACAM6 dans le développement du cancer. Les souris CEABAC peuvent également servir de modèle *in*

vivo pour des études relative à d'autres cancers et infections impliquant la famille des CEACAMs humaines. Ce modèle pourrait également servir à des études de thérapies précliniques.

PREFACE

This thesis includes published and submitted original research articles formatted in accordance to the "Guidelines for Thesis Preparation" provided by the Faculty of Graduate and Postdoctoral Studies. The relevant section states the following:

As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: 1) a table of contents; 2) a brief abstract in both English and French; 3) an introduction which clearly states the rational and objectives of the research; 4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); 5) a final conclusion and summary; 6) a thorough bibliography; 7) Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

6. When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

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8. In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis.

CONTRIBUTIONS OF AUTHORS

All work described in this thesis is entirely my own.

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Chapter 1

CEACAM Family Genes:
Our knowledge after four decades of research

1. Perspectives

The human carcinoembryonic antigen (CEA) was first discovered as a *tumor specific antigen* in circulating blood of patients with colon cancer in 1965 (Gold and Freedman 1965). Since then, CEA has become a widely used clinical marker for various cancers (Fuks et al. 1975; Gold et al. 1978; van Nagell et al. 1978; Shuster et al. 1980; Sikorska et al. 1988; Lamerz 1992; Ballesta et al. 1995; Macdonald 1999; Kim et al. 2000; Ebeling et al. 2002; Molina et al. 2003) and a popular molecular target for novel cancer therapies (Chester et al. 2000; Fong et al. 2001; Koch et al. 2001; Berinstein 2002; Burke et al. 2002; Kousparou et al. 2002; Goldenberg 2003; Marshall 2003) due to its consistent over-expression in as many as 70% of all human cancers, including those in colon, breast and lung (Chevinsky 1991; Ballesta et al. 1995; Jantscheff et al. 2003). With such an overwhelming correlation with cancer and the many potential applications, CEA was studied by many research groups over the past decades. After the cloning of CEA cDNA in 1986, CEA was shown to function as an intercellular adhesion molecule (Benchimol et al. 1989). Other CEA-related cell adhesion molecules (CEACAMs) were also identified in humans, monkeys, cattle, rats, and mice (Hammarstrom et al. 1998; Zimmermann 1998; Beauchemin et al. 1999; Tobi et al. 2000; Zhou et al. 2001; Kammerer et al. 2004b; Naghibalhossaini and Stanners in preparation). During the past two decades, physiological and pathological roles of this large gene family, mainly in cancer, infectious diseases, immunology, and diabetes, have been gradually elucidated in different model systems. Although the growing knowledge on the functions of CEACAMs has already been beneficial to clinical practice and therapy, further understanding of their mechanism of action in associated diseases and discovery of their participation in other diseases may have greater impact on both clinical and biological sciences in the future.

2. CEACAM Family Glycoproteins: Structure, Expression and Functions

2.1. Structures of CEACAM family glycoproteins

The CEACAM family members are highly glycosylated proteins that belong to the immunoglobulin gene superfamily (Paxton et al. 1987). In humans, the CEACAM family

glycoproteins are divided into two subgroups, the CEA- and the PSG (pregnancy specific glycoprotein)-subgroups, based on sequence homology. Family members of the CEA-subgroup are membrane-linked glycoproteins, which are composed of a V-like Ig amino-terminal (N)-domain followed by a variable number (between 0 and 6) of I-like Ig internal domains (named A1, B1, A2, B2, A3 or B3) and are anchored to the cell surface either by a glycophosphatidyl inositol (GPI)-anchor or a transmembrane domain (Fig. 1). The GPI-anchored members include CEA, CEACAM6 (formerly NCA), CEACAM7 (formerly CGM2), and CEACAM8 (formerly CGM6). The transmembrane members include CEACAM1 (formerly BGP), CEACAM3 (formerly CGM1) and CEACAM4 (formerly CGM7). Family members of the PSG-subgroup (PSG1 to PSG11) are secretory glycoproteins that are composed of a V-like N-domain followed by a variable number (between 0 and 3) of I-like Ig domains (Thompson et al. 1991; Hammarstrom et al. 1998).

In mice and rats, the basic classification mentioned above can be applied with a few exceptions: 1) GPI-anchored members are absent in the CEA-subgroup; 2) secretory members are present in the CEA-subgroup; and 3) secretory family members (both CEA- and PSG-subgroups) usually contain more than one N-domains (Zimmermann 1998). In mice, the CEA-subgroup is composed of two transmembrane members (CEACAM1 and CEACAM2) and three secretory members (CEACAM9, CEACAM10 and CEACAM11); the PSG-subgroup consists of eight secretory members (PSG16, PSG17, PSG18, PSG19-4. PSG21-4, PSG22-4, PSG23-4, and PSG28). In rats, the CEA-subgroup is composed of one transmembrane member (CEACAM1) and four secretory members (CEACAM9, CEACAM10, CEACAM11, and CEACAM12); the PSG-subgroup consists of eight secretory members (PSG36, PSG37, PSG38-4, PSG39. PSG40, PSG41-4, PSG42-4, and PSG43-4).

The CEACAM gene families are also present in other mammalian species, such as non-human primates and cattle. In cattle, transmembrane CEACAM1 has recently been identified (Kammerer et al. 2004b) and other CEACAM family members, such as PSGs, may be present as well. As in humans, monkeys possess GPI-anchored CEACAMs, transmembrane CEACAM1 and PSGs (Tobi et al. 2000; Zhou and Hammarstrom 2001; Zhou et al. 2001; Naghibalhossaini and Stanners in preparation). Thus far, GPI-anchored CEACAMs have been detected in primates, but not in lower mammals.

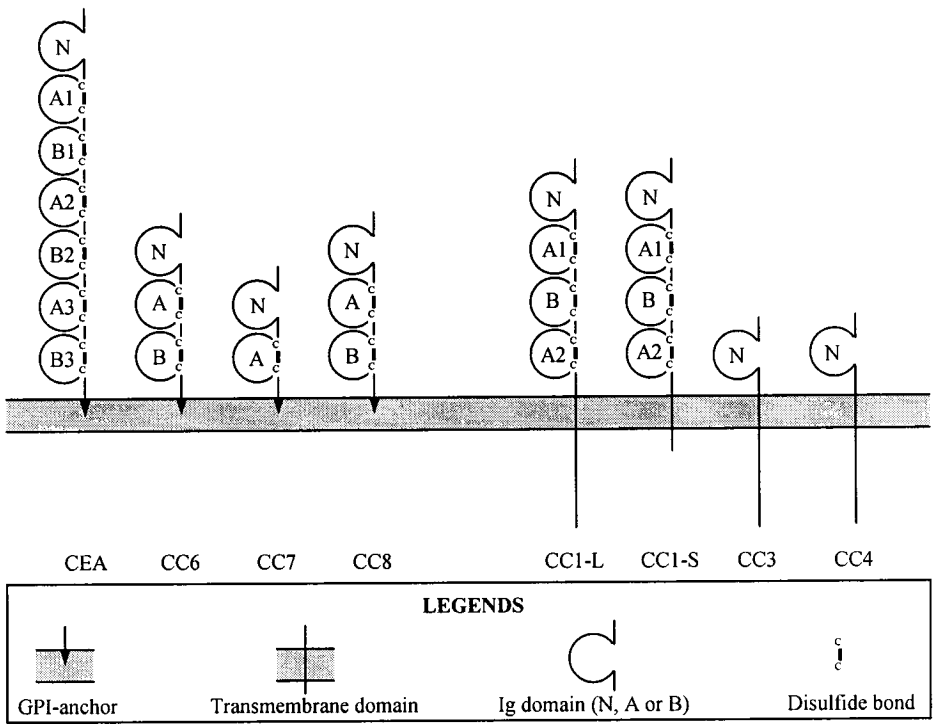

Figure 1: Structures of human CEACAM family glycoproteins. GPI-anchored family members (CEA, CC6 (CEACAM6), CC7 (CEACAM7), and CC8 (CEACAM8)) and transmembrane family members (CC1 (CEACAM1), CC3 (CEACAM3) and CC4 (CEACAM4)) are shown. Note that long form and short form of CC1 are shown as CC1-L (with long cytoplasmic tail) and CC1-S (with short cytoplasmic tail), respectively. Note also that different N-glycosylation sites are not shown.

2.2. Expression pattern of CEACAM family glycoproteins

Each CEACAM family member has a distinct tissue-specific expression pattern which varies during different stages of development. In healthy human adults, the expression of CEA is mostly found in columnar epithelial cells and mucus-secreting cells of the colon (Nap et al. 1988; Jothy et al. 1993; Baranov et al. 1994; Frangsmyr et al. 1999). CEA can also be found at a lower level in secretory epithelial cells present in the stomach and small intestine (Nap et al. 1988; Kinugasa et al. 1998), in squamous epithelial cells of the tongue, esophagus and cervix (Nap et al. 1988), in secretory epithelial and duct cells of the sweat glands (Metze et al. 1996), in epithelial cells of the prostate (Nap et al. 1988), and in transitional epithelial cells of the urinary bladder (Nap et al. 1988). Expression of CEA in lung, breast and pancreas is still controversial. The expression pattern of CEACAM6 is somewhat broader but generally similar to that of CEA with a few exceptions. CEACAM6 is not expressed in small intestine, but in duct cells of the breast and pancreas, in pneumocytes and bronchiole epithelial cells of the lung, and in myeloid cells of the bone marrow, spleen and many other tissues (Kuijpers et al. 1992; Kuroki et al. 1992a; Scholzel et al. 2000). CEACAM1 has the broadest expression pattern. CEACAM1 is expressed in epithelial cells of the colon, small intestine, liver, gallbladder, pancreas, kidney, and prostate (Frangsmyr et al. 1995; Prall et al. 1996; Frangsmyr et al. 1999), in squamous epithelial cells of the cervix (Prall et al. 1996), in glandular epithelial cells of the esophagus and endometrium (Prall et al. 1996), in transitional epithelial cells of the urinary bladder (Prall et al. 1996), in secretory epithelial cells of the sweat and sebaceous glands (Metze et al. 1996), in duct cells of the breast (Prall et al. 1996; Riethdorf et al. 1997; Huang et al. 1998), in endothelial cells in many organs (Prall et al. 1996), in Sertoli cells of the testis (Lauke et al. 2004), and in myeloid and lymphoid cells (Moller et al. 1996; Prall et al. 1996). In contrast, CEACAM7 is highly restricted to columnar epithelial cells of the colon and duct cells of the pancreas (Scholzel et al. 2000); CEACAM3, CEACAM4 and CEACAM8 are expressed only in granulocytes (Kuijpers et al. 1992; Kuroki et al. 1992a; Nagel et al. 1993). PSGs are expressed mostly in the placenta during fetal development (Streydio and Vassart 1990; Lei et al. 1992) and hematopoietic cells (Wu et al. 1993).

Table 1: Tissue-specific expression of human CEACAM family members

Tissues	CEA	CEACAM6	CEACAM7	CEACAM8	CEACAM1	CEACAM3	CEACAM4
Tongue	+	+	–				
Salivary Gland		+	–				
Esophagus	+	+	–		+		
Stomach	+	+	–		–		
Small Intestine	+	–	–		+		
Colorectum	+	+	+	–	+	–	–
Liver	–	–	–		+		
Gallbladder	–	+	–		+		
Pancreas	±	+	+		+		
Kidney	–	–	–		+		
Lung	±	+	–		–		
Endometrium	–	–	–		+		
Cervix	+	+	–		+		
Breast	±	+	–		+		
Prostate	+	+	–		+		
Testis	–	–	–		+		
Urinary Bladder	+	–	–		+		
Sweat Gland	+	+	–		+		
Myelocytes	–	+	–	+	+	+	+
Lymphocytes	–	–	–	–	+	–	–
Endothelium	–	–	–	–	+	–	–

± denotes expression is uncertain. Blank space indicates expression is not determined.

In mice and rats, the expression pattern of CEACAM1 is very similar to that of human CEACAM1 (Zimmermann 1998). CEACAM2, which is only present in mice, has a more restricted expression pattern than CEACAM1. It is expressed in colon, macrophages, spleen, liver and kidney (Nedellec et al. 1994; Robitaille et al. 1999). CEACAM9-12 and PSGs are mostly expressed in placenta during fetal development (Ogilvie et al. 1990; Chen et al. 1992; Rudert et al. 1992; Rebstock et al. 1993; Earley et al. 1996; Finkenzeller et al. 1997; Finkenzeller et al. 2000; Kataoka et al. 2000; Finkenzeller et al. 2003). In addition, CEACAM10 is expressed in the gastrointestinal tract, salivary gland and bone marrow (Keck et al. 1995).

During fetal development, human CEA is highly expressed in the developing gut and to a lesser extent in other endoderm-derived organs around 9-40 weeks of gestation (von Kleist et al. 1986; Nap et al. 1988; Benchimol et al. 1989). von Kleist et al. also showed its expression in other germ layers at the 7th week of gestation (von Kleist et al. 1986). However, little is known about the expression pattern of other human CEACAM family members in developing embryos and fetuses. In mice, mRNA transcripts of

CEACAM1 can be detected from 10.5 days post-coitum (p.c.) to birth and are present in gut, lung, meninges, bone, cartilage, endothelia, and placenta (Huang et al. 1990).

2.3. Functions of CEACAM family glycoproteins

2.3.1. Cell adhesion

Cell surface adhesion molecules are essential for cell-cell and cell-extracellular matrix (ECM) communication, which form the basis of many cellular functions within a multi-cellular organism, including tissue architectural organization and body morphogenesis. Depending on the specialty of individual cells, different sets of adhesion molecules can be expressed. In general, they can be grouped into two main categories. The first group of adhesion molecules binds to substrates or polymerized substrates, termed cell-substrate adhesion molecules (SAMs). The classical example is the integrin family of adhesion molecules. They are heterodimeric transmembrane glycoproteins consisting of different combinations of α and β subunits (Hynes 1992). Each combination has its own substrate specificity and affinity (Hynes and Lander 1992). For example, $\alpha_5\beta_1$ integrins recognize fibronectins, which are present in the ECM, usually in a polymerized state. This cell-substrate interaction can establish the orientation of the cells. The second group of adhesion molecules binds to other cell surface molecules on apposing cells, termed cell-cell adhesion molecules (CAMs). These cell-cell interactions are important for cell sorting during morphogenesis and maintenance of tissue architectural organization after morphogenesis. A classical example is the cadherin family of adhesion molecules (Munro and Blaschuk 1996). They are transmembrane glycoproteins that function as calcium-dependent homophilic adhesion molecules (i.e., binding to the same kind of cadherin molecules). They facilitate cell sorting during morphogenesis and their interactions maintain cell-cell junctions in the polarized epithelia.

The CEACAM gene family belongs to the second group of adhesion molecules (CAMs). In contrast to cadherins, most CEACAMs can mediate both homotypic and/or heterotypic intercellular adhesion through their extracellular domains (i.e., both the same kind and different kind of CEACAM molecules). While the adhesive property of CEACAM1 is dependent on temperature and Ca^{2+} concentration (Rojas et al. 1990; Turbide et al. 1991), binding of CEA and CEACAM6 is independent of temperature and

Ca^{2+} concentration (Rojas et al. 1990; Rojas et al. 1996). In normal polarized epithelia, the CEACAM family members are present primarily in the apical surfaces and hence cannot be involved in the maintenance of cell-cell junctions.

CEA can bind to another CEA molecule from adjacent cells by double reciprocal bonds between the N-domains and A3B3-domains in an anti-parallel fashion (Zhou et al. 1993a). It is believed that CEA can also bind to another CEA molecule on the same cell through a N-N domain interaction in a parallel fashion (Taheri et al. 2003). CEACAM1 and CEACAM6 can bind homotypically to themselves through N-N domain interactions (Rojas et al. 1990; Stanners et al. 1992; Teixeira et al. 1994; Watt et al. 1994; Rojas et al. 1996; Watt et al. 2001) and N-A1B1 domain interactions, respectively (Stanners and Fuks 1998). In addition, CEA, CEACAM1 and CEACAM6 can bind heterotypically to each other (Oikawa et al. 1989; Zhou et al. 1990; Oikawa et al. 1991; Oikawa et al. 1992) although the precise binding mechanism is still unknown. However, CEACAM8 is incapable of homotypic binding and can only bind heterotypically to CEACAM6 through interaction between their N-domains (Oikawa et al. 2000; Kuroki et al. 2001). Hence, heterotypic interactions between various CEACAM family members are likely dependent on N-domain binding, which are always present in different CEACAMs, but confirmation is required.

2.3.2. Cell differentiation

Cell differentiation is an essential process turning stem cells into specialized cells which carry out their proper functions in a multi-cellular organism. In the normal situation, expression of cell adhesion molecules is usually dependent on the differentiation state of the cells, i.e., the expression level varies during cell differentiation, and the developmental stage of the organism. For example, during embryogenesis, CEA, and probably CEACAM6, are expressed in undifferentiated cells of the developing gut at high levels (von Kleist et al. 1986; Nap et al. 1988). In contrast, in the normal adult, CEA and CEACAM6 are only expressed in differentiated cells, but much lower levels in stem cells, of the colon (Jothy et al. 1993; Ilantzis et al. 1997; Scholzel et al. 2000). In fact, based on the latter observation, CEA and CEACAM6 are considered as differentiation markers in many cell types. However, expression levels of CEA and CEACAM6 are inversely

correlated with the differentiation status of colon cancer cells, i.e., higher expression in less differentiated cells, which resemble the situation in embryonic cells (Ilantzis et al. 1997). This leads to a hypothesis that high expression of these adhesion molecules can inhibit cell differentiation in embryonic cells in order to maintain their dividing capacity and increase the stem cell pool during development; cancer cells take advantage of these benefits by over-expressing these molecules (Stanners 1998).

The GPI-anchored CEACAMs, CEA, CEACAM6 and CEACAM7 were in fact shown to block cell differentiation. In L6 rat myoblasts, over-expression of human CEA, CEACAM6 and CEACAM7, but not CEACAM1, was shown to completely inhibit myogenic differentiation (Eidelman et al. 1993; Rojas et al. 1996; Screaton et al. 1997; Zhai and Stanners in preparation). CEA expression in these cells leads to a reversible and viable G_0-like undifferentiated state (Screaton et al. 1997). Expression of CEA and CEACAM6, but not CEACAM1, inhibits colonocyte differentiation (Ilantzis et al. 2002). CEA was also shown to inhibit neurogenic and adipogenic differentiation (Stanners 1998). In contrast, transmembrane CEACAM1 was found to restore mammary cell differentiation in 3D cultures of mammary carcinoma cell lines (Kirshner et al. 2003a), showing an opposite effect to that of the GPI-anchored CEACAMs.

This differentiation block was originally thought to be dependent on the intercellular adhesive properties of the CEA extracellular domain since the ΔN-CEA mutant (which contains a deletion of a major portion of the N-domain) lacks intercellular adhesion properties (anti-parallel interactions) and does not block cell differentiation (Eidelman et al. 1993). Fine mapping of amino acid residues responsible for this effect using CEA mutants with point mutations showed that intercellular adhesion and cell differentiation can be independently blocked (Taheri et al. 2003). CEA subdomains within the N-domain were responsible, leading to the suggestion that CEA-CEA parallel interactions (between CEA molecules on the same cell) were required to block cell differentiation. This suggestion was supported by the observation that the differentiation blocking ability of ΔN-CEA could be restored with cross-linking antibodies (Taheri et al. 2003). Morevoer, the presence of specific GPI-anchor of CEA, which resides in specific membrane microdomains or lipid rafts, was shown to be required in differentiation block (Screaton et al. 2000). Altogether, these results suggested that differentiation block by

CEA is possibly through clustering of multiple CEA molecules on the cell surface and activate downstream signaling cascades (see cell signaling section below).

2.3.3. Cell death

Cells are constantly receiving both survival and death signals which are maintained in a fine balance. When death signals prevail, the cells will undergo apoptosis (programmed cell death), which is a suicidal process designed to remove unwanted cells within an organism. This is a critical process during embryonic development, normal tissue homeostasis/renewal and removal of abnormal cells, such as those with non-reparable DNA damage. If the latter cells escape the death pathway, they may become cancer cells.

Over-expression of CEA and CEACAM6, but not CEACAM1, was shown to inhibit apoptosis and anoikis (apoptosis of cells lacking proper anchorage to extracellular matrix) in L6 rat myoblasts, MDCK epithelial cells, human SW1222, Caco-2 and HT29 colorectal cancer cells, and human PANC1, capan2 and MiaPaCa2 pancreatic cancer cells (Ordonez et al. 2000; Soeth et al. 2001; Wirth et al. 2002; Duxbury et al. 2004a). In contrast, over-expression of CEACAM1 induces apoptosis of mammary carcinoma cell lines (Kirshner et al. 2003a). These findings showed that opposite effects can be mediated by the two groups of CEACAMs, as in cell differentiation. Hence, it is advantageous for the tumor cells to over-express CEA and CEACAM6 and to down-regulate CEACAM1, so that they can escape apoptosis.

2.3.4. Cell signaling

Many cell surface molecules, including receptors and adhesion molecules, can convey information from the milieu to the interior of the cells by means of signal transduction, so that the cells can respond to the changes in the environment. The CEACAM gene family of cell surface adhesion molecules is no exception. The transmembrane CEACAMs have alternate cytoplasmic tails. CEACAM1 has a long form (CEACAM1-L) and a short form (CEACAM1-S), each of which can affect a different set of downstream elements. The cytoplasmic domain of CEACAM1-L containing an ITIM (immunoreceptor tyrosine-based inhibitory motif) can associate with G-actin (Sadekova et al. 2000; Schumann et al. 2001), tropomyosin (Schumann et al. 2001), paxillin (Ebrahimnejad et al. 2000), SHP-1/2

(Beauchemin et al. 1997; Chen et al. 2001), Src family tyrosine kinases (Brummer et al. 1995; Skubitz et al. 1995), and integrin β_3 (Brummer et al. 2001); the shorter cytoplasmic domain of CEACAM1-S that lacks an ITIM can interact with F-actin and annexin II (Schumann et al. 2001; Kirshner et al. 2003b). Thus, they are both linked to actin cytoskeleton. However, CEACAM1-L has a modulatory effect on cell growth due to its association with either SHP-1/2 phosphatases (inhibitory) or Src family kinases (activating) depending on the cellular context (Obrink et al. 2002); CEACAM1-S does not have such effect, but can restore apoptosis in mammary carcinoma cells (Kirshner et al. 2003a). The growth inhibitory effect of CEACAM1-L *in vivo* depends on phosphorylation at serine 503, but not tyrosine 488 (Estrera et al. 2001). An opposite result showing that tyrosine 488 is critical for the growth inhibitory effect (Izzi et al. 1999) further supports the dual role of CEACAM1 in cell growth (Obrink et al. 2002). CEACAM3 has an ITAM (immunoreceptor tyrosine-based activating motif) that can interact with SH-2 containing kinases or adaptor proteins. Src family kinases are the major downstream elements of CEACAM3 and stimulation of Rac GTPase (membrane recruitment and increased GTP loading) and PI3-Kinases (increased production of phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3-phosphate) were also observed (Booth et al. 2003; Schmitter et al. 2004).

While signal transduction mediated by the transmembrane CEACAMs is characterized to some extent, that mediated by the GPI-anchored CEACAMs is less understood. Nevertheless, Screaton et al. suggested that the presence of CEA-specific GPI-anchors, which reside in specific membrane microdomains (lipid rafts), is essential for their cellular functions, at least as inhibitors of cell differentiation (Screaton et al. 2000). Ordonez et al. further suggested that perturbation of integrin functions and integrin-ECM interactions may be the underlying mechanism responsible for their inhibitory effect on anoikis (Ordonez et al. 2000). Unpublished data from our laboratory has shown that co-clustering of cell surface lipid raft-associated CEA/CEACAM6 and integrin $\alpha_5\beta_1$ leads to increased fibronectin binding to the cells and recruitment of downstream signaling elements, including ILK, Src family kinases, PI3K, and Akt/PKB (Camacho-Leal and Stanners in preparation; Ordonez et al. submitted manuscript). A similar observation was made by another group involving CEACAM6 and integrin $\alpha_v\beta_3$

in pancreatic adenocarcinoma cell lines (Duxbury et al. 2004c). Altogether, the GPI-anchored CEACAMs can mediate cell signaling through modulation of integrin functions.

2.3.5. Systemic functions

Human CEACAMs serve as cellular receptors for *Escherichia coli* (Sauter et al. 1991; Berger et al. 2004), *Neisseria gonorrhoeae*, *Neisseria meningitides* (Virji et al. 1996; Bos et al. 1997; Chen et al. 1997; Gray-Owen et al. 1997ab; Bos et al. 1998; de Vries et al. 1998; Virji et al. 1999), *Moraxella catarrhalis* (Hill and Virji 2003), *Haemophilus influenzae* (Virji et al. 2000); mouse CEACAM1 is a cell receptor for murine hepatitis virus (MHV) (Williams et al. 1991; Dveksler et al. 1993; Nedellec et al. 1994; Blau et al. 2001; Hemmila et al. 2004). These pathogens express surface glycoproteins, i.e., fimbriae, bacterial adhesins (Afa/Dr, Opa, Opc), ubiquitous surface protein (Usp), P5 protein, and MHV spike protein, that interact with the N-domains of different CEACAMs (Sauter et al. 1991; Dveksler et al. 1993; de Vries et al. 1998; Hill et al. 2001; Hill and Virji 2003; Berger et al. 2004). In most cases, these interactions allow the pathogens to infect the host, either gaining entry to the host cells or passing through the barrier of polarized epithelial cells with tight junctions. In some cases, these pathogens can be engulfed and eliminated by the CEACAM-expressing phagocytes through similar molecular interactions (Sauter et al. 1991; Billker et al. 2002; McCaw et al. 2004; Schmitter et al. 2004).

CEACAMs are involved in immune regulation of lymphocytes. In the intestinal epithelium, CEA present on epithelial cells can bind directly to CD8 on a subset of intestinal intraepithelial lymphocytes, CD8+ suppressor T-cells, and stimulate their proliferation (Mayers and Stanners in preparation). Absence of this activation and its resultant immune suppressive effect may cause inflammatory bowel disease (Toy et al. 1997). CEACAM1 was found to be an important regulator in T-cells (Morales et al. 1999; Nakajima et al. 2002; Chen and Shively 2004; Iijima et al. 2004), B-cells (Greicius et al. 2003), NK cells (Markel et al. 2002), and dendritic cells (Kammerer et al. 2001). It was shown to be upregulated after lymphocyte activation and to inhibit functions of the activated lymphocytes and further activation of lymphocytes thereafter. It also stimulates

maturation of dendritic cells and leads to induction of the Th1 immune response (Kammerer et al. 2001; Iijima et al. 2004).

Stimulation of granulocytes markedly increases surface expression of CEACAMs (Kuroki et al. 1992b). Anti-CEACAM antibodies and synthetic peptides mimicking N-domains of CEACAMs can activate neutrophils and increase adhesive properties of CD11/CD18, resulting an increase in neutrophil adhesion to endothelial cells (Skubitz et al. 1996; Skubitz et al. 2000). Moreover, CEACAM1 and CEACAM6 were shown to interact with E-selectin, which is required for recruiting granulocytes to sites of inflammation (Kuijpers et al. 1992).

The role of CEACAM in hepatic insulin clearance was recently discovered by a transgenic mouse study with a dominant negative rat CEACAM1 (Poy et al. 2002). Without functional CEACAM1 in the liver, insulin accumulates in the circulation and hyperinsulinemia develops. This leads to secondary insulin resistance with impaired glucose tolerance and random hyperglycemia. This finding links the CEACAM gene family to obesity and diabetes (Najjar 2002; Poy et al. 2002).

3. CEACAM Family Genes: Localization, Regulation and Evolution

3.1. Human CEACAM Family Gene Locus

The human CEACAM gene family is composed of 29 genes/pseudogenes and gene-like sequences that are localized on the long arm of human chromosome 19 in the region of q13.2. (Brandriff et al. 1992; Thompson et al. 1992; Tynan et al. 1992; Hammarstrom et al. 1998; Zimmermann 1998). This large gene family can be divided into the CEA-subgroup (n=12, where 5 of them are pseudogenes), the PSG-subgroup (n=11) and the incomplete non-expressed CGM (CEA gene family member)-subgroup (n=6) (Hammarstrom et al. 1998; Hammarstrom 1999; Frangsmyr et al. 2000). These genes were arranged during evolution into two clusters (250 kb proximal and 850 kb distal clusters in relation to the centromere) separated by 700 kb of genomic DNA containing a few unrelated genes (Hammarstrom et al. 1998). As indicated in Figure 2, CEACAM4, CEACAM7, CEA (CEACAM5), CEACAM6 and CEACAM3 are closely clustered in the proximal cluster; CEACAM1, CEACAM8 and all PSG/CGM genes in the distal cluster.

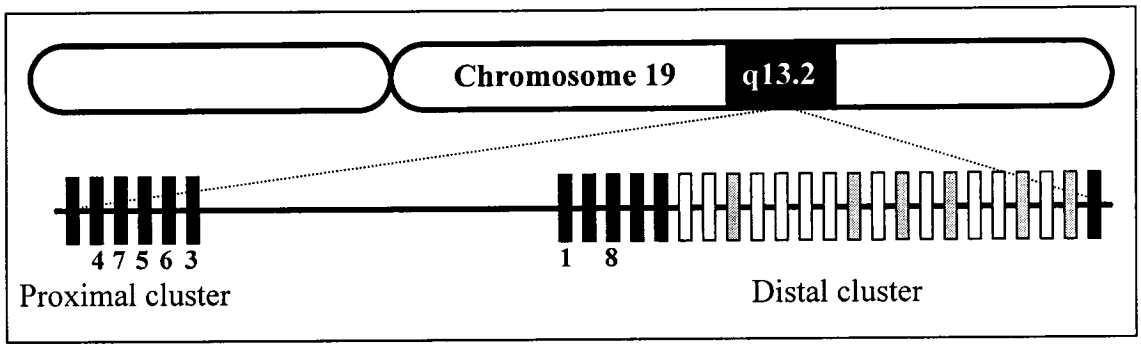

Figure 2: Schematic map of the human CEACAM family gene locus on chromosome 19. Chromosomal region 19q13.2 is highlighted in black on chromosome 19 (upper panel). Higher resolution of a 2 Mb region within 19q13.2, where the CEACAM family gene locus is located, is shown in the lower panel. Vertical bars represent individual genes (CEA-subgroup in black, PSG-subgroup in white and CGM-subgroup in grey). Numbers below the black vertical bars identify expressed genes from the CEA-subgroup (4, CEACAM4; 7 CEACAM7; 5, CEA (CEACAM5); 6, CEACAM6; 3, CEACAM3; 1, CEACAM1; 8, CEACAM8).

3.2. CEACAM Family Gene Loci in other species

The rodent (mouse and rat) CEACAM gene families are presently composed of 19 and 7 genes/pseudogenes, respectively, although these two families are still growing in size as more are identified in the mouse genome. They are localized on mouse chromosome 7 in the region of A2-A3 and rat chromosome 1 in the region of q21, which are syntenic (derived from a common ancestor) to the human chromosomal region 19q13.2 (Zimmermann 1998). In contrast to the human CEACAM gene family, mouse or rat CEA/PSG genes could not easily be separated into two subgroups and no genes corresponding to the CGM genes could be identified (Zimmermann 1998). CEACAM gene families have been detected in non-human primates and cattle as well (Tobi et al. 2000; Zhou and Hammarstrom 2001; Kammerer et al. 2004b; Naghibalhossaini and Stanners in preparation); however, their chromosomal localization in these species has not yet been resolved.

3.3. Transcriptional regulation

Studies on transcriptional regulation of the CEACAM family genes were previously performed with human CEACAM1, CEA and CEACAM6 genes (Schrewe et al. 1990; Hauck et al. 1994; Hauck and Stanners 1995; Koops et al. 1998). Regulation of other CEACAM family genes in humans and other species is little known. The upstream promoter sequences of CEA, CEACAM1 and CEACAM6 genes lack the classical TATA and CCAAT boxes. TATA/CCAAT-less genes can generally be grouped into 1) constitutively active house-keeping genes with relatively G/C-rich promoter regions, SP1 sites and often multiple transcriptional start sites, or 2) genes lacking G/C-rich regions that have tightly clustered transcriptional start sites that are differentially or developmental regulated (Smale and Baltimore 1989). However, in contrast to other TATA/CCAAT-less genes, CEACAM family genes possess features from both groups. They have G/C rich promoter regions and SP1 sites, but they also have clusters of transcriptional start sites and are differentially expressed.

CEA and CEACAM6 promoters show a sequence homology of 80% within the first 230 nucleotides upstream of the translational start site, where they could share the same transcriptional binding factors (Fig. 3). The sequences farther upstream diverge significantly from each other (Koops et al. 1998). The CEACAM1 promoter shows a sequence homology of 79% and 70% to the CEA and CEACAM6 promoters, respectively, within the first 500 nucleotides upstream of translational start site (Hauck et al. 1994). However, the CEACAM1 promoter lacks the SP1 binding sites and the upstream silencer binding element (Hauck et al. 1994). Sequences farther upstream and downstream of the translational start sites of these three genes were not studied extensively; therefore, the presence of additional transcriptional control elements in these regions is probable. The latter speculation can be reinforced by the fact that some genes, especially closely clustered genes, have transcriptional control elements, such as enhancers (Banerji et al. 1981; Wasylyk et al. 1983), locus control regions (Grosveld et al. 1987) and insulators/boundaries (Kellum and Schedl 1991; Dorsett 1999; Gerasimova and Corces 2001; West et al. 2002), up to hundreds of kilobases upstream of the transcriptional start site or downstream of the gene (Bresnick and Felsenfeld 1994; Ganss et al. 1994; Seidl et al. 1999; Tolhuis et al. 2002; Yannoutsos et al. 2004).

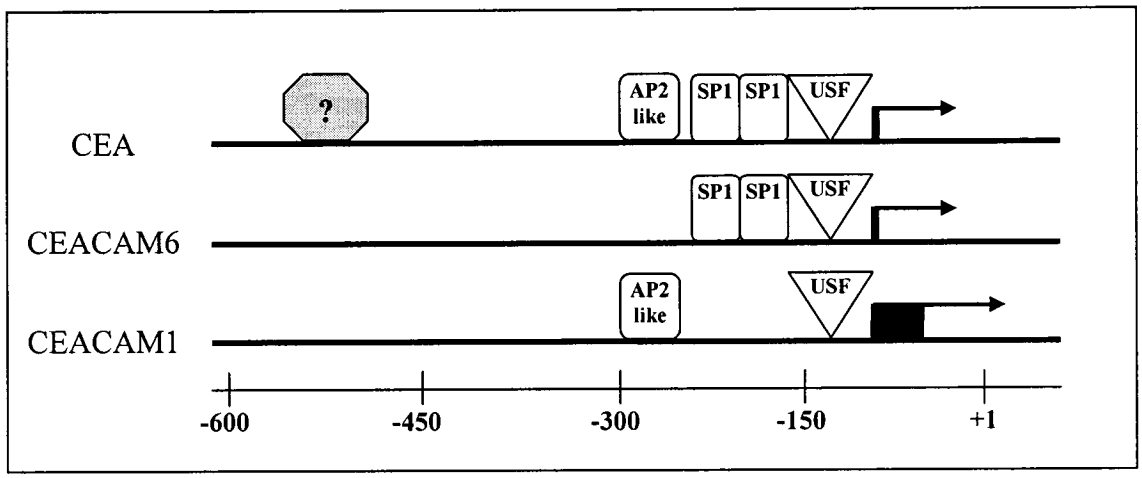

Figure 3: Schematic representation of transcriptional regulation of human CEA, CEACAM6 and CEACAM1 genes. Upstream promoter regions (-600 to +1) of CEA, CEACAM6 and CEACAM1 are shown. +1 indicates the translational start site. Vertical black areas at base of arrows and black areas underneath indicate clusters of transcriptional start sites (CEA, from -110 to -104; CEACAM6, from -112 to -104; CEACAM1, from -107 to -61). Boxes indicate transcriptional activators (USF, SP1 and AP2-like) and an unknown transcriptional silencer (?).

3.4. Evolution of CEACAM Gene Family

Gene duplication, functional divergence and selection are the main driving force of evolution of multigene families in many species (Lynch and Conery 2000; Prince and Pickett 2002; Langkjaer et al. 2003; Papp et al. 2003). As mentioned earlier, the CEACAM families are localized in syntenic chromosomal regions of humans, mice and rats, indicating that they were likely evolved from a common ancestral CEACAM family (Zimmermann 1998). Since only CEACAM1 shares the same domain organization among the three mammalian species, it is believed that all CEACAM family members were evolved from an common ancestral CEACAM1-like gene by duplication and exon-shuffling (Zimmermann 1998). Based on the relative order between the CEACAM1 gene and adjacent marker genes, an inversion took place at the beginning of the primate radiation; however, these loci were well conserved between these species (Zimmermann 1998). As mentioned earlier, since GPI-anchored CEACAM family members are present only in primates, they must be evolved from the transmembrane family member after the

primate radiation. In fact, although not efficiently processed to the cell surface, GPI-anchored family members can be derived artificially from transmembrane family members by introducing a single stop codon in the transmembrane domain at a position corresponding to that of GPI-anchored family members. Another point mutation is sufficient to produce an efficiently processed GPI-anchored family member (Naghibalhossaini and Stanners 2004). Although the evolutionary history of this multigene family has been investigated extensively, the exact selective pressure, especially on evolution of the tumorigenic GPI-anchored members, is debatable. Moreover, since each family member has its distinct spatiotemporal expression pattern, transcriptional regulation must have co-evolved during gene duplication and exon-shuffling, as observed in yeast (Papp et al. 2003); however, the mechanism of such evolution is not known.

4. Mouse Models of CEACAM Family Genes

4.1. Human CEA transgenic mice

As mentioned above, mice do not possess genes determining GPI-anchored CEACAM family members. Transgenic mice expressing human CEA were first created with full-length CEA cDNA driven by the SV40 promoter. CEA was ubiquitously expressed in all tissues examined including brain, thymus, spleen, liver, kidney, and colon (Hasegawa et al. 1991). Quantitative analysis of self-tolerance induced by different amounts of CEA in these mice was performed and showed that the presence of this antigen in the thymus is necessary for the induction of CEA tolerance (Hasegawa et al. 1992). This was followed by the generation of CEA transgenic mice using a 33 kb cosmid clone (cosCEA1) isolated from a human genomic library that contains the CEA gene (complete coding region with 3.3 kb of 5'-upstream regulatory and 5 kb 3'-downstream regulatory flanking sequences). The spatiotemporal expression pattern of CEA was shown to be very similar to that of humans, indicating the conservation of transcriptional control between humans and mice during evolution (Eades-Perner et al. 1994; Clarke et al. 1998). As in humans, these mice do not develop immune responses against CEA-positive tumors, demonstrating their usefulness for studying efficacy and safety of various immunotherapy strategies directed

against this tumor self-antigen (Clarke et al. 1998). These mice were also bred with other tumor-prone transgenic mouse strains, including $Apc^{Min/+}$ (intestinal tumors), SPC-TAg (lung tumors) and MMTV-Neu (breast tumors). CEA was positive in all intestinal tumors, but only focally positive (i.e., no uniform detection in tumors, indicating very low expression levels) in lung and breast tumors; no significant effect of CEA on tumor incidence or growth was observed (Thompson et al. 1997).

Recently, a more human-like mouse model, CEABAC, was generated using a 187 kb human bacterial artificial chromosome that contains genomic sequences of complete genes for CEA, CEACAM6, CEACAM7, and CEACAM3 along with 29.3 kb centromeric and 19.4 kb telomeric flanking sequences (Chan and Stanners 2004). As described in Chapter 2, the spatiotemporal expression pattern of these CEACAM family genes was shown to be very similar to that of humans, indicating the conservation of transcriptional control for all these human CEACAM family members between humans and mice during evolution (Chan and Stanners 2004).

4.2. Other transgenic and knockout models

CEACAM8 transgenic mice were also generated using a 42 kb cosmid clone that contains the human CEACAM8 gene (complete coding region with 13 kb of 5'-upstream regulatory and 12.5 kb 3'-downstream regulatory flanking sequences) and showed expression only in granulocytes (as in humans) indicating that all elements necessary for the granulocyte-specific expression were present (Eades-Perner et al. 1998).

CEACAM1 knockout mice (one partial and one complete) were generated and showed reduced susceptibility and complete resistance to MHV infection, respectively (Blau et al. 2001; Hemmila et al. 2004). Transgenic mice with rat S503A-CEACAM1 (dominant negative mutant, SACC1) driven by the human ApoA1 promoter were generated and developed hyperinsulinemia resulting from impaired hepatic insulin clearance (Poy et al. 2002).

5. CEACAM Family Genes in Human Cancers

5.1. De-regulated expression in common cancers

The human CEACAM family genes are usually de-regulated in many human cancers. CEA is over-expressed in many gastrointestinal cancers (colorectal, esophageal, gastric, pancreatic, hepatocellular, and gallbladder carcinomas), lung cancer, breast cancer, cancers of the female reproductive tract (ovarian, cervical and endometrial carcinomas), medullary thyroid carcinomas, urinary bladder cancer, and prostate cancer (Lloyd et al. 1983; Chevinsky 1991; Ballesta et al. 1995; Hammarstrom et al. 1998; Kinugasa et al. 1998; Frangsmyr et al. 1999; Takano et al. 1999; Genega et al. 2000; Kanthan et al. 2000; Kijima et al. 2000; Bojunga et al. 2001; Castro et al. 2001; Comin et al. 2001; Bhatnagar et al. 2002; Chu et al. 2002; Jantscheff et al. 2003; Logsdon et al. 2003; Nakamura et al. 2003; Ordonez 2003). Similarly, CEACAM6 is over-expressed in colorectal, gastric and pancreatic carncers, lung cancer, breast cancer, cancer of the female reproductive tract (ovarian, cervical and endometrial carcinomas), and acute lymphoblastic and chronic myelocytic leukemia (Allard et al. 1994; Boccuni et al. 1998; Hammarstrom et al. 1998; Kinugasa et al. 1998; Sugita et al. 1999; Carrasco et al. 2000; Scholzel et al. 2000; Jantscheff et al. 2003; Logsdon et al. 2003). CEACAM7 is over-expressed in gastric and pancreatic cancers (Kinugasa et al. 1998; Yoshida et al. 2003), but down-regulated in colorectal cancer (Scholzel et al. 2000). In sum, CEA and CEACAM6 are over-expressed in as many as 70% of all human cancers.

Expression of CEACAM1 in colon cancer is inconsistent. Some studies showed that CEACAM1 is down-regulated (Neumaier et al. 1993; Nollau et al. 1997), while others showed an over-expression and/or correlated with more aggressive cancers (Yeatman et al. 1997; Jantscheff et al. 2003). Otherwise, CEACAM1 is down-regulated in hepatocellular carcinomas, breast carcinomas, endometrial carcinomas, prostate carcinomas, and renal cell carcinomas (Riethdorf et al. 1997; Tanaka et al. 1997; Bamberger et al. 1998; Luo et al. 1999; Kammerer et al. 2004a), but over-expressed in lung cancer, multiple myeloma, melanomas, and perhaps acute lymphoblastic and chronic myelocytic leukemias (Ohwada et al. 1994; Carrasco et al. 2000; Satoh et al. 2002; Thies et al. 2002; Sienel et al. 2003).

5.2. Functional contribution to cancers

The GPI-anchored CEACAMs, CEA and CEACAM6, have a variety of tumorigenic effects on cells cultured *in vitro* and in xenograft mouse models. Over-expression of CEA and CEACAM6 impedes myogenic, adipogenic, neurogenic, and colonic differentiation programs (Eidelman et al. 1993; Rojas et al. 1996; Stanners 1998; Ilantzis et al. 2002), inhibits anoikis/apoptosis in colon and pancreatic cancer cells (Ordonez et al. 2000; Soeth et al. 2001; Wirth et al. 2002; Duxbury et al. 2004a), disrupts cell polarization and tissue architecture (Ilantzis et al. 2002), enhances liver metastasis (Jessup and Thomas 1998; Leconte et al. 1999; Duxbury et al. 2004a), and increases chemoresistance (Duxbury et al. 2004b; Ilantzis et al. unpublished data). Transmembrane CEACAM1 was proposed to be a tumor suppressor since it is down-regulated in many cancers and its forced expression in various cancer cell lines, including prostate, colon and breast, decreases proliferation and increases apoptosis (Hsieh et al. 1995; Luo et al. 1997; Luo et al. 1999; Kirshner et al. 2003a). However, this generalization becomes blurred based on the following observations: 1) detection of its over-expression in various cancers (Ohwada et al. 1994; Yeatman et al. 1997; Carrasco et al. 2000; Satoh et al. 2002; Thies et al. 2002; Jantscheff et al. 2003; Sienel et al. 2003); 2) its contribution to tumor aggressiveness (Yeatman et al. 1997; Thies et al. 2002; Sienel et al. 2003); 3) the nature of its immune suppressive effects (Morales et al. 1999; Kammerer et al. 2001; Markel et al. 2002; Nakajima et al. 2002; Iijima et al. 2004; Kammerer et al. 2004a); and 4) its pro-angiogenic properties (Ergun et al. 2000; Wagener and Ergun 2000).

6. CEACAM Family Genes in Colorectal Cancer

6.1. Types of colorectal cancers

Colorectal cancers are the end result of multiple malignant events in normal epithelia that are constantly undergoing renewal. Depending on the stages of the disease, colorectal tumors are classified histologically as benign polyps (tubular, tubulovillous, villous and serrated adenomas) or malignant lesions (adenocarcinomas). While benign polyps (i.e., adenomas) can potentially become malignant, hyperplastic and hamartomatous polyps are

grouped as non-neoplastic types and are commonly believed not to progress to malignant lesions (Crawford 1994).

Familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC) are the two common types of hereditary colon cancers that account for 3%–7% of all cases annually (Kinzler and Vogelstein 1996; Skibber et al. 2001). The remainder cases (>90%) belong to the sporadic colorectal cancers. Germline mutations (mostly truncating) in APC (adenomatous polyposis coli) gene are commonly (>85%) found in FAP patients (Kinzler and Vogelstein 1996). Recently, recessive mutations of the MYH gene (human homolog of mutY, a base excision repair gene in *Escherichia coli*) have been found in a group of patients, termed MYH-associated polyposis (MAP), that are clinically indistinguishable from FAP patients but usually present with a severe phenotype (Al-Tassan et al. 2002; Bisgaard et al. 2004; Lipton and Tomlinson 2004). Germline mutations in components of the DNA Mismatch Repair (MMR) complex (>70%), such as hMSH2, hMLH1 and hPMS2, causing microsatellite instability (MSI) represent the genetic basis of HNPCC (Kinzler and Vogelstein 1996; Markowitz 2000). The molecular basis of sporadic colon cancer is much more complex. Chromosomal instability, microsatellite instability and epigenetic changes are the fundamental basis of sporadic colon cancers (Markowitz et al. 2002).

6.2. Adenoma-carcinoma sequence of neoplastic transformation

Based on information derived from FAP patients, a simple sequence of neoplastic events was originally constructed, termed the *adenoma-carcinoma sequence* (Kinzler and Vogelstein 1996). Loss of functional APC causes a transition from normal epithelium to aberrant crypt foci (ACF), the earliest detectable tumorigenic change (Bird 1995), followed by K-ras activation (adenoma formation), loss of SMAD2/SMAD4 and p53 inactivation (carcinoma formation). MSI (MLH1, MSH2, PMS2, and PMS1), which can accelerate this whole process, was later added into this picture. With growing knowledge in the genetics of other hereditary and sporadic colorectal cancers, more genes are being placed into this sequence, depending on their contribution: β-catenin, COX2, EGF-R, TGFβ-RII, TGFβ, PRL3, etc. (Markowitz et al. 2002). Nevertheless, APC mutations are

still believed to be the initial step in this malignant transformation by most clinicians/researchers.

6.3. Search for alternative pathways of neoplastic transformation

The necessity for seeking alternative neoplastic pathways was provoked by a few key studies. Smith et al. showed that only 7% of sporadic colon tumors contain mutations in all of APC, K-ras and p53 and only 39% of tumors contain mutations in one of these genes, indicating that the widely accepted sequence of neoplastic transformation is not representative of the majority of sporadic colorectal cancers (Smith et al. 2002). In patients without FAP, Takayama et al. showed that mutations in K-ras (68%) were present at a much higher frequency than APC or β-catenin (downstream element of APC in the Wnt signaling pathway) in both dysplastic and non-dysplastic ACFs, suggesting APC may not be the sole initiating step (Takayama et al. 2001). In fact, this was supported by a transgenic mouse model with K-ras^{V12G} (a constitutively active mutant) that developed spontaneous intestinal tumors without acquiring APC mutations (Janssen et al. 2002). In sum, a more general model of neoplastic transformation should be established with a flexible order of genetic events and perhaps multiple starting points.

6.4. Serrated pathway of neoplastic transformation

Serrated adenomas are one type of adenomas that show serrated or sawtooth-like configuration of crypt epithelium. Serration is likely a result of inhibition of anoikis caused by clonal mutations (Jass et al. 2002). Moreover, crypt serration is commonly found in hyperplastic polyps and mixed hyperplastic polyps. As mentioned earlier, hyperplastic polyps are considered non-neoplastic; therefore the adenomatous component in mixed hyperplastic polyps is only considered to be due to a chance mixture of two different polyps and serrated adenomas to be the usual adenomas with serration. On the contrary, Jass and colleagues have argued that they are part of the same continuum termed *serrated pathway* of neoplastic transformation, based on three key observations (Jass et al. 2002; Jass 2004). First, a mixture of these three types of lesions (i.e., hyperplastic polyps, mixed hyperplastic polyps and serrated adenomas) is often present in a condition called hyperplastic polyposis (Leggett et al. 2001). Second, serrated adenomas

are rare lesions but frequently associate with mixed hyperplastic polyps (Iino et al. 1999). Finally, the same microsatellite mutations can be found in both hyperplastic and adenomatous components within the same mixed hyperplastic polyps (Iino et al. 1999). Thus, hyperplastic polyps are not completely harmless and should not be ignored during diagnosis (Jass 2004).

In general, the *serrated pathway* is started by a general inhibition of anoikis caused by a clonal genetic alteration (Jass et al. 2002). Depending on the alteration, a small fraction of these hyperplastic lesions will progress to serrated adenomas, termed sessile serrated adenomas. BRAF, a member of the RAF family of serine/threonine kinases, has recently been identified as a factor involved in the transformation of hyperplastic polyps to serrated adenomas (Kambara et al. 2004). Further mutations in hMLH1 or MGMT (methylguanine methyltransferase) are postulated to lead to the progression of serrated adenomas to MSI-H (high level of MSI) and MSI-L (low level of MSI) colorectal carcinomas, respectively (Jass 2004).

6.5. To which pathway(s) does CEACAM gene family changes in expression belong?

Although CEA, and to a lesser extent CEACAM6, are consistently over-expressed in most colorectal cancers and that they have a broad range of tumorigenic effects, they have not yet been classified to belong to any proposed pathway. Over-expression of CEA in 30-90% of ACFs from non-FAP patients (Pretlow et al. 1994; Ilantzis et al. 1997) suggest that over-expression of CEA can be an early event, perhaps an alternative to or a facilitation of the selection of APC mutations in the classical *adenoma-carcinoma sequence*. Because of the continual and progressive over-expression of CEA and CEACAM6 in adenomas and adenocarcinomas (Au et al. 1986; Salem et al. 1993; Ilantzis et al. 1997; Scholzel et al. 2000) and their broad tumorigenic effects, they can be associated with later steps in the sequence as well. Their overexpression in hyperplastic polyps and sessile serrated adenomas (Jass et al. 1984; Salem et al. 1993; Scholzel et al. 2000) and their anti-apoptotic ability may suggest their involvement in the *serrated pathway* of neoplasia.

7. CEACAM Family Genes in Cancer Clinics

7.1. Diagnosis and prognostic evaluation of cancers

Since expression of different CEACAMs is usually de-regulated in various cancers, they are used as tumor markers in cancer clinics. However, CEA is the only member that is commonly used because of its consistent over-expression leading to elevated blood levels in many cancers including the three major ones, i.e., colon, breast and lung (Shuster et al. 1980; Lamerz 1992; Yasasever et al. 1994; Ballesta et al. 1995; Eskelinen et al. 1997; Buccheri and Ferrigno 2003; Mujagic et al. 2004). Since cancer patients usually have higher serum CEA levels, serum CEA is a common diagnostic test for the presence of many types of cancers and is used in the management of colon, breast and lung cancer. However, recent studies have criticized its broad utility due to its low sensitivity (serum level usually underestimates the actual level in the tumor and may not reflect the true stage of the disease), possibility of false positives (some cancer-free patients, such as cigarette smokers, can have a higher endogenous serum level) and lack of specificity (many different cancers give the same outcome, i.e., higher serum CEA levels), which creates a major problem: low cost-effectiveness (Falcone et al. 1996; Guadagni et al. 2001; Anan et al. 2002). Despite this fact, CEA, and potentially other CEACAMs, have good prognostic value in many types of cancers: high CEA level is usually associated with poor prognosis, i.e. poor outcome and reduced survival (Gaglia et al. 1988; Ballesta et al. 1995; Sawabata et al. 2002; Jantscheff et al. 2003; Sakao et al. 2004).

7.2. Cancer Therapeutics

Combinations of surgery, radiation therapy and chemotherapy represent the conventional treatment modalities for cancer. With the growing knowledge of tumor molecular biology, however, most novel cancer therapies are focused on tumor specific targets in order to increase efficacy and to minimize systemic toxicity. Due to its ectopic or de-regulated over-expression in up to 70% of all tumors, CEA represents a popular target for novel cancer therapies, including cancer vaccines, cellular immunotherapy, radioimmunotherapy, antibody therapy and gene therapy (Chester et al. 2000; Fong et al. 2001; Koch et al. 2001; Berinstein 2002; Kousparou et al. 2002; Goldenberg 2003;

Marshall 2003). Similarly, CEACAM6 could also be developed as a tumor marker or a cancer therapeutic target (Burke et al. 2002).

8. Aim and Scope of the Present Study

The GPI-anchored CEACAM family members, CEA and CEACAM6, are over-expressed in as many as 70% of all human cancers. This correlation suggests an instrumental role for them in tumor progression, which is supported by extensive results obtained with several model systems *in vitro* and *ex vivo*. Although the tumorigenic effects mediated by CEA/CEACAM6 over-expression should provide growth advantages to tumor cells, their contribution and relevance *in vivo* remains uncertain without further validation. Since mice do not possess homologs of the human CEA and CEACAM6 genes, transgenic mice for the CEA and CEACAM6 genes are necessary for this validation. Although CEA transgenic mice were previously constructed, they did not show any obvious tumorigenic phenotype, even in combination with other activated oncogenes. Hence, this discrepancy may be a consequence of: 1) the absence of CEACAM6 for potential synergetic effects with CEA; and/or 2) the absence of important *cis*-transcriptional regulatory elements upstream or downstream of the CEA transgene used, resulting in an insufficient expression level for tumorigenesis.

To provide a better *in vivo* model, transgenic mice harboring a 187 kb portion of the human CEA family gene locus contained in a bacterial artificial chromosome (CEABAC) that includes CEA, CEACAM3, CEACAM6, and CEACAM7 genes were successfully constructed in this study, as described in Chapter 2. Based on the correct spatiotemporal expression pattern of these genes and the gene dosage-dependent expression, this mouse model can be used for multiple applications (See Chapter 2).

As in CEA-only transgenic mice, no obvious tumorigenic phenotype was evident in the CEABAC transgenics. However, in contrast to the CEA transgenics, the CEABAC mice were found to be more susceptible to develop spontaneous lung tumors (See Chapter 4). Thus far, tumorigenic effects of CEA and CEACAM6 have never been reported for human lung cancers in spite of their over-expression. To evaluate the tumorigenic properties of CEA and CEACAM6 in a more familiar system, the CEABAC mice were

challenged with a colon-specific carcinogen (azoxymethane) to induce sporadic colon tumors (See Chapter 3). The CEABAC mice were more susceptible, as in the lung system.

To investigate the underlying causes of these phenomena, phenotypic changes at the cellular level were studied in Chapter 5. In fact, at low to moderate expression levels of CEA/CEACAM6, a partial block in cell differentiation and a mild to moderate hyperproliferation were evident in the transgenic colon. At higher or tumor-like expression levels, a complete block in cell differentiation, extreme hyperproliferation and inhibition of apoptosis were observed. These mice had reduced survival, growth retardation and chronic diarrhea, and showed massively enlarged colons comprising continuous non-focal cytological and architectural abnormalities, including dysplastic and serrated adenomatous morphology by only 3 months of age. These results suggested that, while moderate expression levels of CEA/CEACAM6 cause an imbalance of tissue homeostasis leading to increased tumor susceptibility, tumor-like expression levels alone produce a severe imbalance leading directly to tumor formation.

Altogether, these results provide direct evidence for an important role of CEA/CEACAM6 in cancer development *in vivo*, especially in the *serrated pathway* of neoplasia. These findings also suggest that CEA/CEACAM6 over-expression may represent an alternative to APC mutations as the initiation step for colorectal cancer, since the Wnt signaling pathway was shown not to be involved (See Chapter 5). Furthermore, these CEABAC mice also provide an *in vivo* model system for studies in other cancers and infectious diseases involving human CEACAM family genes and for pre-clinical trials of CEACAM-based therapies.

9. Publication status of the following chapters

Chapter 2: Construction and Characterization of CEABAC Transgenic Mice is published as: C.H.F. Chan and C.P. Stanners. Novel Mouse Model for Carcinoembryonic Antigen-Based Therapy. Molecular Therapy 9(6): 775-785, 2004.

Chapter 3: Increased Susceptibility of Carcinogen-induced Colon Tumors in CEABAC Transgenic Mice is submitted to Gastroenterology.

Chapter 4: Higher Incidence of Spontaneous Lung Tumors in CEABAC Transgenic Mice will be submitted to Cancer Research.

Chapter 5: Spontaneous Colon Tumors in CEABAC Transgenic Mice will be submitted to Cancer Cell.

Chapter 2

Construction and Characterization of CEABAC Transgenic Mice

ABSTRACT

Many novel cancer therapies, including immunotherapy and gene therapy, are specifically targeted to tumor-associated molecules, among which carcinoembryonic antigen (CEA) represents a popular example. Discrepancies between pre-clinical experimental data in animal models and clinical outcome in terms of therapeutic response and toxicity, however, often arise. Pre-clinical testing can be compromised by the lack of CEA and other closely related human CEACAM family members in rodents, which lack analogous genes for most human CEACAM family members. Here, we report the construction of a transgenic mouse with a 187 kb human bacterial artificial chromosome (CEABAC) that contains part of the human CEACAM family gene cluster including complete human CEA, CEACAM3, CEACAM6 and CEACAM7 genes. The spatiotemporal expression pattern of these genes in the CEABAC mice was found to be remarkably similar to that of humans. This novel mouse will ensure better assessment than previously utilized models for the pre-clinical testing of CEA-targeted therapies and perhaps allow the testing of CEACAM6, which is over-expressed in many solid tumors and leukemias, as a therapeutic target. Moreover, expression of CEACAM family genes in gastrointestinal, breast, hematopoietic, urogenital and respiratory systems could facilitate other clinical applications, such as the development of therapeutic agents against *Neisseria Gonorrhoeae* infections, which use CEACAM family members as major receptors.

INTRODUCTION

Combinations of surgery, radiation therapy and chemotherapy are the conventional treatment modalities for cancer. With the growing knowledge of tumor molecular biology, however, most novel cancer therapies are focused on tumor specific targets in order to increase efficacy and to minimize systemic toxicity. Immunotherapy (Goldenberg 2003; Ribas et al. 2003; Trail et al. 2003; von Mehren et al. 2003) and gene therapy (Kellen 2002), for example, are often directed against tumor-associated molecules and CEA, due to its ectopic or de-regulated over-expression in up to 70% of all tumors, represents a popular target (Chester et al. 2000; Fong et al. 2001; Koch et al. 2001; Kousparou et al. 2002; Goldenberg 2003; Marshall 2003).

Human CEA is the prototypic member of the human CEACAM gene family, a group of highly glycosylated homotypic/heterotypic cell surface intercellular adhesion molecules and part of the immunoglobulin gene superfamily which consists of 7 expressed genes (Hammarstrom et al. 1998; Hammarstrom 1999). These have molecular structures consisting of multiples of highly homologous units and demonstrate a wide range of expression patterns and biological activities. Their extracellular domains all consist of a V-like Ig amino-terminal domain followed by a variable number (between 0 and 6) of I-like Ig internal domains. They can be divided into two groups based on their membrane anchorage: glycophosphatidyl inositol (GPI)-anchored members (CEA, CEACAM6, CEACAM7 and CEACAM8) and transmembrane members (CEACAM1, CEACAM3 and CEACAM4).

The prototype member, CEA, a well-known tumor marker (Ballesta et al. 1995), is expressed mostly in the gastrointestinal tract (Hammarstrom et al. 1998; Hammarstrom 1999) and is over-expressed in many human cancers, including epithelial tumors originating from the gastrointestinal tract, lung, thyroid, breast, prostate, cervix and ovaries (Chevinsky 1991; Hammarstrom et al. 1998; Hammarstrom 1999). Pre-clinical animal studies are commonly performed with athymic immunodeficient mice bearing human xenografted tumors. For CEA-targeted therapies, however, these animal models are inadequate for testing therapeutic response and toxicity because rodents lack the gene coding for human CEA.

Recently established immunocompetent pre-clinical animal models, i.e., human CEA transgenic mice bearing human CEA-transfected mouse xenografted tumors (Mizobata et al. 2000; Xu et al. 2000; Wilkinson et al. 2002), represent an improvement but still have serious shortcomings. For example, anti-CEA antibodies used in various kinds of immunotherapy (Chester et al. 2000; Kousparou et al. 2002; Goldenberg 2003) can show differences in pharmacokinetics in mice versus humans depending on their specificity (Oriuchi et al. 1998). This can be due to cross-reaction with other human CEACAM family members, such as CEACAM6 (formerly NCA), that are absent in mice, resulting in mistargeting to CEACAM6 on circulating and tissue granulocytes and other CEACAM6-expressing tissues in humans (Oriuchi et al. 1998). This may not only limit efficacy of the therapy but may also cause unnecessary toxicity. Construction of a transgenic model with a closer human approximation, i.e. with as many human CEACAM family members as possible having correct spatiotemporal expression, will be necessary to ensure better pre-clinical assessment of CEA-targeted therapies.

Rodents have only genes coding for murine transmembrane CEACAM1 [formerly biliary glycoprotein (BGP)], a homolog of human CEACAM1. CEACAM1 has a broad expression pattern in normal human and rodent tissues and is usually down-regulated in human cancers (Hammarstrom et al. 1998). During the primate radiation, the human CEACAM family genes evolved, presumably from a primordial CEACAM1-like gene, by duplication (Zimmermann 1998), acquired new structures and functions and developed a more restricted expression pattern (Hammarstrom et al. 1998). For example, CEACAM6 is expressed in the gastrointestinal tract, breast and hematopoietic system (Scholzel et al. 2000) and is also over-expressed in a variety of adenocarcinomas (Hammarstrom et al. 1998; Hammarstrom 1999) and leukemias (Boccuni et al. 1998; Hansen et al. 1998); CEACAM7 (formerly CGM2) is expressed mainly in colon and pancreas (Scholzel et al. 2000); and CEACAM3 (formerly CGM1) is expressed only in mature neutrophils (Nagel et al. 1993). In humans these genes are organized into two clusters (250 kb proximal cluster and 850 kb distal cluster, relative to the centromere) on the long arm of chromosome 19 in the region 19q13.2 (Brandriff et al. 1992; Thompson et al. 1992).

Using a well-established protocol for transgenesis with bacterial artificial chromosomes (BAC) (Dewar et al. 1997), we have successfully constructed transgenic

mice with genomic insertions of an intact 187 kb human BAC containing much of the proximal human CEACAM family gene cluster, including CEA, CEACAM3, CEACAM6 and CEACAM7 genes along with 29.3 kb centromeric and 19.4 kb telomeric flanking sequences. While the expression of CEACAM3 is inconclusive, CEA, CEACAM6 and CEACAM7 are expressed much the same as in humans, showing the inclusion and conservation of all *cis*-transcriptional regulatory elements in the CEABAC transgenic mice. Their close linkage ensures co-transmission in mouse matings. Thus the human-like expression pattern of 3 and possibly 4 human CEACAM family genes and the ease of mouse line maintenance make this mouse the model of choice for pre-clinical animal testing for CEA-targeted therapies.

MATERIALS AND METHODS

Primers and Antibodies

Primers specific for the genes and cDNAs of CEA, CEACAM3, CEACAM6, and CEACAM7 are listed in Table 1. Primers specific for the T7 (5'-TACCCGGGGATCCTC TAGAGTC-3') and SP6 (5'-TTCCGGCTCGTATGTTGTGTGG-3') sequences on the pBeloBAC11 vector were used to sequence both ends of the 187 kb CEABAC insert.

Rabbit polyclonal anti-CEA antibody (RbαCEA) recognizes all human CEACAM family members. The mouse monoclonal antibodies used are: D14, specific for CEA (Zhou et al. 1993b); B18, specific for CEA, CEACAM6 and human CEACAM1 (Zhou et al. 1993b); 9A6, specific for CEACAM6 (Scholzel et al. 2000); and BAC2, specific for CEACAM7 (Scholzel et al. 2000). None of these antibodies bind to endogenous mouse CEACAM family members (murine CEACAM1).

Table 1: Primer sequences and expected sizes of PCR products

Gene	5' Primer	3' Primer	Genomic product (bp)	cDNA product (bp)
CEA	5'-GAAATGACACAGCAAGCTAC-3'	5'-ATAGACTGTGATCGTCGTGA-3'	738	338
CEACAM3	5'-AACCCCAGGACAGCAGCTTC-3'	5'-GAGAGGCCTTTGTCCTGACC-3'	587	260(S)/312(L) [b]
CEACAM6	5'-TACTCAGCGTCAAAAGGAAC-3'	5'-AGAGACTGTGATCATCGTGA-3'	794	353
CEACAM7	5'-ATATAGCAGCCTTGGTGTAG-3'	5'-CTACTGGGCAATACAACAGT-3'	4039	1423
CEACAM7 [a]	5'-TGATCCTCCTGATTGTCACA-3'	5'-CTACTGGGCAATACAACAGT-3'	535	535
B-Actin	5'-ATATCGCTGCGCTGGTCGTC-3'	5'-GATGGGCACAGTGTGGGTGA-3'	1008	485

[a] Primer set, which does not flank an intron, was used only for genotyping.
[b] Splice variants of CEACAM3 (S denotes the one with short cytoplasmic tail and L denotes the one with long cytoplasmic tail).

Confirmation of CEABAC Sequence

The CEABAC (GenBank accession no. BC627193), cloned in the HindIII site of the 7.4 kb pBeloBAC11 vector (Kim et al. 1996), was obtained from a human BAC library (Research Genetics Inc., Huntsville, AL). The CEABAC clone was verified by PCR using primers specific for CEA, CEACAM3, CEACAM6 and CEACAM7 and by restriction mapping using pulse-field gel electrophoresis (PFGE; Bio-Rad Laboratories Inc., Hercules, CA). The CEABAC ends were subcloned by digestion with restriction enzyme AvrII (New England Biolabs Inc., Beverly, MA), which cuts the CEABAC but not the

vector, followed by ligation. The CEABAC ends were then sequenced with an automated DNA nucleotide sequencer using T7 and SP6 primers and the sequences were compared with the DNA sequence of human chromosome 19q13.2 (GenBank accession no. NT_011139) to identify the exact location of the CEABAC.

Generation of CEABAC Transgenic Mice

The CEABAC insert was removed from the pBeloBAC11 construct by digestion with NotI (New England Biolabs Inc., Beverly, MA), separated from the vector by PFGE and purified by β-Agarase digestion (Invitrogen Corp., Carlsbad, CA). The DNA solution was dialyzed and adjusted to 1 ng/ul with microinjection buffer (10 mM Tris pH7.4, 0.1 mM EDTA). This DNA solution was microinjected minimizing shearing forces into pronuclei of FVB embryos, which were then implanted into pseudo-pregnant CD-1 females, as described previously (Huxley 1998). CEABAC transgenic founders were identified by PCR on genomic DNA using CEA, CEACAM3, CEACAM6 and CEACAM7 specific primers and tested for expression by ELISA with specific monoclonal antibodies applied to fecal protein extracts, as described below. Gene copy-numbers were determined by Southern blots of genomic DNA digested with EcoRI calibrated with known levels of CEABAC-end DNA digested with EcoRI, and detected with ^{32}P-labeled DNA probes, generated by random priming on the CEABAC ends using the High Prime DNA Labeling Kit (Roche Diagnostics Inc., Laval, QC). Intactness of the integrated CEABAC sequences was determined by long-range Southern blots of genomic DNA digested with PacI (New England Biolabs Inc., Beverly, MA) in agarose blocks and resolved with PFGE, probing with ^{32}P-labeled DNA probes generated by random priming on the CEA cDNA that lacks the Alu sequence at the 3'-end using the High Prime DNA Labeling Kit (Roche Diagnostics Inc., Laval, QC).

Tissue Protein Extraction, Immunoblot Analysis and ELISA

Tissues were homogenized on ice and lysed with SDS lysis buffer (100 mM Tris, pH8.0, 10% glycerol, 2% SDS). 50 ug of tissue extracts were resolved by SDS-PAGE and Western blot analyses were performed using B18 and 9A6 antibodies as described previously (Screaton et al. 1997). Mouse fecal pellets were homogenized on ice with PBS

containing 1% TX100 and sonicated for 30 sec. 200 ul of fecal extracts were analysed by antibody-sandwich ELISA (Hornbeck 1991) using D14, 9A6 and BAC2 as coating antibodies and RbαCEA as detecting antibody.

Tissue RNA Extraction and RT-PCR

Tissues were removed from animals and preserved in RNALaterTM (Ambion Inc., Austin, TX) at -20°C. Bone marrow, spleen and pancreas were prepared fresh for RNA extraction. 20-60 mg of tissues were homogenized and RNA was isolated using the RNAqueousTM extraction kit (Ambion Inc., Austin, TX) following the manufacturer's instructions and stored at -80°C. Human RNA samples were purchased and stored at -80°C (Ambion Inc., Austin, TX). 0.5-1 μg total RNA was used for each RT-PCR reaction by MMTV-RT (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's procedures.

Immunohistochemical Analyses

Tissues were cut into pieces not thicker than 5 mm, fixed with 4% paraformaldehyde for 16 hours, infused with PBS-0.5 M sucrose for 12 hours at 4°C and quickly frozen with isopentane at –70°C. 7-10 um frozen sections were obtained using a cryostat at –20°C to –30°C. Immunohistochemical staining was performed using Envision Reagents (DAKO Diagnostics Canada Inc., Mississauga, ON) with RbαCEA antibody at a dilution of 1:2,500 to 1:10,000. Sections were developed with DAB (3'3'-diaminobenzidine) for 1 to 10 min and were counterstained with Mayer's hematoxylin (Sigma-Aldrich Canada Ltd., Oakville, ON).

RESULTS

Generation and Identification of CEABAC Transgenic Mice

After sequencing the CEABAC ends, restriction mapping and aligning with the known sequence of human chromosome 19, the CEABAC shown in Figure 1A was determined to have complete coding sequences for the CEA, CEACAM3, CEACAM6 and CEACAM7 genes and 29.3 kb centromeric and 19.4 kb telomeric flanking sequences. A NotI DNA fragment containing the 187 kb CEABAC insert was used to generate transgenic mice. Although over a thousand embryos were microinjected with the CEABAC insert, only 30 mice were born and only three of them (CEABAC-891, -892 and -1747) were positive for the CEABAC transgene by PCR using primers specific for CEA, CEACAM3, CEACAM6, and CEACAM7 (Fig. 2). However, CEABAC-891 lacks part of the CEACAM7 gene (Fig. 2) and is also a germline negative mosaic that did not give any CEABAC-positive offspring (0/8). CEA, but not CEACAM6 or CEACAM7, could be detected in fecal extracts from CEABAC-891 by ELISA, whereas the expression of CEA, CEACAM6 and CEACAM7 were detected in fecal extracts from CEABAC-892 and CEABAC-1747 (Fig. 3). Thus the CEABAC-892 and -1747 transgenics with complete CEABAC transgenes express all three CEACAM family members tested. CEABAC-891 lacking a complete CEACAM7 gene, as expected, expressed no CEACAM7; its failure to express CEACAM6 as well suggests that regulatory elements for this family member could be present within the CEACAM7 gene. This suggestion requires confirmation with more direct assays.

Southern blot analyses showed that the CEABAC inserts were intact in CEABAC-892 and -1747 (Fig. 1B) with approximately 2 and 10 transgene copies, respectively, in a head-to-tail configuration (Fig. 1C). CEABAC-892 and -1747 gave 50% (3/6) and 13% (6/46) positive F1 pups, respectively, indicating that CEABAC-1747 was a germline mosaic. Upon breeding the F1 and F2 generations with wild-type FVB mice, CEABAC-892 and -1747 lines gave 52% (16/31) and 47% (60/127) positive offspring, respectively, following a Mendelian pattern of inheritance thus showing removal of mosaicism from CEABAC-1747 line and confirming close clustering of the multiple CEABAC inserts in each line. Also, in the CEABAC-1747 heterozygotes, only one labelled nuclear spot

could be detected by fluorescence in situ hybridization (data not shown). These two lines (CEABAC-892 and -1747) were used to establish the CEABAC transgenic lines for further studies.

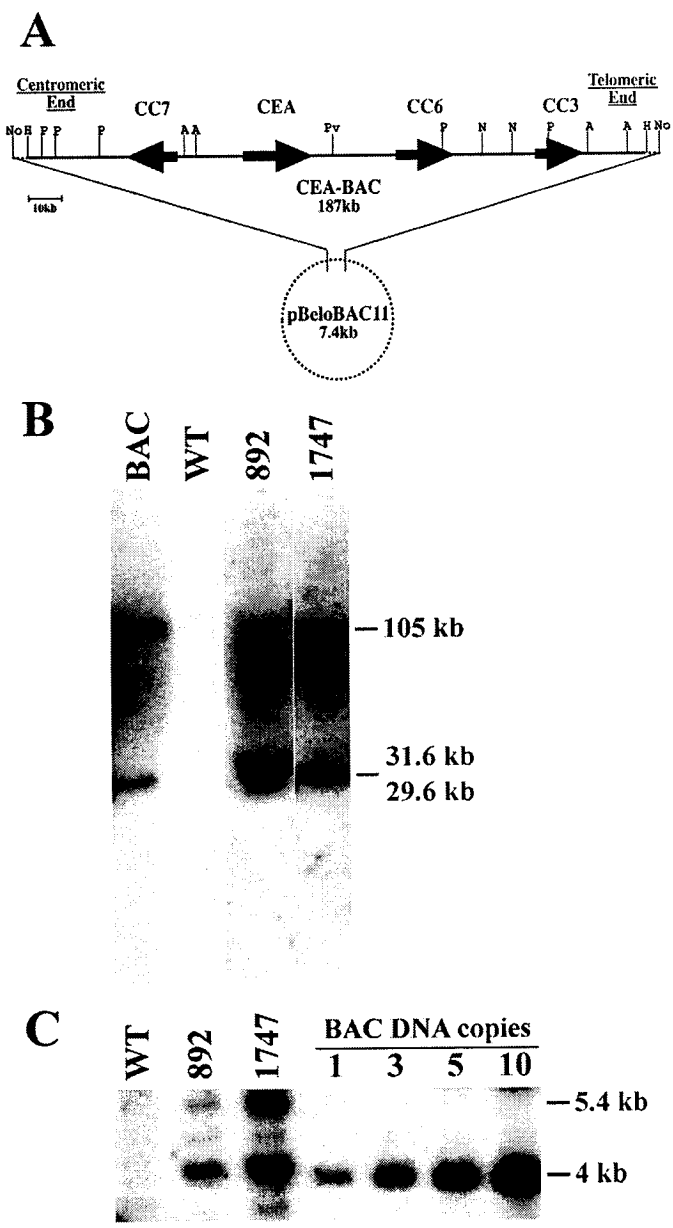

Figure 1: CEABAC DNA construct and Southern blots of CEABAC transgenic mouse genomic DNA. A) The human CEABAC DNA was cloned into the pBeloBAC11 vector at the HindIII restriction site which is flanked by two NotI restriction sites. Dotted and solid lines denote pBeloBAC11 vector (7.4 kb) and CEABAC (187 kb) sequences, respectively. The CEABAC contains four known genes (arrows pointing in the direction of transcription) that belong to the human CEACAM family, starting from the centromeric end: CEACAM7 (CC7, 44.2 kb - 29.3 kb); CEA (64.7 kb - 86 kb); CEACAM6 (CC6, 111.5 kb - 127.9 kb); and CEACAM3 (CC3, 152.4 kb - 167.7 kb). Restriction enzyme sites: No (NotI), H (HindIII), P (PacI, 2 kb, 7.9 kb, 20.7 kb, 125.8 kb and 157.4 kb), A (AatII, 47.5 kb, 51 kb, 169.2 kb and 181.8 kb), Pv (PvuI, 92.1 kb), and N (NruI, 137.6 kb and 146.7 kb)

are shown. The CEABAC end sequences were: (centromeric end) AAGCTTATTTATGTTCCACCTAAAGTCAGTTTTGGGAAACACTGA...(CEABAC)...GTAG CTATGGCAGTGGCAGAAGATTTTAATTAGAAAACAAAGCTT (telomeric end) where AAGCTT is the HindIII cloning site. **B)** Long range Southern blot of agarose-embeded fibroblast genomic DNA (5-30 µg) from CEABAC heterozygous mice and BAC DNA digested with Pac1, resolved by PFGE and probed with ^{32}P-labelled random primers generated from CEA cDNA. Lanes (from left to right): BAC DNA control, negative mouse control, CEABAC-892, and CEABAC-1747. As indicated in panel **A**, three bands (a 105 kb-band containing the CEACAM7, CEA and part of the CEACAM6 gene, a 31.6 kb-band containing part of the CEACAM6 gene and a 29.6 kb-band containing the CEACAM3 gene) were expected due to high homology between these family members. Note that the latter two bands co-migrated in the unresolvable region of PFGE. Also note that the 29.6 kb-band would become 31.6 kb in the CEABAC mice due to the head-to-tail configuration of multiple BAC copies. **C)** Southern blot of genomic DNA (10 ug) and quantified BAC end DNA digested with EcoRI and probed with ^{32}P-labelled random primers generated from BAC end DNA (3'-end only). Lanes (from left to right): negative mouse control, CEABAC-892, CEABAC-1747, and BAC end copy-number control (1, 3, 5 and 10 copies). Note the expected 4 kb-band (3'-end) and a 5.4 kb-band (5'-end + 3'-end) that was due to incomplete digestion of the CEABAC concatemers in a head-to-tail configuration. By densitometric quantification, the transgene copy-number of CEABAC-892 and CEABAC-1747 was estimated to be 2.3±0.1 and 9.7±0.2 copies (by combining the two bands), respectively.

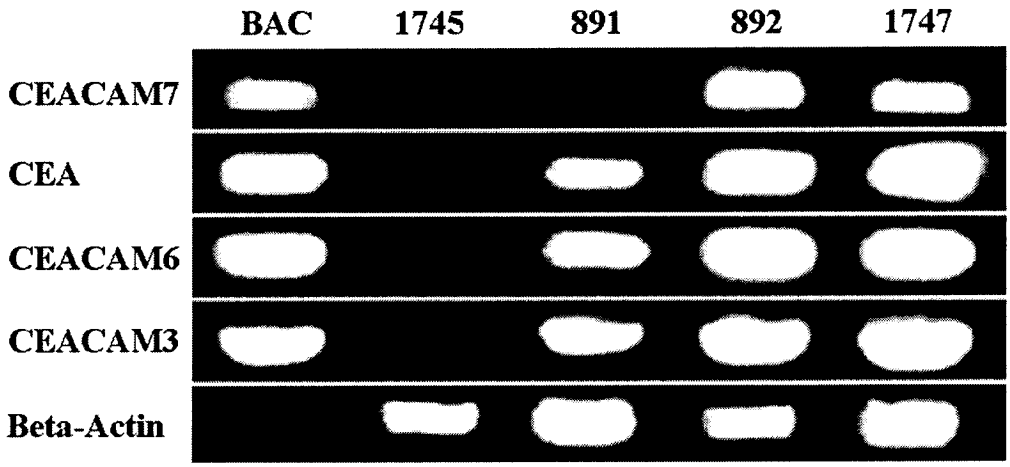

Figure 2: PCR Analyses of CEABAC transgenic mouse tail DNA. Columns from left to right: CEABAC DNA control, negative mouse (1745) and transgenic founders (891, 892 and 1747). Rows from top to bottom: CEACAM7 specific primers (535 bp), CEA specific primers (738 bp), CEACAM6 specific primers (794 bp), CEACAM3 specific primers (584 bp) and ß-actin (DNA level control) specific primers (1008 bp).

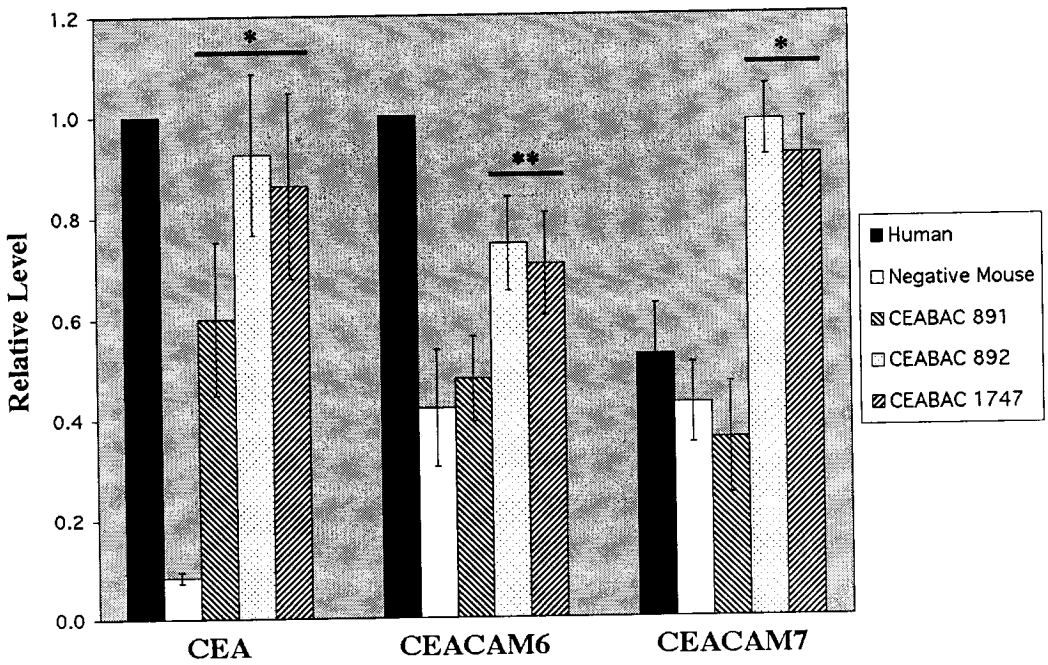

Figure 3: Expression of CEACAM family members from ELISA assays of fecal extracts. Fecal levels of CEA, CEACAM6 and CEACAM7 were detected by antibody-sandwich ELISA using RbαCEA and mono-specific antibodies (D14, 9A6 and BAC2, respectively). Relative levels in each experiment were calculated by normalizing to the highest absorbance at 405 nm and were averaged for 3 independent experiments. Black, white and patterned series denote fecal samples collected from human, negative mouse and CEABAC transgenic founders (891, 892 and 1747), respectively. Mean±SD was plotted for each series. Fecal CEA, CEACAM6 and CEACAM7 could be detected in both 892 and 1747 transgenics whereas only CEA could be detected in 891 transgenic fecal extracts. * and ** denote significant differences between CEABAC transgenic founders and negative mice (p-values < 0.005 and < 0.05, respectively). Error bars are not shown for CEA and CEACAM6 levels in the human samples since they represented the highest levels (used for normalizing the other levels) in all three experiments (SD=0).

Expression of CEA and CEACAM6

The expression levels of CEA and CEACAM6 in various tissues of CEABAC-1747 (10 transgene copies) were shown by immunoblots to be higher than that of CEABAC-892 (2 transgene copies) by approximately 5-fold using serial dilutions of colon protein extracts (Fig. 4). Although both transgenics exhibited similar spatiotemporal expression patterns, immunoblot results for CEABAC-1747 only are shown (Fig. 5). While CEA was highly expressed in part of the gastrointestinal tract and vagina (Fig. 5, low exposure), CEACAM6 was highly expressed in the bone marrow and vagina (Fig. 5, low exposure). CEA could also be detected at a much lower level in the trachea, salivary gland, penis, cervix, and breast (Fig. 5, high exposure). Similarly, CEACAM6 could be detected at lower levels in the trachea, lung, part of the alimentary tract, cervix, breast, and spleen (Fig. 5, high exposure). The much higher level of CEA relative to CEACAM6 expression in colonic tissue in the CEABAC mice is mirrored in CEA vs CEACAM6 expression in normal human colonic tissue (Cournoyer et al. 1988).

Molecular weights of the CEACAM family members expressed in human cells and in transfectants of various animal cell lines have often been found to be variable due to variable glycosylation (Yamahita and Kobata 1996), giving multiple broad bands on immunoblots. In the CEABAC mice, the molecular weights of CEA in most tissues (180 KDa) were comparable to known human values (Hammarstrom et al. 1998). The molecular weight of CEACAM6 in bone marrow (50 & 90 KDa) was also found to be similar to that of human granulocytes (Hammarstrom et al. 1998). The molecular weight of the major band of CEACAM6 in many tissues, however, was found to be 75-90 KDa, which is broader than that found in human tissues (Hammarstrom et al. 1998). In rectal tissue, both CEA and CEACAM6 had surprisingly higher molecular weights than expected (Hammarstrom et al. 1998). Since splice variants giving variable molecular weights have never been observed for CEA and CEACAM6, these results are most likely indicative of higher levels of glycosylation. Thus, although the spatiotemporal expression pattern of CEA and CEACAM6 was highly conserved between humans and mice, machinery responsible for post-translational modifications may not be completely conserved, leading to differences in glycosylation of some CEACAM family members in some tissues.

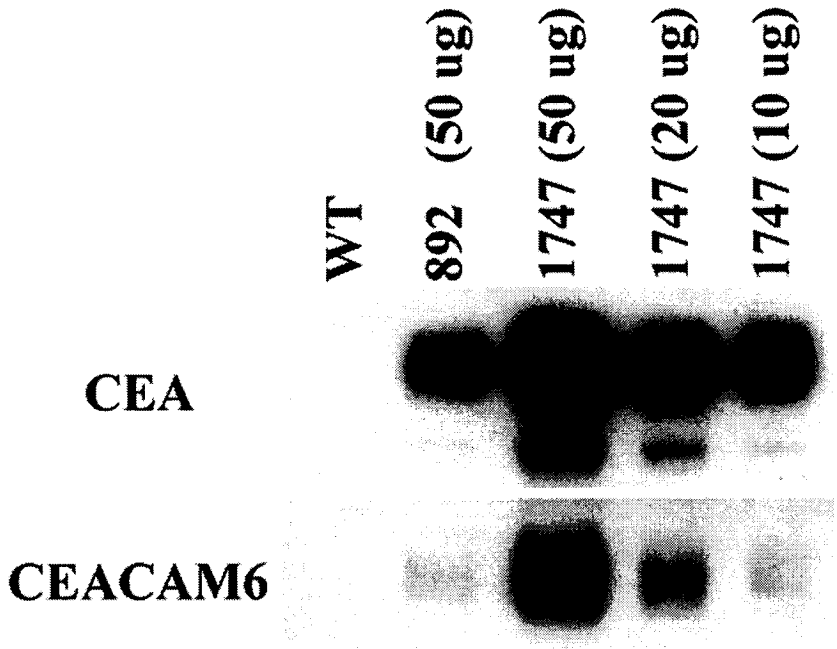

Figure 4: Gene dosage-dependent expression of CEA and CEACAM6. Immunoblots using mAb B18 (specific for both CEA and CEACAM6) for detection of CEA (upper panel) and CEACAM6 (lower panel) in protein extracts of WT, CEABAC-892 and CEABAC-1747 mouse colons. Lane 1: WT colon control; Lane 2: CEABAC-892 colon protein extract (50 ug); Lane 3-5: different dilutions of CEABAC-1747 colon protein extract (50, 20 and 10 ug, respectively). Note that the expression levels of CEA and CEACAM6 are approximately 5-fold higher in CEABAC-1747 than CEABAC-892 by comparing the intensity of bands obtained from lane 2 and lane 5.

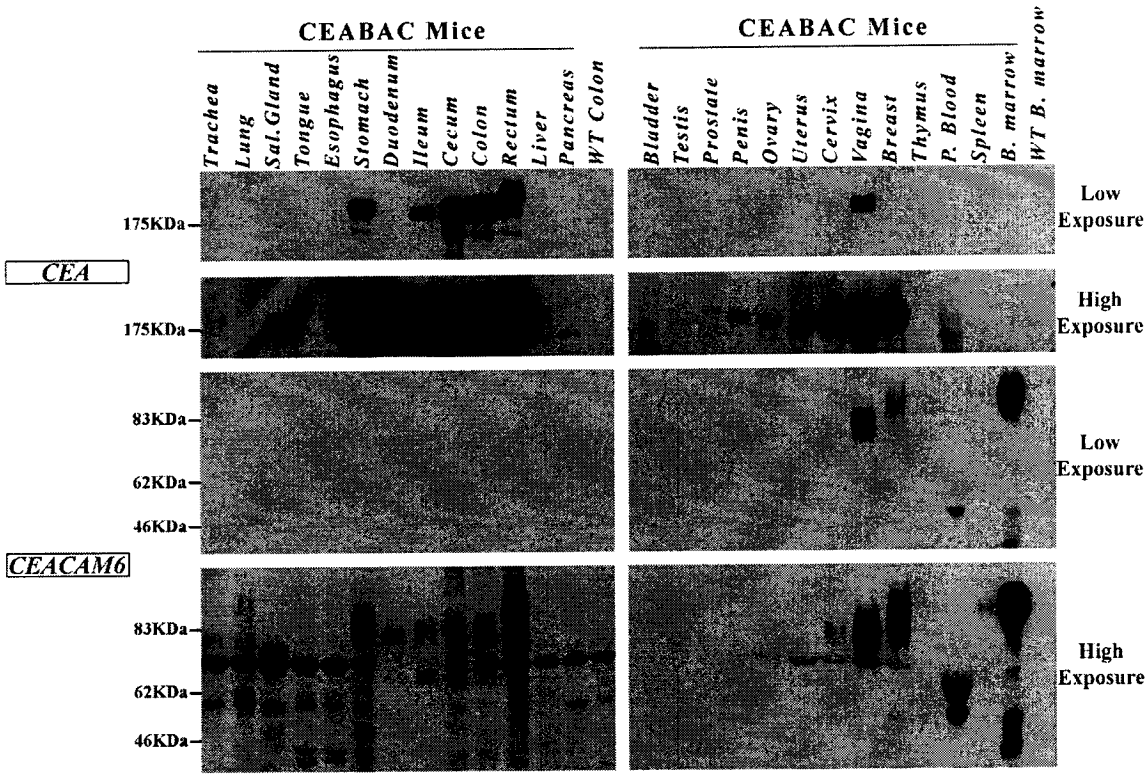

Figure 5: Tissue specific expression of CEA and CEACAM6 from immunoblots. Lanes 1 to 13 and 15 to 27: immunoblots using mAb B18 (specific for both CEA and CEACAM6) for detection of CEA (the lower M_r CEACAM6 region of the blot is not shown; and identical but much more weakly detectable bands in higher M_r region were obtained using a CEA-specific mAb, data not shown), and mAb 9A6 for detection of CEACAM6, after electrophorectic resolution of 50 µg protein from extracts of the indicated tissues (200 µg protein from peripheral blood) of CEABAC mice. Lanes 14 and 28: protein extracts of colon and bone marrow, respectively, from wild-type (WT) control littermates. The lower panels of each of the CEA and CEACAM6 immunoblots represent longer film exposures of the same blots by a factor of 300 for CEA and 300 (left panel) or 30 (right panel) for CEACAM6. The presence of a prominent background band (~70 KDa) in most of the samples (except small intestine) including the WT controls (lanes 14 and 28) is due to ubiquitous endogenous mouse IgG molecules (not detected when rabbit anti-CEA Ab and anti-rabbit secondary antibody was used – not shown).

Expression of CEACAM3 and CEACAM7

CEACAM3 and CEACAM7 were expected to be expressed at a very low level in mature neutrophils and colonocytes, respectively, and in fact could not be easily detected by immunoblots of total protein extracts (data not shown). Hence, the expression pattern of CEACAM3 and CEACAM7 was assessed by RT-PCR in selected tissues. CEACAM3 could not be detected in any tested tissues including spleen where the proportion of mature neutrophils is high relative to other hematopoietic organs (data not shown). Since CEACAM3 mRNA could not be detected in a human spleen RNA sample using the same RT-PCR conditions, the expression of CEACAM3 in mature neutrophils was inconclusive. In the gastrointestinal tract, CEACAM7 could be detected only in the colon (Fig. 6). CEA and CEACAM6 RT-PCR results are included in Figure 6 as positive controls. Thus, the expression of CEACAM7 was highly restricted, as observed in humans (Hammarstrom et al. 1998).

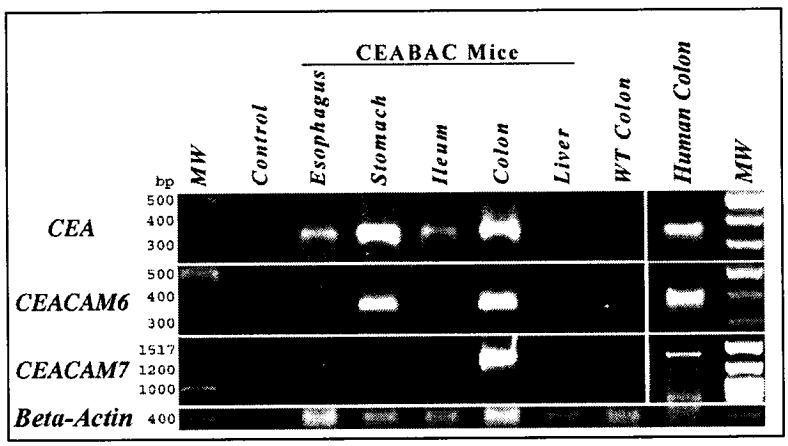

Figure 6: Tissue-specific expression of CEACAM family members from RT-PCR. From left to right: 100 bp ladder marker (MW); RT-PCR buffer control; RT-PCR products of RNA extracted from esophagus, stomach, ileum, colon and liver of CEABAC mice respectively; RT-PCR control of RNA extracted from the colon of a wild-type control littermate; RT-PCR control of human colon RNA; MW markers. From top to bottom: RT-PCR for CEA mRNA (expected size: 338 bp) – positive for esophagus, stomach, small intestine, and colon; RT-PCR for CEACAM6 mRNA (expected size: 353 bp) – positive for stomach and colon; RT-PCR for CEACAM7 mRNA (expected size: 1423 bp) – positive for colon only; and RT-PCR for mouse β-actin mRNA (expected size: 405 bp) – positive for mouse samples only.

Immunohistochemical Analysis of CEACAM Family Expression

Although individual CEACAM family members could be distinguished using specific mouse monoclonal antibodies, ubiquitous endogenous mouse immunoglobulins rendered their localization technically difficult. Therefore, immunohistochemistry was carried out using polyclonal rabbit anti-CEA antibody which detects all human CEACAM family members, but fails to bind to any of the mouse CEACAM1 proteins (Fig. 5, WT colon and bone marrow lanes and data not shown), with results shown in Figure 7; the actual family member(s) detected can be deduced by referral to the immunoblots of Figure 5. These results were confirmed using mouse monoclonal antibodies specific for CEA and CEACAM6 with special provision for the presence of mouse immunoglobulins but are not shown here due to the persistence of some background. The highest expression of CEACAM family members was found in colon followed by stomach and vagina (in agreement with levels detected by immunoblots). The expression of CEA (CEACAM6 and CEACAM7 are expressed at much lower levels in the colon – Figs. 5 & 6) was found to be localized to the apical surface of differentiated colonocytes at the top of colonic crypts; expression decreased towards the proliferative zone at the base of the crypts (Fig. 7H). Along the alimentary tract (Fig. 7A-H), CEA and/or CEACAM6 were also detected on the fusiform epithelium of the tongue (A), the luminal surface of the salivary glands (B) and esophagus (C), the mucosal surface of the gastric stomach (D), pits of the pyloric stomach (E), and crypts and villi of the small intestine [with a higher level in the duodenum (F) than the ileum (G), contrary to the immunoblot result]. CEA and/or CEACAM6 were also detected in other epithelial tissues (Fig. 7J-R): the ductal epithelium of the female breast (J) in the bronchial epithelium of the trachea (K) and lung (L), the mucosal surface of the urinary bladder (M), the glandular epithelium of the cervix (N), the squamous epithelium of the vagina (O), the luminal surface of the lateral and dorsal prostatic glands (P), and the epithelia of the male penile urethra (Q) and skin (R). Importantly, CEACAM6 can be detected in the bone marrow where positive immature myelopoietic cells and a small number of mature monocytes and neutrophils were found (Fig. 7S, T and U). Based on the nuclear morphology, cell size and presence of the azurophilic granules (appearing purplish with Wright's stain, data not shown), CEACAM6, similar to human expression (Watt et al. 1991), was first seen at low levels

in the early promyelocyte stage (Fig. 7S), then at gradually increasing levels during neutrophilic differentiation (Fig. 7T), and finally back to low levels in mature neutrophils (Fig. 7U). CEACAM6 could also be detected in cells of the monocytic lineage, but not in other lineages (data not shown). No staining (except for neutrophils that were present) could be detected in other tissues, including kidney, heart, thymus and liver (data not shown).

The tissue-specific expression patterns of the 3 CEACAM family members are summarized in Table 2. They show remarkable similarity to the human pattern, with a few notable exceptions that are discussed below.

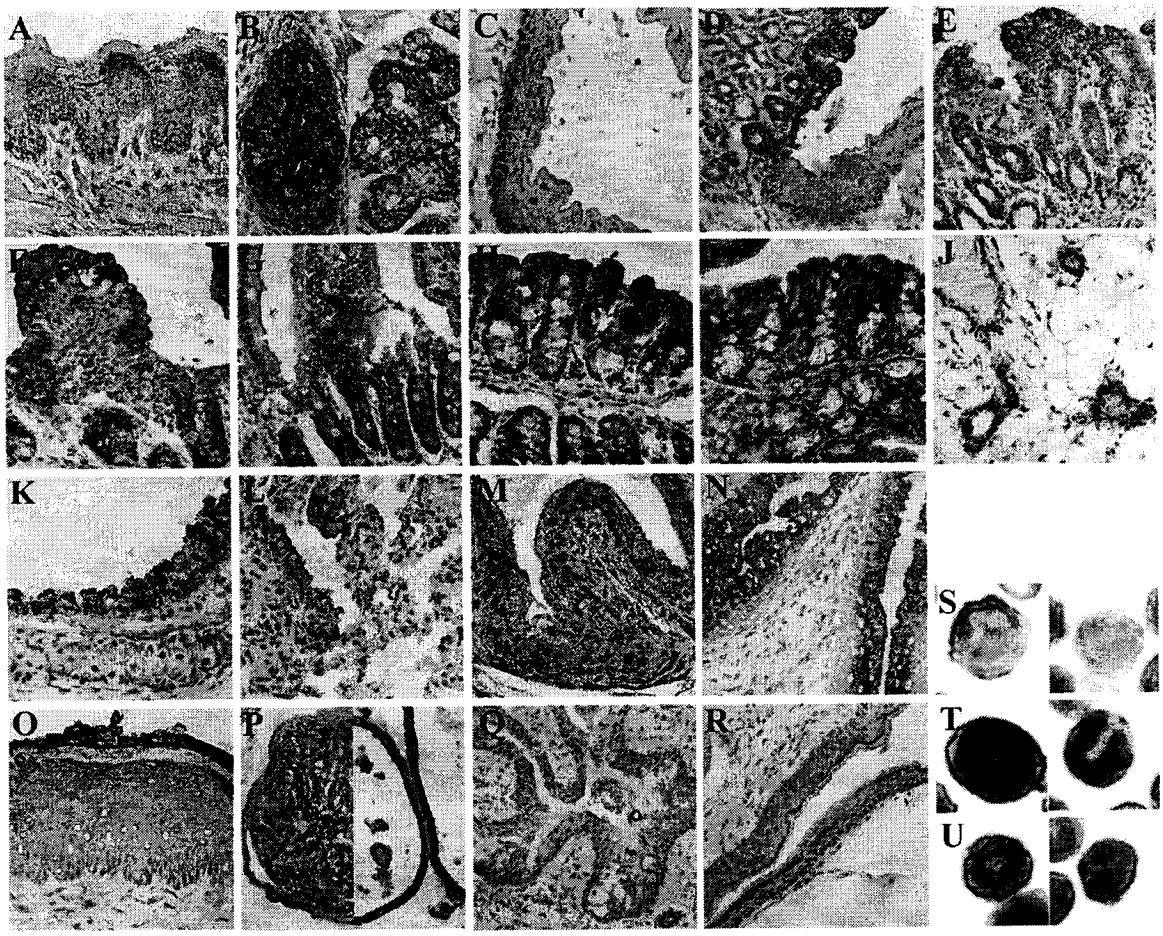

Figure 7: Patterns of tissue expression from immunohistochemistry. All sections were incubated at room temperature with RbαCEA as primary antibody, HRP-conjugated anti-rabbit antibody as secondary antibody and DAB substrate then counterstained with

hematoxylin. RbαCEA antibody dilution: 1:2500 (**A-C**, **G**, **J-N**, **P-U**), 1:5000 (**D-F** and **O**), and 1:10,000 (**H** and **I**). Original magnification: 400X (**A-R**) and 1000X (**S-U**). **A)** Tongue epithelium – positive cluster of cells in the fusiform epithelium; **B)** Serous salivary glands (left) and mucous salivary gland (right) – positive epithelial cells; **C)** Esophagus – weak positive luminal surface; **D)** Gastric stomach adjacent to esophagus – positive mucosal surface only; **E)** Pyloric stomach – positive staining only in the pits, but not in the glands; **F)** Duodenum adjacent to the pyloric stomach – positive staining throughout the epithelium of the crypts and villi; **G)** Ileum – positive staining on the apical surface of the epithelial cells throughout the crypts and much less in the villi; **H)** Colonic epithelium – intense mucosal surface staining and only on the apical surface of the cells with decreasing gradient from top to bottom; **I)** Colonic epithelium from a wild-type control littermate – no background staining; **J)** Breast – positive epithelial cells and luminal content; **K)** Trachea – positive epithelial cells; **L)** Lung – positive bronchiole; **M)** Urinary bladder – positive staining of the cuboidal cells of the transitional epithelium; **N)** Cervix – positive glandular epithelium; **O)** Vagina – positive stratified squamous epithelium; **P)** Dorsal prostate (left) and lateral prostate (right) - positive staining on the luminal surface of the prostatic glands; **Q)** Male urethra – weak positive staining on the luminal surface; **R)** Penile skin – clusters of positive cells in the stratified squamous epithelium; **S)** Bone marrow immature granulocyte (early promyelocyte stage) – weak surface staining (left) and wild-type control (right); **T)** Bone marrow immature neutrophil (band cell) – strong surface staining (left) and wild-type control (right); and **U)** Bone marrow mature neutrophil – intermediate surface staining (left) and wild-type control (right). Note that only wild-type controls for colon and immature or mature granulocytes are presented; however, parallel staining of all tissue sections from the wild-type control mice were completely negative (data not shown).

TABLE 2: Tissue-specific expression of the human CEACAM family members in the CEABAC transgenic mice

Tissues Examined	CEA			CEACAM6			CEACAM7			Localization
	Human	CEABAC		Human	CEABAC		Human	CEABAC		
		Protein	RNA		Protein	RNA		Protein	RNA	
Gastrointestinal										
Tongue	+	+(?)		+	+(?)		-			Cluster in fusiform epithelium
Salivary Gland		+		+	+		-			Apical surface of epithelium cells
Esophagus	+	+(?)	+	+	+(?)	+/-	-			Apical surface of epithelium cells
Stomach	+	++	+	+	+	+	-			Mucosal surface (gastric stomach); pit (pyloric stomach)
Duodenum	+	+		-	+		-			Enterocytes of crypt and villi
Jejunum/Ileum	-	+	+	-	+	-	-			Apical surface of enterocytes of crypt and villi
Cecum	+	+++		+	+					Apical surface of enterocytes and goblet cells, mucosal surface of crypts
Colorectum	+	+++	+	+	+	+	+	+	+	Apical surface of enterocytes and goblet cells, mucosal surface of crypts
Pancreas	+/-	-	-	+	-	-	+		-	
Liver	-	-	-	-	-	-	-			
Urogenital										
Kidney	-	-		-	-					
Urinary Bladder	+	+(?)		-	+(?)					Cuboidal cells of the transitional epithelium
Prostate	+	+(?)		+	+(?)					Luminal surface of dorsal and lateral prostatic glands
Penis		+			-					Mucosal surface of the urethra; epithelia of the skin
Uterus	-	-		-	-					
Cervix	+	+		+	+					Glandular epithelial cells
Vagina		++			++					Squamous epithelial cells
Ovaries	-	+(?)		-	+(?)					Follicular cells of a small number of ova
Testes	-	-		-	-					
Seminal Vesicle		-		-	-					
Hematopoietic										
Thymus	-	-		-	-					
Bone Marrow	-	-		+	+++	+				Immature granulocytes
Spleen	-	-		+	+	+				Neutrophils
Lymphocytes	-	-		-	-					
Granulocytes	-	-		+	+					Cell Surface
Monocytes	-	-		+	+					Cell Surface
Others										
Trachea	+	+	+		+	+				Epithelial cells
Lung/Bronchi	+/-	-	+	+	+	+				Bronchiolar epithelial cells
Heart	-	-		-	-					
Female Breast	+/-	+		+	+					Epithelial cells of the mammary ducts, luminal contents
Adrenal Gland	-	-		-	-					
Thyroid Gland	-	-		-	-					
Muscle	-	-		-	-					
Brain	-	-		-	-					

Expression in human was summarized from literature (Hammarstrom et al. 1998; Scholzel et al. 2000), whereas expression in CEABAC mice was determined by immunohistochemistry using RbαCEA, immunoblotting using mAb B18 (CEA and CEACAM6 specific) and 9A6 (CEACAM6 specific) and RT-PCR using CEA-, CEACAM6-, and CEACAM7- specific primers. Expression levels of CEA and CEACAM6 in different tissues of CEABAC mice were compared by immunohistochemistry and/or immunoblotting: high level (+++), medium level (++), detectable level (+), and undetectable (-). +(?) denotes inconclusive result due to indistinguishable detection between CEA and CEACAM6 by immunohistochemistry; +/- denotes uncertain expression due to very low level; blank space denotes untested tissue.

DISCUSSION

The CEACAM gene family is a large group of homotypic/heterotypic intercellular adhesion molecules (Hammarstrom et al. 1998), each of which has a specific tissue distribution with presumably specific functions in various tissues. CEA, over-expressed in as many as 70% of all human cancers (Chevinsky 1991; Hammarstrom et al. 1998; Hammarstrom 1999), is a popular target for novel cancer therapies, including cancer vaccines, cellular immunotherapy, radioimmunotherapy, antibody therapy and gene therapy (Chester et al. 2000; Fong et al. 2001; Koch et al. 2001; Kousparou et al. 2002; Goldenberg 2003; Marshall 2003). Although pre-clinical animal data can show promising effects, clinical outcomes have commonly been unfavorable, such as low therapeutic response or the presence of associated toxicity. This discrepancy suggests improper or insufficient assessment by present animal models. Although previously constructed transgenic mice bearing the CEA gene alone can improve the validity of pre-clinical tests (Mizobata et al. 2000; Xu et al. 2000; Wilkinson et al. 2002), a transgenic model with a closer human approximation is necessary to ensure valid pharmacokinetics of test agents and to avoid possible treatment toxicity and/or reduced efficacy from cross-reaction with other highly homologous human CEACAM family members.

Although the minimal transcriptional promoter (included in the 3.3 kb 5'-upstream sequences used in previous CEA transgenic mice) of the CEA gene was shown to confer spatiotemporal expression in previous studies (Eades-Perner et al. 1994; Clarke et al. 1998), the promoters/enhancers of the CEACAM6 gene (Koops et al. 1998) and other family members are incompletely or not at all characterized, not to mention the possibility of *cis*-interactions of transcription factors between these closely linked and highly homologous genes. Thus, the use of the entire human CEACAM family gene locus in transgenics should provide more accurate models than those obtainable by crossing multiple transgenic lines and, in any event, no CEACAM6 gene transgenic has been reported. Although multiple linked-gene transgenesis can be achieved using YACs (yeast artificial chromosomes) (Huxley 1998), a 187 kb BAC that holds part of the proximal CEACAM family gene cluster containing 4 of the 7 expressed CEACAM family genes was used for the following reasons. BAC clones, unlike YAC clones, are rarely hybrids of

unrelated genomic regions and do not usually undergo deletions or rearrangements when propagated in bacteria (Kim et al. 1996; Song et al. 2001). YAC DNA, being usually longer, is also more prone to fragmentation during purification, microinjection and genomic integration (Huxley 1998). Like YAC clones, BAC expression is unlikely to be influenced by the genomic integration site in transgenics, simply because of size (Huxley 1998). Moreover, the CEABAC contains most of the human family genes except CEACAM1 (although the mouse homologue of this gene is present in the transgenics), CEACAM8 (another GPI-anchored family member, formerly denoted CGM6, and expressed only in granulocytes) and CEACAM4 (formerly denoted CGM7 and similar in structure and limited expression pattern to CEACAM3) (Hammarstrom et al. 1998). Thus, although some human CEACAM family genes were omitted, those that are more widely expressed and at higher levels are all included.

Two CEABAC transgenic lines (892 and 1747) expressing CEACAM family members were successfully generated and individual identification in mouse line maintenance could be easily achieved by PCR using CEA-specific primers, since CEA-positive offspring would inherit the entire CEABAC locus. The expression pattern of CEA, CEACAM6, and CEACAM7 in both CEABAC transgenic lines was shown to be very similar to that observed in humans, both spatially and in relative level. Expression levels were proportional to their CEABAC copy numbers indicating, as expected, little or no influence of their different integration sites. In brief, CEA was found to have a more restricted expression pattern, with the highest levels in colon, stomach and vagina, than CEACAM6, which was shown to have a broader expression pattern with the highest levels in bone marrow (primarily immature myelocytes) and vagina. Expression of CEACAM7 was shown to be highly restricted to colorectum. The presence of CEACAM3 in mature neutrophils remained inconclusive due to the fact that it is expressed at low levels in a small population of cells in a given tissue in humans and, presumably in the CEABAC transgenics. However, the fact that CEACAM3 was weakly detected in occasional tissue neutrophils using CEACAM3-specific monoclonal antibody suggests that it is, in fact, expressed in CEABAC mouse neutrophils (data not shown). The expression of human CEA and CEACAM6 in the penile urethra and vagina of the CEABAC mice was unexpected and suggests the possibility of similar expression in

humans, where apparently no such studies have been reported. However, unlike the human situation, CEA and CEACAM7 could not be detected in the pancreas. This may be due to the sensitivity of the detection methods employed or intrinsic differences between humans and mice. Actually, the anatomical structures of the pancreas of humans (a well-defined organ) and mice (ill-defined fat-like tissue) are radically different so that different expression patterns, either qualitative or quantitative, are perhaps not surprising. Based on the conservation of the complex spatiotemporal expression pattern, presumably due to the conservation in evolution of most, if not all, of the relevant *trans*- and *cis*-transcriptional regulating elements, our CEABAC mice are suitable for the pre-clinical trials of CEA-targeted agents using existing protocols (Mizobata et al. 2000; Xu et al. 2000; Wilkinson et al. 2002), as explained earlier.

Since CEACAM6 is also over-expressed in a variety of adenocarcinomas (Hammarstrom et al. 1998) and leukemias (Boccuni et al. 1998; Hansen et al. 1998), CEACAM6-targeted therapies, such as radioimmunotherapy for acute leukemia using radiolabeled anti-CEACAM6 antibodies (Burke et al. 2002), are possible. Hence, this mouse model can be used for pre-clinical trials of novel CEACAM6-targeted cancer therapy.

The expression of the CEACAM family genes in gastrointestinal, breast, respiratory, urogenital and hematopoietic systems in the CEABAC mice could facilitate the search for therapeutic agents against various kinds of cancer and other diseases associated with the human CEACAM family and also provides an adequate model for basic studies. Examples include: 1) CEA and CEACAM6 have been shown to contribute directly to tumorigenesis (Stanners 1998; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002) and metastasis (Jessup and Thomas 1998; Kim et al. 1999; Leconte et al. 1999; Wirth et al. 2002) in various model systems. Over-expression of CEA and CEACAM6 blocks cellular differentiation (Stanners 1998), disrupts cellular polarization and normal colonic tissue architecture (Ilantzis et al. 2002), and leads to an inhibition of anoikis (Ordonez et al. 2000; Soeth et al. 2001), i.e., death by apoptosis of cells detached from their extracellular matrix. Thus, reversal of these effects represents a novel therapeutic approach and can be exploited in this present mouse model. 2) *Neisseria Gonorrhoeae* infection, a sexually transmitted disease, uses CEACAM family members

as major binding receptors (Dehio et al. 1998). Thus agents, which inhibit this interaction *in vivo*, may be useful for prevention and their effectiveness can be assessed in this mouse model. 3) Human CEACAM family members can activate neutrophils and increase their adhesion to endothelial cells and extracellular matrices (Kuijpers et al. 1992; Nair and Zingde 2001; Skubitz et al. 2001). They can also induce cytokine release from immune cells (Gangopadhyay et al. 1996; Filella et al. 2001). These properties suggest their role in inflammatory reactions and their potential use as targets for anti-inflammatory drugs.

In conclusion, the human-like expression pattern of 3 and possibly 4 human CEACAM family genes along with a simple strategy for mouse line maintenance should make this novel transgenic mouse the model of choice for therapeutic trials of CEA-targeted therapies. This may also lead to better understanding of various diseases associated with these human-specific CEACAM family members, and eventually, development of more successful therapies.

Chapter 3

Increased Susceptibility of Carcinogen-induced Colon Tumors in CEABAC Transgenic Mice

ABSTRACT

Human carcinoembryonic antigen (CEA), a widely used clinical tumor marker, and its close relative, CEACAM6, are often over-expressed in many cancers. This correlation suggests a possible instrumental role in tumor progression, which is supported by extensive results obtained with several systems *in vitro* and *ex vivo*. The implication that these results could also apply *in vivo*, however, requires confirmation. Since mice do not possess homologs of the CEA and CEACAM6 genes, we have constructed transgenic mice harboring a 187 kb portion of the human CEACAM family gene locus contained in a bacterial artificial chromosome (CEABAC) that includes CEA and CEACAM6. In this study, we treated the CEABAC mice and their wild-type littermates with azoxymethane (AOM) in order to induce colon tumor formation. Untreated CEABAC transgenics do not develop colonic tumors but, at 20 weeks post-treatment, the AOM-treated CEABAC transgenics showed more than a 2-fold increase in mean tumor load relative to the AOM-treated wild-type littermates, a highly significant difference ($p<0.0001$). Cell surface expression of CEA and CEACAM6 increased by 2 and 20 fold, respectively, in colonocytes from the tumors relative to colonocytes from non-AOM treated transgenics and a de-regulated spatial pattern of CEA/CEACAM6 expression was found in "normal" crypts adjacent to the tumors, thus mimicking closely the situation in human colon tumorigenesis. This study supports the hypothesis that CEA and CEACAM6 over-expression can have an instrumental role in colon tumorigenesis *in vivo* and also presents a useful mouse model for the development of CEA-based therapies.

INTRODUCTION

Human carcinoembryonic antigen (CEA) family genes, that encode a group of highly glycosylated homotypic/heterotypic intercellular adhesion molecules, are closely clustered on chromosome 19q13.2 and represent a subset of the immunoglobulin superfamily (Hammarstrom et al. 1998). The glycophosphatidyl inositol (GPI)-anchored family members, CEA and CEACAM6, are over-expressed in as many as 70% of all human tumors, a fact that underlies their widespread use as tumor markers in the cancer clinic (Chevinsky 1991; Ballesta et al. 1995; Jantscheff et al. 2003). This overwhelming correlation suggests an instrumental role for these molecules in tumorigenesis. In human patients, over-expression of CEA and CEACAM6 is often associated with poor prognosis (Ballesta et al. 1995; Jantscheff et al. 2003). Over-expression of CEA and CEACAM6 *in vitro* was also shown to elicit various tumorigenic effects, i.e., blockage of cell differentiation (Eidelman et al. 1993; Rojas et al. 1996; Ilantzis et al. 2002), inhibition of anoikis/apoptosis (Ordonez et al. 2000; Soeth et al. 2001; Duxbury et al. 2004a) and disruption of tissue architecture (Ilantzis et al. 2002). More direct evidence utilizing *in vivo* models, however, has been difficult to acquire due to the lack of informative animal models, since GPI-anchored CEACAM family genes are present only in primates (Hammarstrom et al. 1998; Tobi et al. 2000; Zhou et al. 2001; Naghibalhossaini and Stanners in preparation). CEA-only transgenic mice were previously constructed to test this hypothesis but showed no obvious tumorigenic phenotype (Eades-Perner et al. 1994; Clarke et al. 1998) nor synergistic effects by mating with other tumor-prone mouse models, including those bearing APC mutations (Thompson et al. 1997). This could be due to expression levels that were below a threshold level required for tumorigenesis or to the absence of other human GPI-anchored CEACAM family members, notably CEACAM6.

A mouse model that better mimics the human situation, in which part of the human CEACAM family gene locus, including genes coding for CEA, CEACAM6, CEACAM7 and CEACAM3, propagated in a 187 kb bacterial artificial chromosome (CEABAC), has been constructed recently on the FVB genetic background (Chan and Stanners 2004). Two independent transgenic lines showed remarkably similar expression

patterns of these CEACAM family genes to that of humans (Chan and Stanners 2004). Gastrointestinal tumors could not be found in aging wild-type FVB mice (Mahler et al. 1996) and the colonic expression of the human CEACAM family genes, at least at the levels seen in these two CEABAC transgenics, had no easily detectable effect on the incidence of colon tumors.

Azoxymethane (AOM), a colon-specific carcinogen, induces focal tumors in murine colons. The guanine bases in genomic DNA can be methylated by AOM forming O^6-methylguanine adducts. The latter can either be repaired by methylguanine methyltransferase (Zak et al. 1994) or trigger apoptosis (Hickman and Samson 2004). Actively proliferating cells that escape these two protective mechanisms will acquire G→A base conversions after two rounds of DNA replication (Wali et al. 1999). Mutations that provide survival or growth advantage will be selected for and will be found at high frequency in tumors. For example, mutations in Kras and β-catenin are frequently found in AOM-induced tumors (Yamada et al. 2003). Hence, this carcinogen provides a useful tool to assess colon tumor susceptibility in different murine strains, including transgenics and knockouts, whose cells may have strain-specific different rates of apoptosis, proliferation and repair.

To evaluate the tumorigenic effects of CEA and CEACAM6 anticipated by the *in vitro* studies, the CEABAC mice were given weekly injections of AOM for 6 weeks. A highly significant 2-fold increase in tumor load was obtained in the CEABAC mice at 20 weeks post-treatment. These additional tumors were likely derived from small tumors observed to be present at a 4-fold increased incidence in the CEABAC mice at an early time point. A de-regulated cryptal expression pattern of CEA/CEACAM6 and a 2- and 20-fold over-expression of CEA and CEACAM6, respectively, were observed in these tumors, as seen in human colon cancers. Altogether, these results show that the expression of human CEA and CEACAM6 genes predisposes mice to sporadic colon tumors. This suggests that they could play a significant role in the development of human colon tumors.

MATERIALS AND METHODS

Animals

The generation of the CEABAC transgenic mice harboring a 187 kb bacterial artificial chromosome that contains complete human CEA, CEACAM6, CEACAM7, and CEACAM3 genes was reported elsewhere (Chan and Stanners 2004). These mice were generated and maintained on the FVB (Harlan Bioproducts for Science Inc., Indianapolis, IN) genetic background. The CEABAC2 and CEABAC10 transgenic lines possess 2 and 10 copies of the transgenes, respectively, in a head to tail orientation in a single cluster (Chan and Stanners 2004). Age-matched wild-type controls were obtained from breeding between CEABAC and FVB mice. All mice were housed under controlled conditions of a 12 hour light/dark cycle, 23 ± 2 °C room temperature and 50 ± 10 % relative humidity. Food and water were available *ad libitum*.

Azoxymethane treatment and analytical methods

For tumor induction, 3 to 4 month-old CEABAC2, CEABAC10 and WT mice were treated with 6 weekly intra-peritoneal injections of azoxymethane (National Cancer Institute, Bethesda, MD) at a dose of 10 mg/kg body weight. Untreated 3 to 4 month-old CEABAC2, CEABAC10 and WT mice were the negative controls for the experiment. Animals were sacrificed at 6 and 20 weeks after the last injection. Colons were cut open longitudinally, fixed with 4% paraformaldehyde for 16 hours at 4°C, stained with 2% methylene blue and examined under a dissecting microscope. Tumor number and volumes (length × width × height) were recorded for each AOM-treated mouse.

Histological and immunohistochemical analysis

For histological analysis, freshly excised tumors were fixed in Glyo-Fixx (20% ethanol, 5% glyoxal, 1% propanol and 1% methanol) for 48 hours at room temperature and processed for embedding in paraffin blocks for sectioning. Sections were stained with hematoxylin and eosin. For immunohistochemical analysis, tissues were fixed with 4% paraformaldehyde for 16 hours at 4°C and quickly frozen with isopentane at –70°C. 7 μm frozen sections were obtained using a cryostat at –25°C. Frozen sections were stained for

CEACAM family members using rabbit polyclonal anti-CEA antibody at a dilution of 1:10,000 and anti-rabbit Envision Reagent (DAKO Diagnostics Canada Inc., Mississauga, ON). Sections were developed with DAB (3'3'-diaminobenzidine) for 5 min and were counterstained with Mayer's hematoxylin (Sigma-Aldrich Canada Ltd., Oakville, ON).

FACS analysis

Colon tumors, normal colonic and normal spleen tissues were obtained from freshly resected AOM-induced tumors, untreated normal mucosa and spleen, respectively. Tissues were cut into fine pieces and treated with collagenase solution as described previously (Ilantzis et al. 1997). Single cell suspensions were fixed with 2% paraformaldehyde for 16 hours at 4°C. Cells were labeled with mouse monoclonal antibodies, A20 (specific for CEA) or 9A6 (specific for CEACAM6) and FITC-conjugated goat-anti mouse antibodies. Labeled cells were analysed by FACScanP®P (Becton Dickinson, Bedford, MA).

Statistical analysis

Mean tumor loads and mean tumor volumes of different animal groups were compared using the unpaired two-tailed Student's *t*-tests. The results were considered statistically significant if the *P* values were less than 0.05.

RESULTS

Human CEACAM family gene expression increases AOM-induced tumor incidence

To assess the effect of GPI-anchored CEACAM family members on colon tumorigenesis, WT and CEABAC transgenic mice were given 6 weekly i.p. injections of AOM. FVB mice, the strain used in this study, do not normally develop spontaneous colon tumors but are susceptible to AOM-induced colon tumor formation (Singh et al. 2000). Fifty two WT and 60 CEABAC mice were treated with AOM and 38 WT and 51 CEABAC mice were left untreated as controls. As expected, the untreated WT and CEABAC mice showed no evidence of colon tumors. Many of the AOM-treated mice, however, had bloody stools or diarrhea and some had prolapse of the rectum. While the AOM-treated WT mice bore on average 6.2 ± 0.2 tumors/colon, the AOM-treated CEABAC mice bore 14.4 ± 0.3 tumors/colon (Table 1), which is a highly significant difference ($P < 0.0001$).

Table 1: Tumor Load in AOM-treated animals at 20 weeks post-treatment

Mouse Strain [a]	Number of animals	Tumors/colon [b] (Mean ± SEM)	Tumors/colon [b] (Range)	Mean tumor volume [b] (mm^3 ± SEM)
WT	19	6.2 ± 0.8	1 – 14	16.6 ± 3.1
CEABAC2	13	13.9 ± 1.6 [d]	2 – 22	
CEABAC10	12	15.0 ± 2.2 [d]	4 – 25	
CEABAC total [c]	25	14.4 ± 1.3 [d]	2 – 25	15.6 ± 1.9 [e]

[a] Mice were treated with 6 weekly i.p. injections of 10 mg/kg AOM and sacrificed at 20 weeks after the last injection. No lesions were found in untreated mice. A low proportion (6%) of the treated mice (7/112) were found dead during the 6 week injection period, probably due to acute drug toxicity. At 20 weeks post-treatment, 82% of the AOM-treated mice (64/78) survived relative to 100% for the untreated animals.

[b] Scored for tumors sized ≥ $1mm^3$; SEM = standard error of the mean.

[c] CEABAC2 + CEABAC10.

[d] $P < 0.0001$, compared with WT.

[e] $P > 0.05$, compared with WT.

This difference in incidence is most apparent in a plot of single animal tumor burden, demonstrating that most of the AOM-treated WT mice had less than 6 tumors/colon, whereas most of the AOM-treated CEABAC mice had more than 12 tumors/colon with some bearing as many as 25 tumors/colon (Fig. 1). The mean tumor volumes for the WT vs CEABAC mice, however, showed no statistically significant difference (Table 1).

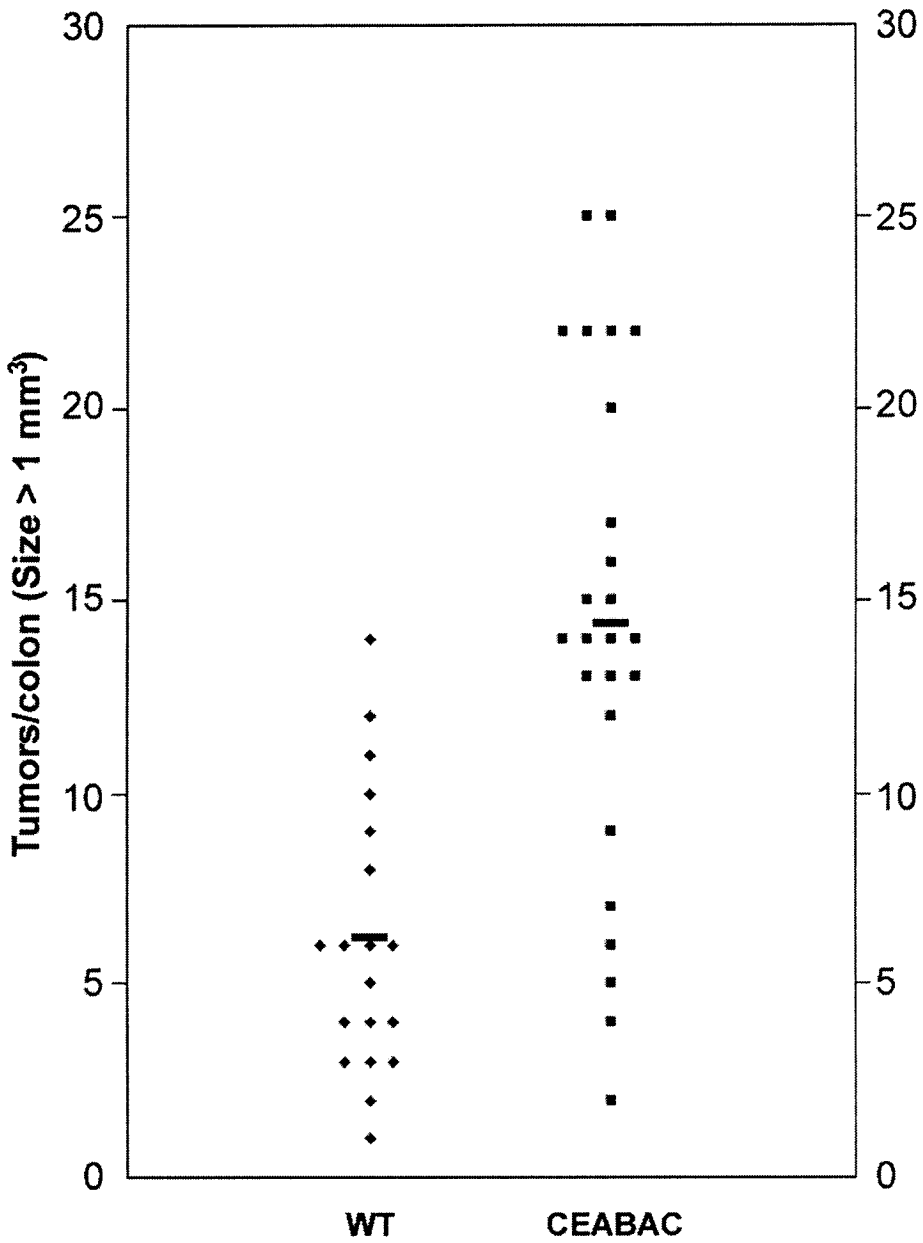

Figure 1: Tumor load of individual animals. Each dot represents the number of colon tumors (with tumor volume > 1 mm^3) in a single animal (WT, ◆; CEABAC2 or CEABAC10, ■) at 20 weeks post-treatment of AOM. The horizontal bar represents the average tumor load for each group of animals (WT, 6.2 tumors/colon; CEABAC, 14.4 tumors/colon). Note that most WT mice had less than 6 tumors/colon and most CEABAC mice had more than 12 tumors/colon.

The increase in incidence was observed for both CEABAC2 and CEABAC10 mice, two independent transgenic lines, ruling out possible effects of the integration site of the transgenes. There was no apparent difference in the tumor load between male and female animals ($P >> 0.05$); no correlation between body weight or cumulative AOM dose (i.e., total amount of AOM injected over the 6 weeks treatment period) and tumor load ($R^2 << 0.95$) could be shown (data not shown). All AOM-induced tumors were present in the distal colon and rectum, as previously reported (Singh et al. 2000). Histologically, the tumors were adenomas and adenocarcinomas in both WT and CEABAC mice (Fig. 2). Thus, the presence of human GPI-anchored CEACAM family genes in mouse colon increases tumor formation induced by carcinogen treatment.

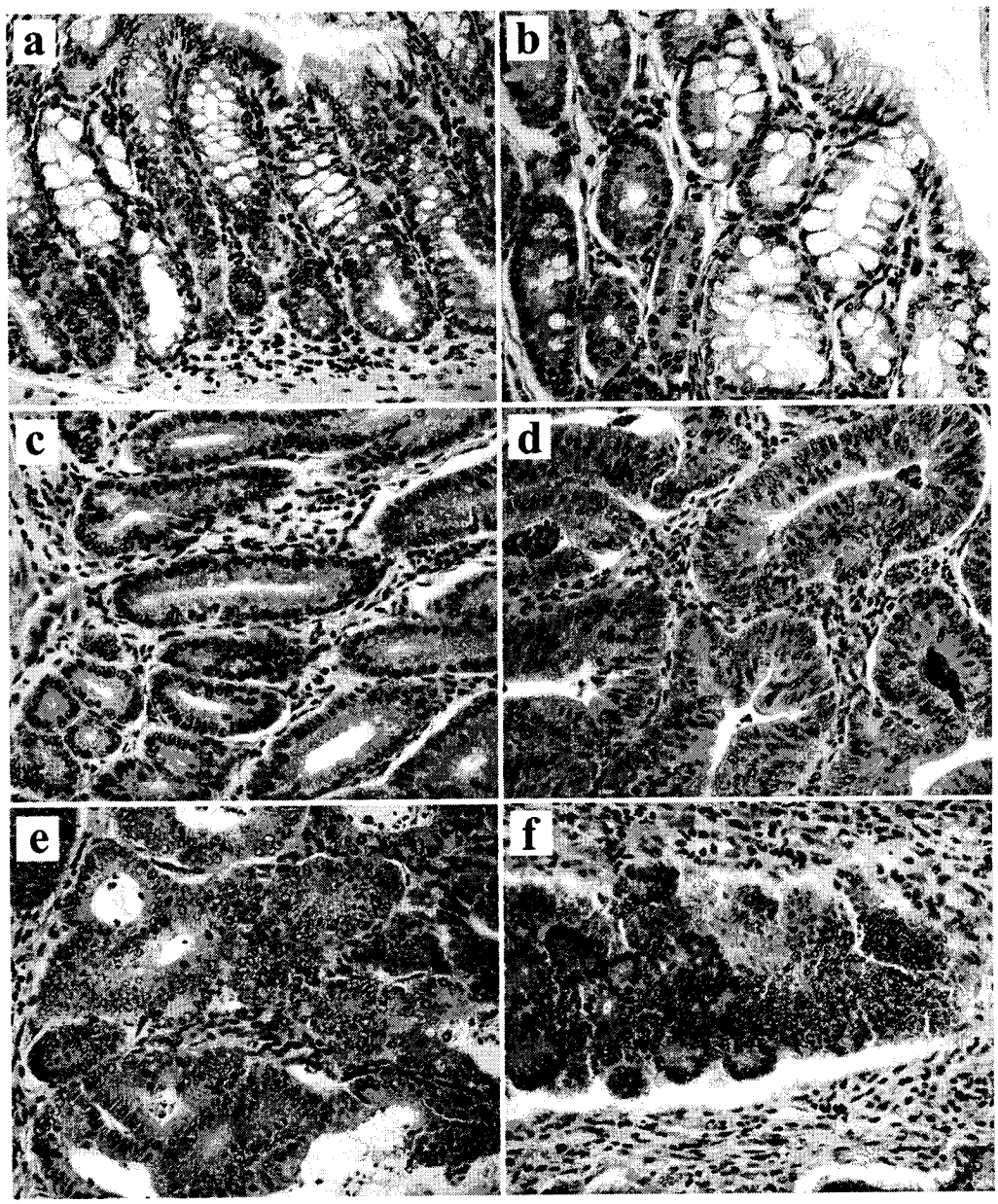

Figure 2: Histology of AOM-treated colonic epithelia. All sections were taken from an AOM-treated CEABAC10 mouse colon at 20 weeks post-treatment but similar histology was also found in AOM-treated WT and CEABAC2 mouse colons (data not shown). Light microscopy of H&E stained paraffin-embedded sections (×400). **a)** Normal colonic epithelium adjacent to tumor. **b)** Junction between early neoplastic lesion (left) and normal colonic crypts (right). **c)** Adenoma with low-grade dysplasia. **d)** Adenoma with high-grade dysplasia. **e, f)** adenocarcinomas.

CEABAC mice bear significantly more small tumors at an early time point

To further investigate the origin of the additional tumors in the CEABAC mice, AOM-treated mice were sacrificed at earlier time points. Using the same criteria to score for tumors (i.e., volume > 1 mm^3), there was only a slight difference between WT and CEABAC mice at 6 weeks post-treatment, which was statistically insignificant (Table 2). However, the quantity of small lesions (volume < 1 mm^3 but with protrusion into the lumen), known as microadenomas, was significantly higher (4 fold) in the CEABAC mice (Table 2). Interestingly, the sum of large and small tumors at 6 weeks yields a similar tumor load to that observed at 20 weeks post-treatment (Table 2), suggesting that the additional tumors in the latter originated from these small lesions. Histologically, the latter were small adenomas similar to that shown in Figure 2c.

Table 2: Tumor Load in AOM-treated animals at 6 weeks post-treatment

Mouse Strain [a]	Number of animals	Large tumors/colon [b] (Mean ± SEM)	Small tumors/colon [c] (Mean ± SEM)	Total tumors/colon (Mean ± SEM)
WT	6	3.2 ± 0.7	2.7 ± 1.1	5.8 ± 1.1
CEABAC [d]	8	5.3 ± 1.1 [e]	10.6 ± 2.5 [f]	15.9 ± 2.3 [g]

[a] Mice were treated with 6 weekly i.p. injections of 10 mg/kg AOM and sacrificed at 6 weeks after the last injection; No lesions were found in untreated mice.
[b] Tumors with size ≥ 1mm^3; SEM = standard error of the mean.
[c] Tumors with size < 1mm^3 and protruding into lumen of the gut; SEM = standard error of the mean.
[d] CEABAC2 + CEABAC10.
[e] $P > 0.05$, compared with WT.
[f] $P < 0.03$, compared with WT.
[g] $P < 0.005$, compared with WT.

Expression of CEA and CEACAM6 are de-regulated after AOM treatment

In human colon tumors, CEA and CEACAM6 are often over-expressed (Ballesta et al. 1995; Jantscheff et al. 2003). To determine whether this important feature of human tumors was also present in AOM-induced tumors in the CEABAC mice, single colonocyte suspensions of the colorectal tumors and control tissues (normal colon epithelium and spleen) were obtained from both WT and CEABAC mice. Cell surface expression levels of CEA and CEACAM6 were determined by FACS analysis using specific monoclonal antibodies. Since CEACAM6 is expressed in the neutrophils (Chan and Stanners 2004) that are present in the normal colonic epithelia and colon tumors,

splenocytes (which include neutrophils) were obtained and subjected to FACS analysis in order to allow removal of any such contribution from the colonocyte FACS profiles (Fig. 3a, 3b & 3c). As expected, all single cell suspensions from WT tissues were negative for CEA and CEACAM6, i.e., similar to background levels (data not shown). In the AOM-induced tumors from the CEABAC mice, mean cell surface expression levels of CEA and CEACAM6 in the tumor colonocytes were 2- and 20-fold higher, respectively, than the levels in normal colonocytes from untreated CEABAC mice (Fig. 3d & 3e). In fact, these cell surface ratios were observed to be similarly higher for CEACAM6 than for CEA in purified colonocytes from human colorectal carcinomas (Ilantzis et al. 1997).

In normal human colonic epithelium, CEA and CEACAM6 are mostly expressed in fully differentiated cells near the mucosal surface and on the apical surface of the cells (Ilantzis et al. 1997), which was also observed in the CEABAC mice (Fig. 4a). However, this cryptal expression pattern of CEA and CEACAM6 is usually lost, i.e., similar expression levels are observed throughout the crypts, not only in tumors but also prior to or during tumor formation in "normal" human epithelium adjacent to the tumor (Ilantzis et al. 1997). Moreover, in adjacent "normal" human epithelium, expression of CEA and CEACAM6 is no longer restricted to the apical surface of colonocytes, but seen on the basolateral surface as well and, in some cases, in the cytoplasm (Ilantzis et al. 1997). This de-regulated expression pattern, known as the "field effect" (Jothy et al. 1996), was also observed in the AOM-treated CEABAC mice (Fig. 4).

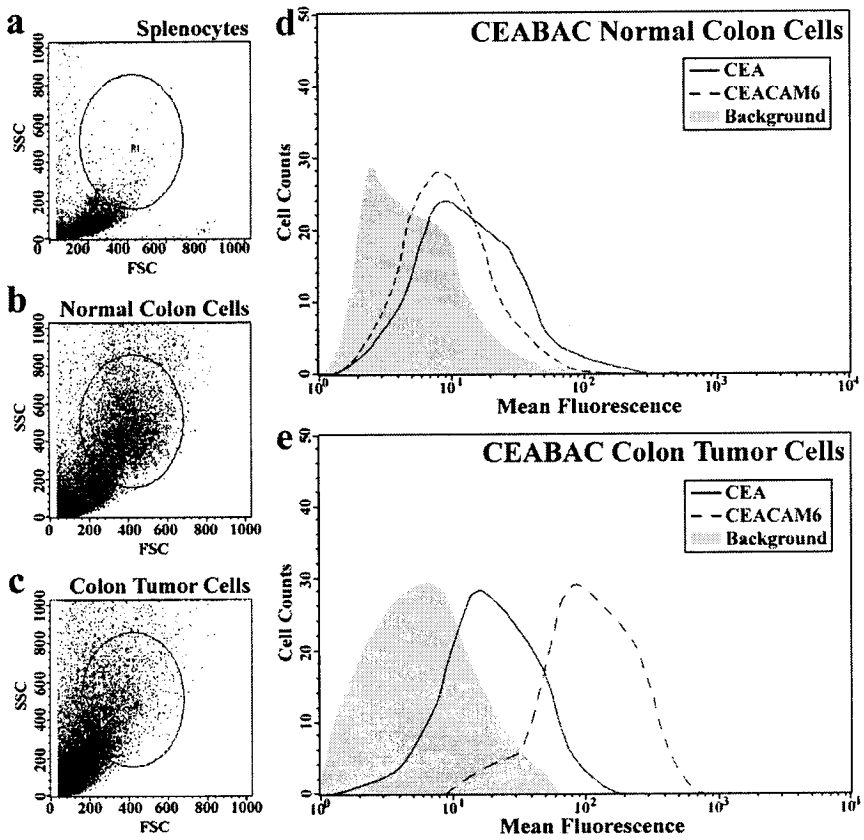

Figure 3: Cell surface expression of CEA and CEACAM6. Dot plots relating side scatter (SSC), i.e., cell volume, to forward scatter (FSC), i.e., cell shape, of **a)** splenocytes, **b)** normal colon cells, and **c)** colon tumor cells. The circles represent the cell populations (R1) gated for analysis, which were set to exclude hematopoietic cells that could include CEACAM6-expressing neutrophils present in colonic samples. **d, e)** FACS profiles of the CEABAC normal (non AOM-treated) colon cells (**d**) and colon tumor cells (**e**). Solid line = CEA cell surface level; Dotted line = CEACAM6 cell surface level; Shaded area = background fluorescence from cell membrane. Note that the profiles were smoothed for clear presentation. For normal colon cells (**d**), mean fluorescence = 8.9 (background), 20.3 (CEA) and 15.1 (CEACAM6). For colon tumor cells (**e**), mean fluorescence = 10.0 (background), 30.3 (CEA) and 135.9 (CEACAM6). Subtracting background, this represents 2.7- and 20-fold increases in CEA and CEACAM6 expression, respectively. The average increases in CEA and CEACAM6 expression for 5 different tumors were 2.1 and 20 fold, respectively.

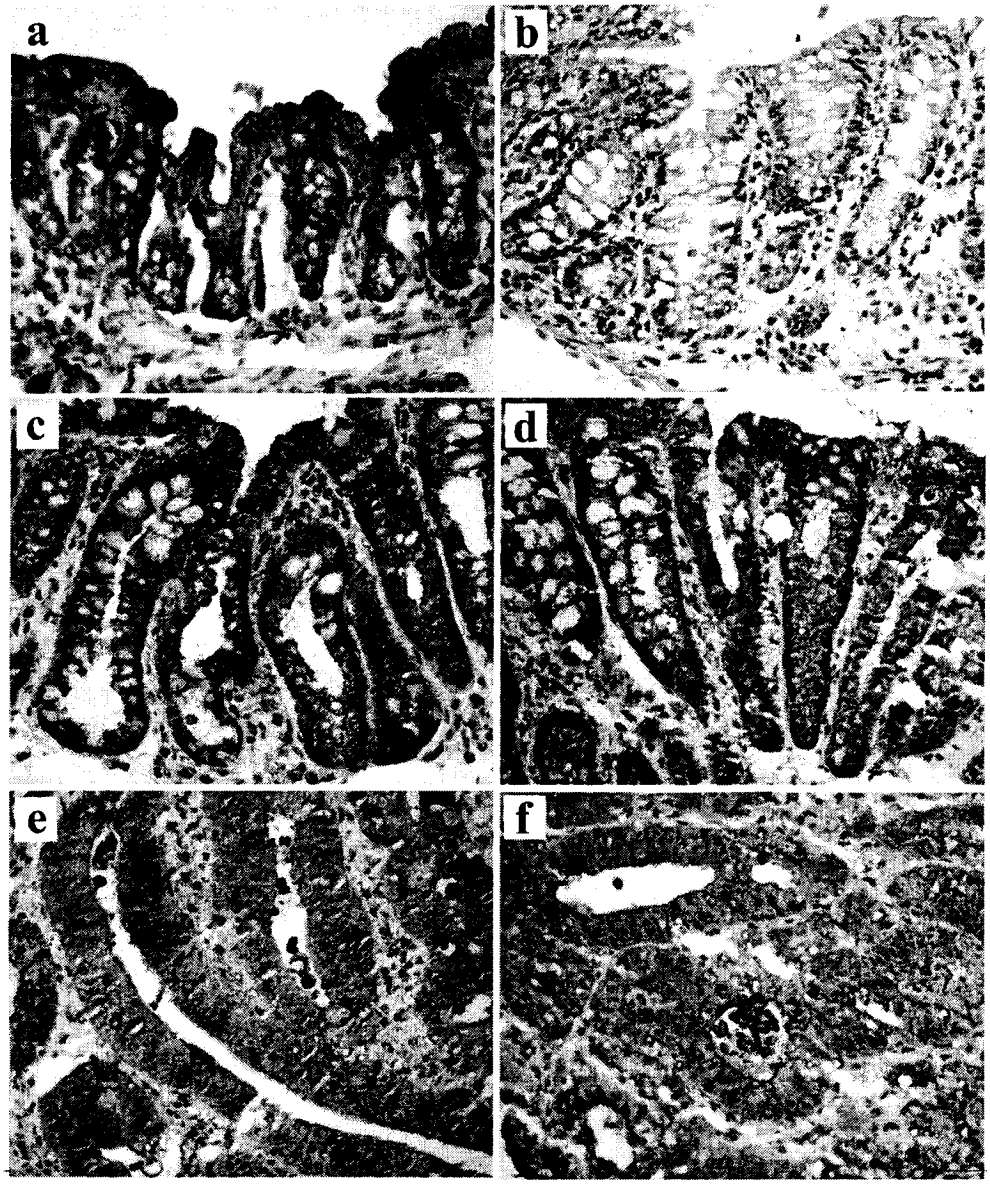

Figure 4: Immunohistochemistry for human CEACAM family gene expression. Cryosections were stained with polyclonal rabbit anti-CEA antibody detecting all human CEACAM family members and counterstained with hematoxylin (×400). **a)** Normal colon epithelium from an untreated CEABAC10 mouse. Human CEACAM family members are localized mainly on the apical surfaces of colonocytes towards the top of the crypts and on the mucosal surface. **b)** Normal epithelium adjacent to a colon tumor from an AOM-treated WT mouse. No staining was present as expected. **c, d)** "Normal" epithelia adjacent to colon tumors from two AOM-treated CEABAC10 mice. Human CEACAM family members are expressed throughout the crypts, in contrast to untreated epithelium (**a**), thus showing a field effect of the tumors. **e, f)** Tumors obtained from AOM-treated CEABAC10 mice.

DISCUSSION

Human GPI-anchored CEACAM family genes, CEA and CEACAM6, were demonstrated to be instrumental for tumor progression in various *in vitro* model systems (Eidelman et al. 1993; Rojas et al. 1996; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002; Duxbury et al. 2004a). However, these tumorigenic effects, including inhibition of cell differentiation and anoikis/apoptosis and the disruption of tissue architecture, which were shown to increase tumorigenicity by animal assays using human and murine cells transfected *in vitro*, had not yet been demonstrated *in vivo*, mainly because of the absence of adequate animal models. Mice lack GPI-anchored CEACAM family members and, by supplementing the mouse genome with a part of the human CEACAM family gene locus containing CEA, CEACAM6 and CEACAM7 genes by means of BAC transgenesis, human-like colonic expression of CEA, CEACAM6 and CEACAM7 was obtained in the CEABAC transgenic mouse colon (Chan and Stanners 2004); transmembrane CEACAM1 was also expressed in mouse colon from the endogenous murine gene. As in the WT FVB mice, neither tumors nor aberrant crypt foci (ACF), precursor lesions for colon tumors (Bird 1995), could be found in colons from the CEABAC mice at any age (Chan and Stanners 2004), as was previously reported for CEA-only transgenic mice (Eades-Perner et al. 1994; Clarke et al. 1998). This may not be surprising since, in our case at least, the expression pattern of these human CEACAM family genes within the colonic tissue was normal, i.e., minimal expression in undifferentiated cells with proliferative capacity at the bottom of colonic crypts, gradually increasing expression in differentiating cells in the middle of colonic crypts and highest expression in fully differentiated cells on the top of colonic crypts (Ilantzis et al. 1997; Chan and Stanners 2004). Over-expression of CEA and CEACAM6 in undifferentiated cells is hypothesized to lead to tumorigenic phenotypes *in vivo* (Ordonez et al. 2000; Ilantzis et al. 2002). A more spatially uniform cryptal expression of CEA seen by immunohistochemistry was reported for CEA-only transgenics (Eades-Perner et al. 1994). We suggest that this was due to the higher sensitivity of CEA detection achieved by these workers resulting in higher apparent levels in the lower proliferative regions of the crypts and saturated levels in the upper differentiated regions; in fact, even intracellular CEA expression was

observed in crypt colonocytes indicating extremely high sensitivity (Eades-Perner et al. 1994).

In this study, the CEABAC mice were shown to have a highly significantly increased susceptibility to AOM-induced colon tumor formation relative to WT FVB mice, which are known to produce colorectal carcinomas after AOM treatment (Singh et al. 2000). Since increased susceptibility was observed in both transgenic lines, the presence of human GPI-anchored CEACAM family genes, rather than a rare insertional mutation due to transgene integration, was apparently responsible for the phenotype. These results, in concordance with previous *in vitro* studies reported by different groups (Eidelman et al. 1993; Rojas et al. 1996; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002; Duxbury et al. 2004a), strongly support a tumorigenic role for human GPI-anchored CEACAM family members *in vivo*. We suggest that the failure to see a significant increase in the carcinogen-induced tumor incidence in the 10 vs the 2 copy CEABAC transgenics was due to a lower threshold for the increased AOM-induced colon carcinogenesis effect than for other tumorigenic effects for which gene dosage-dependent results were seen (See Chapter 5).

Over-expression of CEA and CEACAM6 (2 and 20 fold, respectively) could be shown on the cell surface of colonocytes from the AOM-induced tumors in the CEABAC10 mice relative to normal colonocytes from untreated CEABAC10 mice. Moreover, normal-looking epithelium adjacent to the tumors in CEABAC mice showed an abnormal expression pattern of CEA/CEACAM6, i.e., an absence of the aforementioned expression gradient within the colonic crypts which, in human colorectal carcinomas, has been attributed to a field effect emanating from the tumor (Jothy et al. 1996). The magnitude of over-expression and abnormal cryptal expression pattern of CEA/CEACAM6 thus mimic very closely these features seen in colonic tumor specimens taken from human patients. This de-regulated expression could be a primary focal effect exerted by the AOM treatment on the CEA/CEACAM6 gene transcriptional and/or post-transcriptional controls or a secondary effect exerted by the developing tumor, either of which could account for the increased incidence of tumors in the CEABAC mice, given the tumorigenic effects of CEA/CEACAM6 over-expression observed in other systems (Eidelman et al. 1993; Rojas et al. 1996; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et

al. 2002; Duxbury et al. 2004a). It is known that certain cytokines, such as IFN-γ, IL-6 and IFN-α, can increase CEA and CEACAM6 expression in various human colon cancer cell lines *in vitro* (Kantor et al. 1989; Hinoda et al. 1997; Verhaar et al. 1999) and these could be increased as a consequence of primary mutations induced by AOM. Rearrangement, amplification or mutations of the CEA and CEACAM6 genes have never been identified in human tumors over-expressing CEA and/or CEACAM6; hence, these mechanisms are unlikely to be responsible for the observed increase of mRNA levels (Boucher et al. 1989). Promoter hypomethylation of the CEA gene leading to transcriptional de-repression has been observed and represents a more likely explanation (Tran et al. 1988; Boucher et al. 1989). In any case, in CEA/CEACAM6-overexpressing colorectal carcinoma cells obtained from human patients, mRNA levels of CEA/CEACAM6 are increased but not in direct proportion to the increase in protein levels, indicating de-regulated expression at both transcriptional and post-transcriptional levels (Boucher et al. 1989). Regardless of the mechanism of over-expression, de-regulated expression of CEA/CEACAM6 similar to human colorectal cancers was obtained in the AOM-treated CEABAC mice and may be essential for the observed increase in tumor formation.

At early times (6 weeks) post AOM treatment, the number of tumors of volume > 1 mm^3 was actually about the same in CEABAC vs WT animals. Smaller lesions, i.e., microadenomas with volume < 1 mm^3, however, were about 4-fold more numerous in the CEABAC mice. The fact that the sum of the tumors plus microadenomas showed the same two-fold ratio in CEABAC vs WT mice at 6 weeks as the total tumor ratio at 20 weeks post AOM treatment suggests that the additional tumors in the CEABAC mice at 20 weeks were derived from the microadenomas seen at 6 weeks. If so, human CEA and CEACAM6 expression may have a delayed effect in stimulating AOM-initiated tumor formation due to a time lag either in the up-regulation of CEA/CEACAM6 expression or in a subsequent group of molecular events required for tumor progression. A more sophisticated experimental design would be required to investigate this question. Alternatively, the cellular responses to AOM in the WT and CEABAC mice could be different. If the CEABAC colonocytes are more proliferative and more resistant to apoptosis induced by overwhelming O^6-methylguanine adducts and/or less efficient in

repairing O^6-methylguanine adducts in the CEABAC mice, this could increase the chance for a dividing cell to acquire and maintain milder mutations. Increased cryptal proliferation and anoikal/apoptotic resistance of colonocytes in the CEABAC mice has been documented (See Chapter 5); the consequence of these effects is currently under investigation.

In summary, our results strongly support a positive role for human GPI-anchored CEACAM family genes, CEA and CEACAM6 (and possibly CEACAM7), in tumor formation *in vivo*. From a basic standpoint, understanding the cellular and molecular basis for this effect could be important to the field of clinical cancer, given the high proportion of human cancers showing over-expression of these molecules. In addition, the induction of CEA/CEACAM6 over-expressing tumors in the CEABAC mice using AOM represents a useful animal model system for the development and pre-clinical testing of the CEA-based therapies.

Chapter 4

Higher Incidence of Spontaneous Lung Tumors in CEABAC Transgenic Mice

ABSTRACT

Human carcinoembryonic antigen (CEA) and its close relative, CEACAM6, are often over-expressed in many cancers. While CEA and CEACAM6 were shown to play a role in colon and pancreatic cancers, their relevance to lung cancer is completely unknown although CEA is commonly used for diagnosis and prognostic evaluation of patients with cancers at this site. To investigate their role in lung cancer, we have recently constructed transgenic mice (CEABAC mice) bearing human CEA and CEACAM6 genes with the FVB genetic background, which is susceptible to spontaneous lung tumors. Significantly higher incidence of lung tumors was found in the CEABAC mice and incidence was correlated with transgene dosage. These results implicate the tumorigenic role of over-expression of the human CEA and CEACAM6 in lung cancers as seen in colon and pancreatic cancers. Thus, the understanding of their mechanism of action may improve current treatments for lung cancers as well as colon and pancreatic cancers.

INTRODUCTION

Lung cancer is currently the most common cause of cancer deaths for both males and females (Jemal et al. 2004). Small cell carcinomas, large cell carcinomas, adenocarcinomas, and squamous cell carcinomas are the four major types of lung cancers. The latter three types are collectively termed non-small cell lung carcinomas (NSCLC). Carcinoembryonic antigen (CEA) is over-expressed in over 80% of lung cancers (Chevinsky 1991; Kim et al. 1992; Nakamura et al. 2003; Ordonez 2003). Serum levels of CEA and its close relative, CEACAM6, were also higher in many lung cancer patients suggesting that both CEA and CEACAM6 were overproduced by the tumors and released to the circulation (Allard et al. 1994; Kuroki et al. 1999; Nisman et al. 1999; Nakamura et al. 2003). Gradually increasing expression of CEA was also documented in epithelial cells progressing from normal epithelia to atypical adenomatous hyperplasia, and to bronchioloaveolar adenocarcinomas, a subtype of adenocarcinomas (Kitamura et al. 1996). Over-expression of CEA and CEACAM6 may be present in other precursor lesions as well. In any case, the relevance of this over-expression to the development of lung cancer is currently unknown.

Human CEACAM family genes, encoding a group of highly glycosylated homotypic/heterotypic intercellular adhesion molecules that belong to the immunoglobulin superfamily, are closely clustered on chromosome 19q13.2 (Hammarstrom et al. 1998). The glycophosphatidyl inositol (GPI)-anchored family members, CEA and CEACAM6, are over-expressed in as many as 70% of all human tumors, including colon, lung and breast (Chevinsky 1991; Allard et al. 1994; Ballesta et al. 1995; Scholzel et al. 2000; Jantscheff et al. 2003; Nakamura et al. 2003; Ordonez 2003). Over-expression of CEA and CEACAM6 was shown to elicit various tumorigenic effects *in vitro*, i.e., blockage of cell differentiation (Eidelman et al. 1993; Rojas et al. 1996; Ilantzis et al. 2002), inhibition of anoikis/apoptosis (Ordonez et al. 2000; Soeth et al. 2001; Wirth et al. 2002; Duxbury et al. 2004a) and disruption of tissue architecture (Ilantzis et al. 2002). However, none of these studies were done with lung cancer cell lines. Since the GPI-anchored CEACAM family genes are only present in humans and non-human primates (Zhou et al. 2001; Naghibalhossaini and Stanners in preparation),

CEA-only transgenic mice were previously constructed and crossed with a lung tumor mouse model, i.e., mice transgenic for TAg driven by the SP-C promoter; unfortunately, CEA expression was not up-regulated and no synergistic effect could be shown in lung tumor formation (Thompson et al. 1997). This may be due to the absence of CEACAM6 or the lack of CEA over-expression.

A more human-like mouse model in which part of the human CEACAM family gene locus, including genes coding for CEA, CEACAM6, CEACAM7 and CEACAM3, propagated in a 187 kb bacterial artificial chromosome (CEABAC) has been constructed recently on the FVB genetic background (Chan and Stanners 2004). Two independent transgenic lines (CEABAC2 and CEABAC10, with 2 and 10 transgene copies, respectively) were established and showed a remarkably similar expression pattern of these CEACAM family genes to that of humans and a gene dosage-dependent expression (Chan and Stanners 2004). CEA and CEACAM6 are expressed in CEABAC lung tissues, mostly in tracheal and bronchiolar epithelia (Chan and Stanners 2004). These mice express CEA and CEACAM6 in the colon and are more susceptible to azoxymethane-induced colon tumor formation (See Chapter 3).

Previous studies have shown that 10-25% of aging FVB mice develop spontaneous lung tumors (Mahler et al. 1996); thus, to evaluate the tumorigenic role of CEA and CEACAM6 in lung cancers, cohorts of 28 wild-type (WT), 30 CEABAC2 and 32 CEABAC10 mice were used to score for lung tumor incidence at 10 months of age. Lung tumors were detected in 11% of WT, 20% of CEABAC2 and 50% of CEABAC10 mice. Higher tumor incidence observed in CEABAC10 than CEABAC2 mice indicates a gene dosage- or expression level-dependent process. Hence, these results show that the presence of human CEA and CEACAM6 genes predisposes mice to sporadic lung tumor formation and suggest that they may also have an important role in the development of lung cancer as well as colon cancer.

MATERIALS AND METHODS

Animals

Generation of the CEABAC transgenic mice harboring a 187 kb bacterial artificial chromosome that contains complete human CEA, CEACAM6, CEACAM7, and CEACAM3 genes was reported elsewhere (Chan and Stanners 2004). These mice were generated and maintained in the FVB genetic background. CEABAC2 and CEABAC10 possess 2 and 10 copies of transgenes in a single head-to-tail cluster, respectively (Chan and Stanners 2004). Age-matched wild-type controls were obtained from breeding between CEABAC and FVB mice (Harlan Bioproducts for Science Inc., Indianapolis, IN). All mice were housed under controlled conditions of a 12 hour light/dark cycle, 23 ± 2 °C room temperature and 50 ± 10 % relative humidity. Food and water were available *ad libitum*. At 10 months of age, mice were sacrificed in a CO_2 chamber and their chests were carefully opened to avoid mechanical damage of the lungs. Lungs were then thoroughly examined visually for the presence of tumor masses (i.e., appearance of white nodules).

Histological analysis

Fresh tumors were fixed in Glyo-Fixx (20% ethanol, 5% glyoxal, 1% propanol and 1% methanol) for 48 hours at room temperature and processed for embedding in paraffin blocks for sectioning. Sections were stained with hematoxylin and eosin.

Statistical analysis

Tumor incidences of different animal groups were compared with the two-tailed Fisher's test. The results were considered statistically significant if the P values were less than 0.05.

RESULTS

Human CEACAM family gene expression increases the incidence of spontaneous lung tumors

Since FVB mice are prone to develop spontaneous lung tumors, cohorts of 28 WT, 30 CEABAC2 and 32 CEABAC10 mice were used to look for possible effects of CEA and CEACAM6 expression on lung tumor incidence. At 10 months of age, 11% (3/28) of WT mice developed a single lung nodule with a size ranging from 1 to 10 mm^3; 20% (6/30) for CEABAC2 mice and 50% (16/32) for CEABAC10 mice developed lung tumors of this size. In comparing to WT mice, relative risks for developing a lung tumor are 1.9 and 4.7 for CEABAC2 and CEABAC10 mice, respectively, indicating that CEABAC mice are approximately 2 to 5 times more likely to have a lung tumor by 10 months of age (Table 1). The tumor incidence in CEABAC10 mice was significantly higher than that of WT mice ($P < 0.0001$). Since the difference between WT and CEABAC 2 mice was smaller, a much larger sample size (estimated to be over 150 mice per group) would be required to achieve statistical significance in this case. Nevertheless, an increase in tumor incidence was observed in both CEABAC transgenic lines, indicating independence of the site of transgene integration and a direct effect caused by the presence of human CEA and CEACAM6 genes. Moreover, a linear relationship ($R^2 > 0.995$) can be deduced between gene copy number and tumor incidence or relative risk (Fig. 1), suggesting that the expression level of CEA and CEACAM6 in lung tissues determined the magnitude of the increase in lung tumor formation.

Table 1: Tumor Incidence in WT and CEABAC animals at 10 months of age

Mouse Strain	Number of animals	Number of animals with lung tumor	% Incidence	Relative Risk
WT	28	3	10.7	1
CEABAC2	30	6	20.0	1.9
CEABAC10	32	16	50.0 [a]	4.7

[a] $P = 0.0018$, compared with WT.

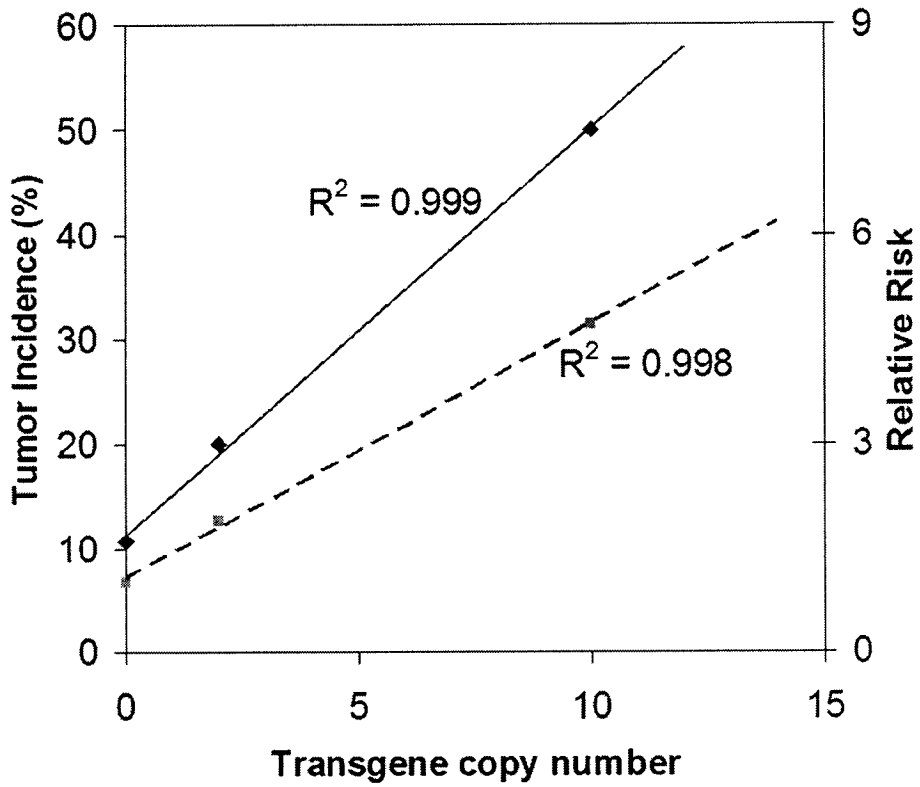

Figure 1: Gene dosage-dependent increase of tumor incidence and relative risk. Percent tumor incidence (■, solid line) and relative risk (◆, dotted line) are plotted against transgene copy number (0 for WT, 2 for CEABAC2 and 10 for CEABAC10). Linear relationships with $R^2 > 0.995$ indicate significant correlations in both cases.

Non-small cell lung carcinomas in CEABAC mice

Based on histological assessment of the tumors resected from WT and CEABAC mice, they both develop mainly undifferentiated large cell carcinomas (a subtype of NSCLC) with occasional glandular structures (Fig. 2d and e). The nuclei of the tumor cells were more "active" (i.e., clear nucleoplasm with multiple nucleolar structures) compared to those of normal bronchiolar epithelium (Fig. 2c and f). The tumor cells are also present in the lymphatic vessels adjacent to the bronchiole (Fig. 2b), indicating a metastatic potential of these cells.

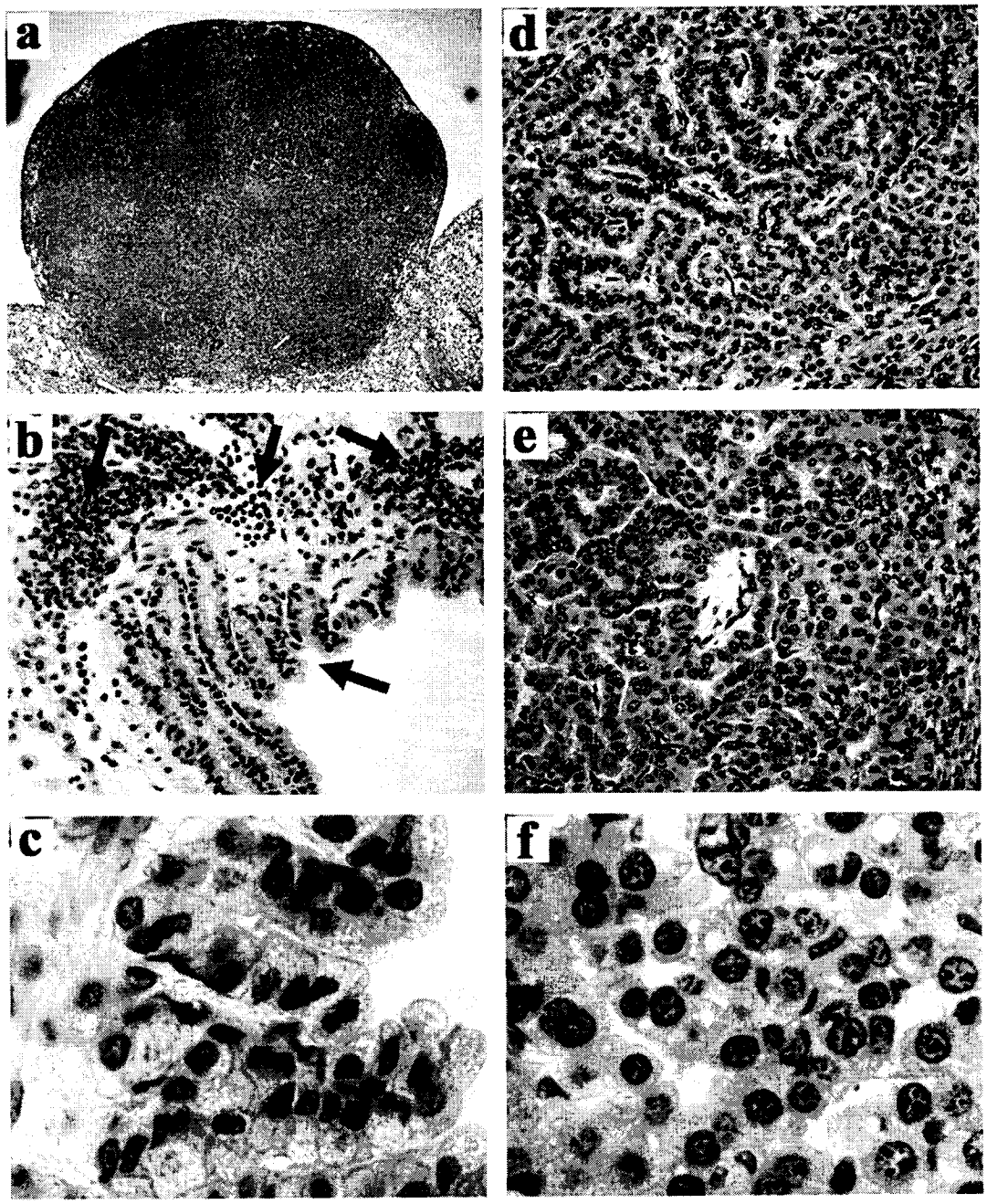

Figure 2: Histology of spontaneous lung tumors. a) Cross section of a peripheral NSCLC (solid mass) in lung tissue (underlying sponge-like tissue). **b)** Normal bronchiolar epithelium (blue nuclei and pinkish cytoplasm, black arrow) adjacent to the tumor shown in a. Note that there are masses of tumor cells (clusters of cells with blue nuclei and pinkish cytoplasm, red arrows) and lymphocytes (cluster of cells with only blue nuclei, green arrow) in the lymphatic vessel. **c)** Higher magnification of normal bronchiolar epithelium

shown in **b**. **d, e)** Different regions of the tumor shown in **a**. Note the glandular structure in **d** and less organized structures in **e**. **f)** Higher magnification of tumor cells present in tumor shown in **a**. Note that nuclei are more active (i.e., clear nucleoplasm with multiple nucleolar structures) compared to normal epithelial cells in **c**. Original magnification: **a** (×100); **b**, **d** and **e** (×400); **c** and **f** (×1000).

DISCUSSION

Human GPI-anchored CEACAM family genes, CEA and CEACAM6, are over-expressed in many cancers including colon and lung cancers (Chevinsky 1991; Kim et al. 1992; Allard et al. 1994; Ballesta et al. 1995; Kitamura et al. 1996; Hammarstrom et al. 1998; Kuroki et al. 1999; Nisman et al. 1999; Jantscheff et al. 2003; Nakamura et al. 2003; Ordonez 2003). De-regulated over-expression of these genes was demonstrated to be instrumental for tumor progression in various *in vitro* model systems (Eidelman et al. 1993; Rojas et al. 1996; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002; Wirth et al. 2002; Duxbury et al. 2004a). However, these tumorigenic effects (cell differentiation block, inhibition of anoikis/apoptosis and disruption of tissue architecture) have not yet been shown to apply to lung cancer. The progressive over-expression of CEA, and probably CEACAM6, in lung cancer development (Kitamura et al. 1996) and its prognostic indication of worse outcome (Nisman et al. 1999; Bandoh et al. 2001; Sawabata et al. 2002; Sakao et al. 2004) are similar to the situation in colon cancer. This leads to the hypothesis that CEA and CEACAM6 could play an instrumental role in lung cancers as in colon cancers (Stanners 1998).

Since mice lack all human GPI-anchored CEACAM family genes and bear only orthologous genes corresponding to the transmembrane CEACAM1, CEABAC transgenic mice were constructed with a part of the human CEACAM family gene locus including CEA and CEACAM6 genes by means of BAC transgenesis. Human-like expression of the CEACAM family genes, CEA and CEACAM6, was obtained in the CEABAC transgenic mouse lung, i.e., focally in alveoli and mostly in tracheal and bronchiolar epithelia (Chan and Stanners 2004). In this study, the CEABAC mice were shown to have a higher incidence of spontaneous lung tumors than the WT FVB mice, which are known to be prone to develop spontaneous lung tumors (Mahler et al. 1996). Since increased incidence was observed in both transgenic lines, the presence of human GPI-anchored CEACAM family genes, rather than a rare insertional mutation due to transgene integration, was responsible for the apparent phenotype. Moreover, the dependency of lung tumor incidence on transgene copy number or expression level of CEA and CEACAM6 indicates that over-expression of CEA and CEACAM6 is directly

involved in the tumorigenic process. In fact, a similar increase in tumor susceptibility was shown in the CEABAC mouse colons after carcinogen challenge (See Chapter 3). These results, in concordance with previous *in vitro* studies reported by different groups with different cell types (Eidelman et al. 1993; Rojas et al. 1996; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002; Wirth et al. 2002; Duxbury et al. 2004a), strongly suggest that human GPI-anchored CEACAM family members play an important role in lung tumor formation *in vivo*.

Although CEA and CEACAM6 are over-expressed in the majority of lung cancers (Chevinsky 1991; Kim et al. 1992; Allard et al. 1994; Kuroki et al. 1999; Nakamura et al. 2003; Ordonez 2003), their functional contribution to the development of lung cancer is presently unknown. Thus far, the tumorigenic effects of these two genes were well documented for human colon cancer. They were shown to block cell differentiation, inhibit apoptosis/anoikis and disrupt tissue architecture (Eidelman et al. 1993; Rojas et al. 1996; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002; Wirth et al. 2002). In normal colonic crypts, precursor stem cells are dividing at the crypt bases (the proliferative zone); committed stem cells migrate upward while they are undergoing differentiation; and fully-differentiated cells undergo apoptosis and are sheded off from the top of the crypts on the mucosal surface of the colon (Stappenbeck et al. 1998). CEA and CEACAM6 are normally expressed at the highest levels in the fully-differentiated cells at the top of the crypts but minimally in those populated in the proliferative zone (Ilantzis et al. 1997; Ilantzis et al. 2002). However, in the pre-cancerous lesions, CEA and CEACAM6 are commonly over-expressed in cells occupied in the proliferative zone. Thus, de-regulated over-expression of CEA/CEACAM6 in the undifferentiated cells that maintain a dividing capacity (i.e., those in the proliferative zone) is proposed to be an important criterion for CEA/CEACAM6 to exert their tumorigenic effects (Ilantzis et al. 1997; Ilantzis et al. 2002). In contrast, renewal/regeneration of the lung epithelia is presently not well-established. Nevertheless, basal cells and Clara cells were found to be the precursor stem cells of the lung epithelia (Hong et al. 2001; Hong et al. 2004). Moreover, CEA was shown to be over-expressed in the Clara cells of lung tumors (Gemma et al. 1991). Altogether, these studies suggested that CEA/CEACAM6 can also be over-expressed in the proliferating cells of lung epithelia in which they may perform

their tumorigenic functions in the same manner as in colon cancer, i.e., block of cell differentiation, inhibition of apoptosis and disruption of tissue architecture. Hence, the tumorigenic properties of CEA/CEACAM6 should be further investigated in the lung system. Should similar tumorigenic effects be shown, it will suggest that CEA and CEACAM6 are general tumorigenic molecules in cancer development at multiple sites.

In summary, our results strongly support an instrumental role for the over-expression of human GPI-anchored CEACAM family genes in tumor formation at multiple sites *in vivo*. Thus, further understanding their role in various cancers and their mechanism of action are necessary to improve medical practices and cancer therapies. Moreover, this animal model system may be useful for the development and pre-clinical testing of the CEA-based therapies.

Chapter 5

Spontaneous Colon Tumors in CEABAC Transgenic Mice

ABSTRACT

Human carcinoembryonic antigen (CEA) and its closely related family member CEACAM6 are over-expressed in as many as 70% of all human cancers, suggesting an instrumental role for these intercellular adhesion molecules in tumorigenesis. This hypothesis has been supported by studies involving cell lines and transfectants expressing CEA/CEACAM6 in various model systems. To test its validity *in vivo*, we constructed a transgenic mouse harboring part of the human CEACAM family gene locus, including the CEA and CEACAM6 genes in a 187 kb bacterial artificial chromosome insert (CEABAC). Mice have no paralogs of these human GPI-anchored CEACAM family members but the newly introduced genes show tissue-specific expression which closely resembles that of humans. These CEABAC transgenics showed higher susceptibility for the development of spontaneous lung tumors and carcinogen-induced colon tumors compared with normal non-transgenic littermates. Here we report the functional contribution of CEA and CEACAM6 over-expression to colon tumor formation in the CEABAC mice. At low to moderate expression levels of CEA/CEACAM6 in two independent transgenic lines with 2 copies (CEABAC2) and 10 copies (CEABAC10) of the CEABAC transgene, a partial block in cell differentiation and a mild to moderate hyperproliferation were evident in the transgenic colon; however, these mice did not develop sporadic colon tumors. At higher expression levels, similar to those of human colorectal carcinomas, produced by increasing the CEABAC copy number to 20, a complete block in colonocyte differentiation, an extreme hyperproliferation and a marked inhibition of apoptosis/anoikis were observed. These mice showed reduced survival, growth retardation and chronic diarrhea. Importantly, they showed massively enlarged colons comprising continuous non-focal cytological and architectural abnormalities, including dysplastic and serrated adenomatous morphology, by only 3 months of age. Similar to human serrated adenomas and unlike conventional human adenomas, the Wnt signaling pathway was not perturbed. These results suggest that, while moderate expression levels of CEA/CEACAM6 cause an imbalance of tissue homeostasis leading to increased tumor susceptibility, tumor-like expression levels alone produce a severe imbalance leading directly to tumor formation.

INTRODUCTION

Tumor-associated markers can be used for diagnosis and prognostic evaluation of many cancers. Carcinoembryonic antigen (CEA), a well-known tumor marker since 1965 (Gold and Freedman 1965), is used extensively in the cancer clinics (Shuster et al. 1980; Ballesta et al. 1995; Jantscheff et al. 2003) and is a popular molecular target for novel cancer therapies (Chester et al. 2000; Fong et al. 2001; Koch et al. 2001; Burke et al. 2002; Kousparou et al. 2002; Goldenberg 2003; Marshall 2003) due to its consistent over-expression in as many as 70% of all human cancers including those in colon, breast and lung (Chevinsky 1991; Ballesta et al. 1995; Jantscheff et al. 2003). CEACAM6, its closely related family member, is also over-expressed in many cancers (Allard et al. 1994; Kinugasa et al. 1998; Sugita et al. 1999; Carrasco et al. 2000; Scholzel et al. 2000; Jantscheff et al. 2003). With such an overwhelming correlation, one could easily ask a simple question: could this over-expression play a role in the development of human cancers?

Over-expression of CEA/CEACAM6 in human cancers has been generally considered a tumor-related event without functional significance. On the other hand, CEA and CEACAM6 were shown to have a variety of tumorigenic effects on cells cultured *in vitro* and in xenograft mouse models. Over-expression of CEA and CEACAM6 was found to block myogenic and colonic differentiation (Eidelman et al. 1993; Rojas et al. 1996; Ilantzis et al. 2002), inhibit anoikis/apoptosis in colon and pancreatic cancer cells (Ordonez et al. 2000; Soeth et al. 2001; Wirth et al. 2002; Duxbury et al. 2004a), disrupt cell polarization and tissue architecture (Ilantzis et al. 2002), enhance liver metastasis (Gangopadhyay et al. 1996; Leconte et al. 1999; Duxbury et al. 2004a), and increase chemoresistance (Soeth et al. 2001; Duxbury et al. 2004b). Moreover, a higher incidence of spontaneous lung tumors and increased susceptibility to carcinogen-induced colon tumors were observed (See Chapters 3 and 4) in our recent transgenic mouse model (CEABAC), which contains part of the human CEACAM family gene locus (187 kb), including complete genes for CEA, CEACAM6, CEACAM7, and CEACAM3 (Chan and Stanners 2004). Altogether, these studies suggest that CEA/CEACAM6 over-expression could play an important role in the development of cancer.

Two independent CEABAC transgenic lines (CEABAC2 and CEABAC10, with 2 and 10 transgene copies, respectively) were established and showed remarkably similar expression patterns of these CEACAM family genes to that of humans (Chan and Stanners 2004). In the CEABAC mice, the expression levels of CEA and CEACAM6 are correlated with the transgene copy number. Interestingly, the incidence of spontaneous lung tumors is also correlated with transgene copy number (See Chapter 4). In addition, the expression of CEA and CEACAM6 was shown to increase by 2 and 20 fold, respectively, in the carcinogen-induced colon tumors relative to the *normal* colonic epithelium in CEABAC10 mice (See Chapter 3). These observations suggest that expression levels of CEA and CEACAM6 could be a critical factor for their functional contribution to tumor development *in vivo*.

To investigate the latter question in the present study, we have established a correlation between the expression levels of CEA and CEACAM6 and their effects on the colonic epithelia of the CEABAC mice. Colon epithelium undergoes continuous renewal, and colonic crypt homeostasis is governed by a fine balance between proliferation, differentiation and apoptosis of colonocytes through the crypt axis (Stappenbeck et al. 1998). However, this balance was found to be shifted towards a more proliferative state in the CEABAC mice, an effect that was correlated with the expression levels of CEA and CEACAM6. Moreover, a further increase in CEA and CEACAM6 expression to human colorectal carcinoma-like levels disturbed this balance completely and massive continuous non-focal tumors were obtained. Our results therefore suggest that, while moderate expression levels of CEA/CEACAM6 cause an imbalance in tissue homeostasis leading to increased tumor susceptibility, tumor-like expression levels alone can produce a severe imbalance leading directly to tumor formation. Thus, CEA and CEACAM6 could contribute significantly to the development of human cancer in the 70% of cancers where they are over-expressed.

MATERIALS AND METHODS

Mouse strains and genotyping

CEABAC transgenic mice (both lines CEABAC2 and CEABAC10) were produced and maintained on the FVB strain background. Genotypes of mice were determined by PCR as described elsewhere (Chan and Stanners 2004). CEABAC20 mice were produced by mating two CEABAC10 mice. Mice homozygous for the 10 head-to-tail CEABAC concatemer were identified by fluorescence *in situ* hybridization of splenocytes (see Results).

Antibodies and immunoblot analysis

Colonic mucosa were scraped off the muscular layer of entire colons and homogenized in ice-cold lysis buffer (20 mM Tris-pH 8.0, 140 mM NaCl, 2 mM EDTA, 1 mM sodium vanadate, 1 mM sodium floride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 100 μg/ml PMSF). Immunoblots were performed as described elsewhere (Chan and Stanners 2004) using the following antibodies: mouse monoclonal anti-CEA/CEACAM6 (B18); rabbit polyclonal anti-β-catenin (Medicorp Inc., Fremont, California); mouse monoclonal anti-keratin 18 (RGE53, Chemicon Inc., Temecula, California); and rabbit polyclonal anti-PARP (H-250, Santa Cruz Biotechnology Inc., Santa Cruz, California).

Histological analysis and immunohistochemistry

For cryosections, freshly collected tissues were fixed with 4% paraformaldehyde and processed as described elsewhere (Chan and Stanners 2004). For paraffin-embedded sections, freshly excised tissues were fixed in Glyo-Fixx (20% ethanol, 5% glyoxal, 1% propanol and 1% methanol) for 48 hours at room temperature and processed for embedding in paraffin blocks for sectioning. For general histopathology, 5-μm frozen sections were stained with hematoxylin (Lynch et al. 1969) or 5-μm paraffin-embedded sections were stained with hematoxylin and eosin (Lynch et al. 1969). To assess colonic differentiation, 5-μm frozen sections were stained by the "alcian blue-periodic acid Schiff" method to detect mucin, followed by methyl green staining to detect nuclei (AB-PAS-MG) (Lynch et al. 1969). For immunohistochemistry, 5-μm frozen sections were

probed with rabbit polyclonal pan-human CEA antibodies and rabbit polyclonal anti-β-catenin antibodies (Medicorp Inc., Fremont, California) as described elsewhere (Chan and Stanners 2004). Actively proliferating cells were detected using peroxidase-conjugated mouse monoclonal anti-PCNA antibody (clone PC10, DAKO Diagnostics Canada Inc., Mississauga, Canada).

Fluorescence In Situ Hybridization (FISH) and TUNEL assays

For FISH, freshly isolated splenocytes were treated as previously described (Spector et al. 1998); DIG-labeled CEA cDNA probes were generated using the High Prime DNA Labeling Kit (Roche Diagnostics Inc., Laval, Canada), and detection was performed using the Fluorescent Antibody Enhancer Set for DIG Detection (Roche Diagnostics Inc., Laval, Canada). TUNEL assays were performed with frozen tissue sections using the ApoAlert® DNA Fragmentation Assay Kit (BD Biosciences, Mississauga, Canada).

Measurement and treatment of data

Animals were sacrificed exactly 21 days postnatally to minimize growth variation. Total body weights were measured with a precision of ±0.005 g. Whole colons (from the ileo-cecal junction to the anus), whole small intestine (from the pyloric sphincter to the ileo-cecal junction) and kidneys were dried and weighed with a precision of ±0.05 mg. % crypt fission was scored as the percentage of forked dividing crypts over the total number of crypt bases for about 100 crypts. Proliferating index was scored as the number of PCNA-positive nuclei divided by total number of nuclei of about 10 individual crypts. The level of differentiation was scored as the number of large goblet cells (mucin vacuole of more than 10 μm) over the total number of crypt bases for about 100 crypts.

RESULTS

Phenotypic differences between wild-type and CEABAC mice

Two independent CEABAC transgenic lines [CEABAC2 and CEABAC10, with 2 and 10 transgene copies in a single cluster (see Fig. 3b), respectively] were established in the FVB genetic background (Chan and Stanners 2004). In humans and CEABAC mice, three human GPI-anchored CEACAMs (CEA, CEACAM6 and CEACAM7) are expressed in the colonic epithelium (Hammarstrom 1999; Chan and Stanners 2004). In the CEABAC colons, the expression level of CEA was determined to be the highest, whereas CEACAM6 was expressed at a lower level and CEACAM7 at a very low level (Chan and Stanners 2004). These relative expression levels of CEACAMs mimic very well the human situation (Hammarstrom 1999). A higher susceptibility to carcinogen-induced colorectal tumors and these tumors showed over-expression of CEA and CEACAM6 relative to untreated CEABAC mice (See Chapter 3).

At three weeks of age, CEABAC mice had consistently lower total body weight ($P < 0.01$) and a higher colon-to-kidney weight ratio than wild-type (WT) littermates ($P < 0.05$). The weight ratio for the small intestine was unchanged (Table 1). Instead of using absolute organ weights, weight ratios were compared to normalize growth variation between animals. Cross sections of colons revealed that the epithelial content was higher in the CEABAC mice, contributing to the overall increase in colon weight (Fig. 1a). To investigate the factors contributing to the increase in epithelial content, the differentiation and proliferation status of the CEABAC colonic crypts were assessed.

Table 1: Weight measurements of 3 weeks old animals

Mouse Strain	Number of animals	TBW [a] (Mean ± SEM)	Colon/Kidney [b] (Mean ± SEM)	SI/Kidney [c] (Mean ± SEM)
WT	32	11.9 ± 0.14	1.08 ± 0.019	3.43 ± 0.044
CEABAC2	17	11.1 ± 0.19 [e]	1.15 ± 0.024 [d]	3.52 ± 0.102
CEABAC10	14	11.0 ± 0.18 [f]	1.25 ± 0.032 [g]	3.47 ± 0.043
CEABAC20	4	7.5 ± 0.33 [g]	1.66 ± 0.105 [g]	3.80 ± 0.090 [e]

[a] Total body weight in grams. SEM = standard error of the mean.
[b] Colon-to-kidney weight ratio. SEM = standard error of the mean.
[c] Small intestine-to-kidney weight ratio. SEM = standard error of the mean.
[d] $P < 0.05$, compared with WT.
[e] $P < 0.01$, compared with WT.
[f] $P < 0.005$, compared with WT.
[g] $P < 0.0001$, compared with WT.

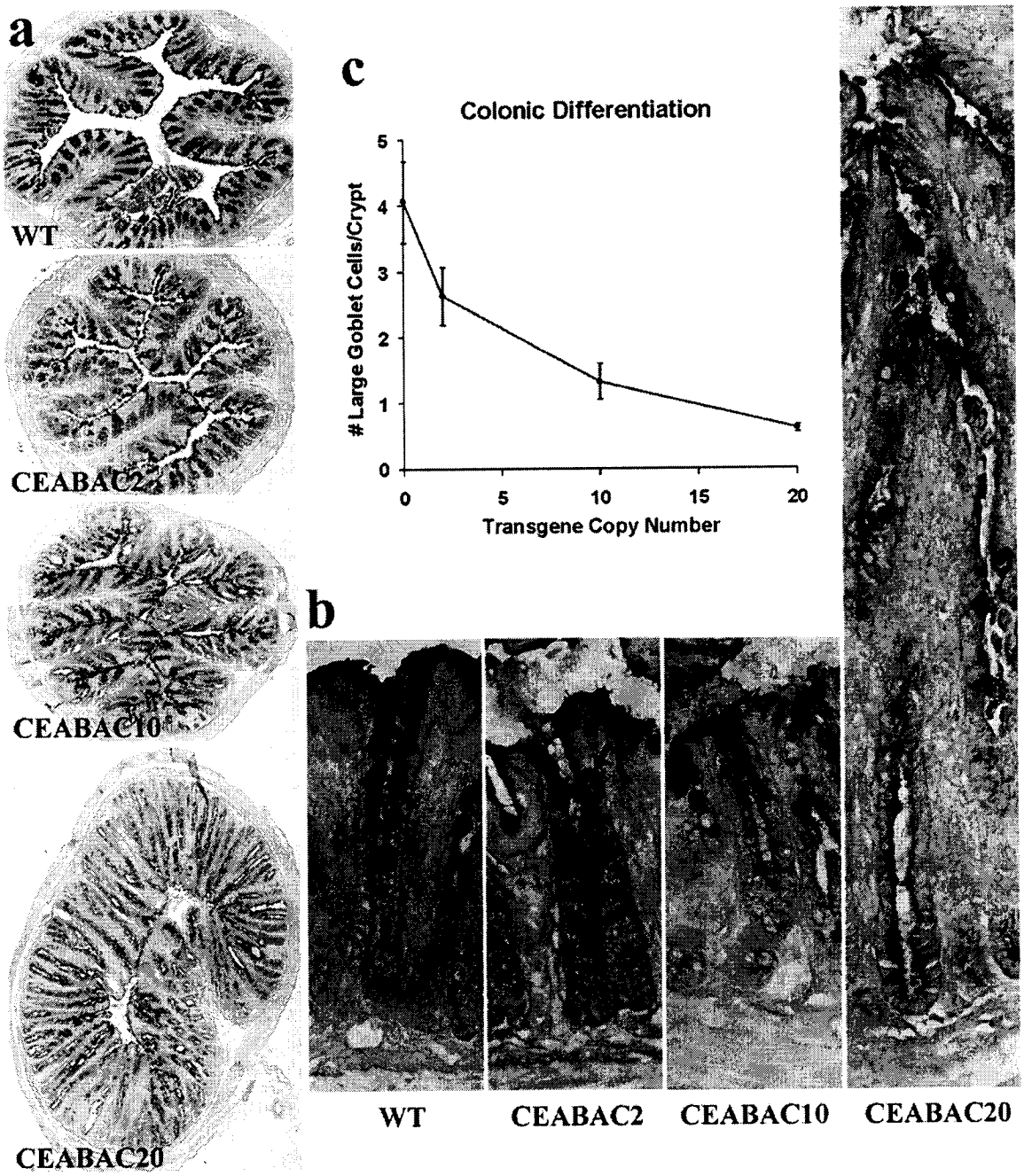

Figure 1: Differentiation block in CEABAC mice at 3 weeks of age. WT denotes wild-type littermate. **a)** cross sections of colons of indicated mice; note the overall increase in epithelial content and reduction of purplish color representing mucin from top to bottom. **b)** colonic crypts; note the absence of purplish differentiated goblet cells (indicated by an arrow in the CEABAC2 colonic crypt), lengthening of crypts, poor cryptal alignment, and increase of peri-cryptal stroma in CEABAC20; note also the absence of bluish cytoplasm,

abundance of small purplish mucinous vacuoles and elongation of nuclei in CEABAC20, indicating hyperplastic changes; **c)** number of normal-size goblet cells per crypt plotted against transgene copy-number; each data point is significantly different from the others ($P < 0.0001$). 35%, 67% and 85% (95% after considering the 3-fold increase in crypt length) reduction was obtained for CEABAC2, CEABAC10 and CEABAC20 mice, respectively. Error bars = SD. Original magnification: × 100 (**a**), × 400 (**b**). Staining: AB/PAS/MG.

Goblet cells are one of the fully differentiated cell types present in the colonic epithelium. Mature goblet cells usually contain large mucin-containing vacuoles, which can be detected by Periodic Acid-Schiff's reagent (PAS). PAS-staining on tissue sections of colonic epithelia derived from WT and CEABAC mice showed that the number of mature goblet cells was significantly reduced in the CEABAC mice (35-67% reduction), indicating an inhibition of cell differentiation (Fig. 1b and c).

Crypt fission (a binary bottom-top fission of a crypt) is the basic mechanism for the growth of colonic epithelium. A dividing crypt can be observed as a crypt with two crypt bases. CEABAC mice were shown to have significantly higher (2-3 fold) crypt fission index (i.e., percent dividing crypts), suggesting that CEABAC colonic crypts duplicate at a much faster rate (Fig. 2a). Furthermore, proliferating cells can be detected by anti-PCNA (proliferating cell nuclear antigen) antibodies on tissue sections, since PCNA is only present in nuclei of actively dividing cells. The proliferative zone of the colonic crypts is usually at the bottom third of the crypt axis with a proliferation index (i.e., PCNA-positive colonocytes/total number of colonocytes) of 0.33. However, extension of this zone to the bottom two-thirds of the crypt axis with a proliferation index of 0.54 was observed in CEABAC10 mice (Fig. 2b). Together with the increase of crypt fission index, the CEABAC colons can be considered hyperproliferative.

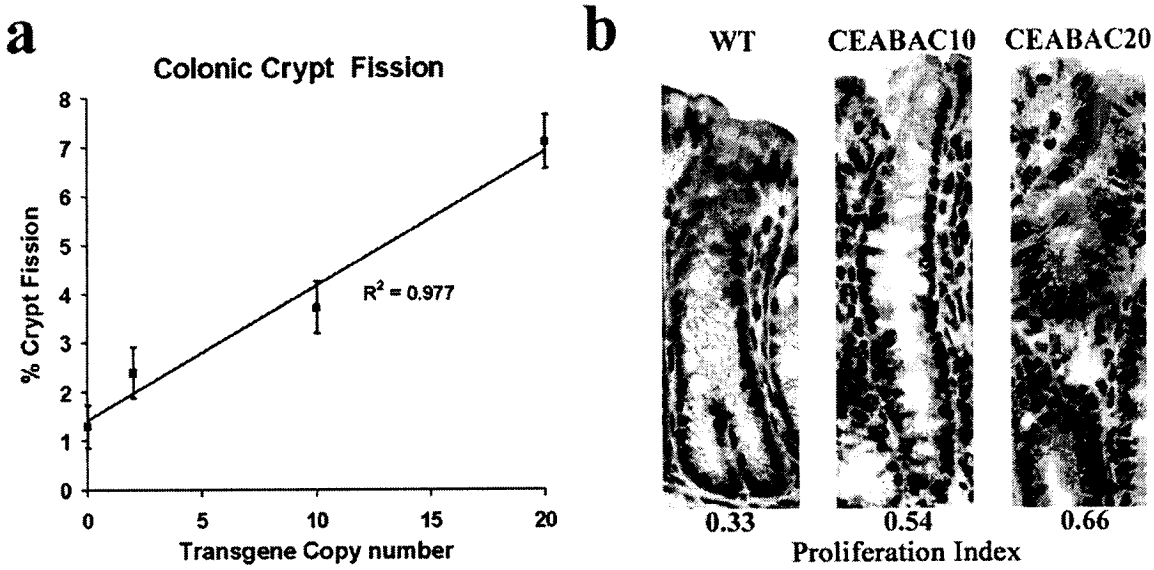

Figure 2: Hyperproliferation in CEABAC mice at 3 weeks of age. WT denotes wild-type littermate. **a)** % crypt fission plotted against transgene copy-number. A linear curve is obtained with a correlation coefficient of 0.977; each data point is significantly different from the others ($P < 0.0001$). 2-, 3- and 6-fold increases were obtained in CEABAC2, CEABAC10 and CEABAC20 mice, respectively. Error bars = SD. **b)** extension of proliferative zones and increase in proliferating indices shown for both CEABAC10 and CEABAC20 by PCNA staining. Note that only the upper part of CEABAC crypts were shown due to increased crypt length. Standard deviations for proliferating indices are 0.04, 0.04 and 0.09 for wild-type, CEABAC10 and CEABAC20 mice, respectively. Original magnification: × 400. Staining: Hematoxylin.

Extended phenotypic changes in CEABAC20 mice

The expression levels of CEA and CEACAM6 were determined to be approximately five fold higher in CEABAC10 than CEABAC2 colons, indicating a gene dosage dependent expression (Chan and Stanners 2004). Interestingly, the observed phenotypic changes in CEABAC mice seemed to be gene dosage dependent as well since CEABAC2 mice always showed intermediate phenotypic changes between wild-type and CEABAC10 mice (Table 1, Figs. 1 and 2). If this is the case, homozygous CEABAC10 (CEABAC20) mice generated by crossing two CEABAC10 mice should give an even more pronounced

phenotype. All CEABAC10 and CEABAC20 offspring were identified by polymerase chain reaction (PCR) assays of genomic DNA and CEABAC20 mice were distinguished from CEABAC10 mice by fluorescence *in situ* hybridization (FISH) analysis. Since the CEABAC transgene was integrated as a single tandem repeat into the mouse genome (Chan and Stanners 2004), the presence of two CEABAC loci was used to identify homozygous CEABAC20 mice by FISH, using probes against the CEABAC transgene (Fig. 3b). CEABAC20 mice also expressed higher levels of CEA and CEACAM6 in their colons as shown by immunoblots (Fig. 3a). Importantly, these levels were actually somewhat less than those observed in human colorectal carcinomas at comparable stages of progression by immunohistochemistry (Fig. 4). Interestingly, premature death [55% (26/47) survived at weaning, 26% (7/27) by 3 months and very few by 6 months], significant growth retardation and chronic diarrhea were observed in the CEABAC20 mice. This phenotype had never been associated with breeding of WT, CEABAC2 and CEABAC10 mice.

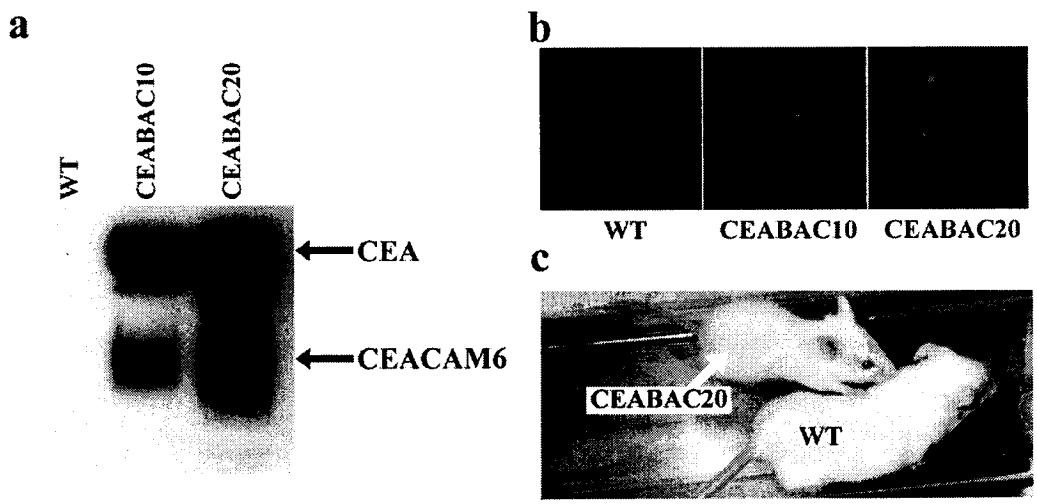

Figure 3: Growth retardation of CEABAC20 mice was correlated with the increased expression of CEA and CEACAM6. a) immunoblot of colon protein extracts showed increased expression of CEA and CEACAM6 in CEABAC20 relative to CEABAC10 mice. **b)** images of fluorescence *in situ* hybridization (FITC-labeled CEA cDNA probes and DAPI-stained nuclei) showed one nuclear spot for CEABAC10 and two for CEABAC20. Original magnification: × 1000. **c)** significant reduction visually of body size of CEABAC20 mice at weaning. WT denotes wild-type littermate.

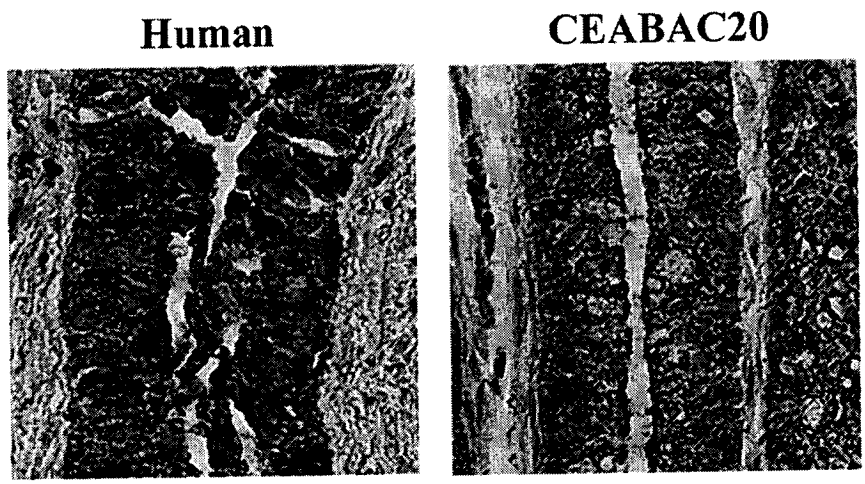

Figure 4: Comparison of CEA/CEACAM6 expression in human and CEABAC20 tumors. Expression levels of CEA/CEACAM6 (brown staining) are somewhat higher in a human carcinoma (left panel) than in dysplastic lesion obtained in CEABAC20 mice at 3 months of age (right panel). Both lesions are at comparable stages of neoplastic progression.

At three weeks of age, CEABAC20 mice demonstrated further progression of the phenotypic changes observed in CEABAC2 and CEABAC10 mice. Total body weight was 37% lower than WT mice (Table 1 and Fig. 3c). The colon-to-kidney weight ratios were 47% higher than those of WT mice (Table 1). Cross sections of the colon showed a dramatic increase in epithelial content (Fig. 1a) and a 6-fold increase in the crypt fission index (Fig. 2a). A complete block of cell differentiation (Fig. 1b and c) and a full extension of the proliferative zone in crypts, with a proliferation index of 0.66, were observed (Fig. 2b). These extended phenotypic changes render the colonic epithelium even more hyperproliferative and lead to a 3-fold increase in crypt length and distorted crypt architecture (Fig. 1b). These poorly formed colons, presumably non-functional, can cause obstruction, mal-absorption, neonatal distress, chronic diarrhea, significant growth retardation and premature death. Although these animals had poor survival and somewhat underdeveloped reproductive organs, both male and female CEABAC20 mice are fertile. However, all lactating females (5/5) died with rectal prolapse within 2 weeks after delivery regardless of their age. In fact, during their short lifespan (usually ranging from 1

week to 3 months, and rarely up to 6 months), all virgin mice (8/8) also died with rectal prolapse.

Continuous non-focal tumors with dysplastic and serrated adenomatous morphology in CEABAC20 mice

Whereas the mild to moderate hyperproliferative colons in CEABAC2 and CEABAC10 mice only made them more susceptible to carcinogen-induced colon tumor formation (See Chapter 3), the extreme hyperproliferative colons seen in 3 week-old CEABAC20 mice continued to grow and progressed to the point where the entire colon could be considered a tumor, characterized by a non-focal mosaic of neoplastic lesions and a strikingly larger diameter, due to the presence of aberrant crypts that were up to ten times normal length (Fig. 5). The crypt architecture had progressed to a more advanced tumorigenic stage combining severe hyperplasia, crypt serration and dysplasia (Fig. 6). In fact, the combination of crypt lengthening, serration and branching observed in several human lesions, including mixed hyperplastic polyps, serrated polyps and serrated adenomas (Jass et al. 2002) that are subtypes of pre-cancerous lesions (Jass 2004), present a striking parallel with CEABAC20 mice. Interestingly, these lesions showed increased expression of CEA (Jass et al. 1984; Baker et al. 2004).

Numerous clumps of colonocytes, positive for CEA/CEACAM6, were present in the crypt lumen, which should normally be eliminated by anoikis/apoptosis (Fig. 6b and f). However, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) assays showed that no DNA fragmentation was present in these anchorless cells, indicating that they were not apoptotic (Fig. 7b and c). TUNEL assays also showed a general reduction of apoptosis (Fig. 7a-d). Moreover, cleavage of PARP [poly(ADP-ribose)polymerase], which is a hallmark of apoptosis, was shown to be reduced in CEABAC20 colons by immunoblots (Fig. 7e).

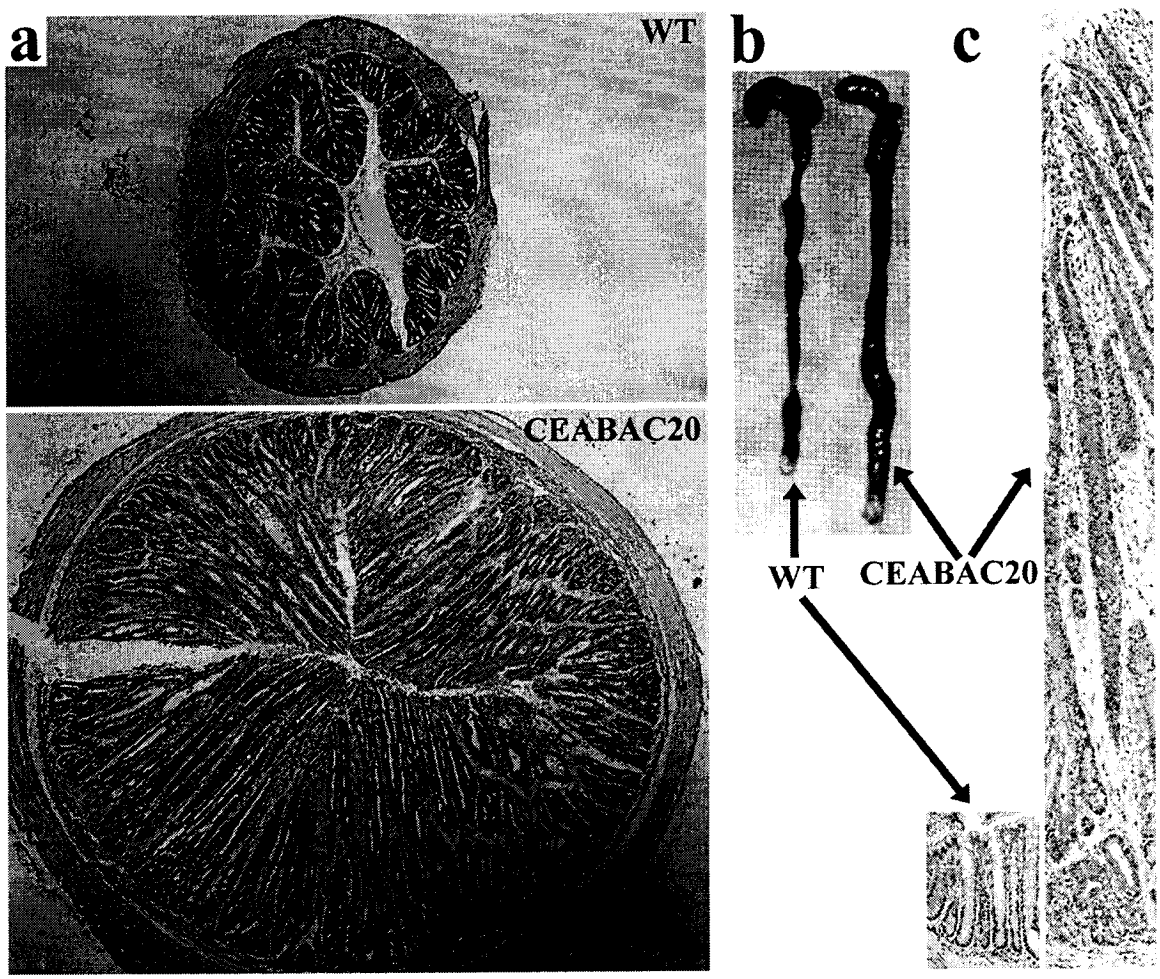

Figure 5: Massive colonic tumors in 3 month-old CEABAC20 mice. a) cross sections of colon; note the much larger size of CEABAC20 vs WT colon. **b)** dramatic increase of colon mass and absence of fecal pellets in CEABAC20 over the entire colon. **c)** colonic crypts; note the dramatic lengthening of intensely stained crypts in CEABAC20. Original magnification: x 40 (**a**), x 100 (**c**). Staining: hematoxylin (**a, c**). WT denotes wild-type littermate.

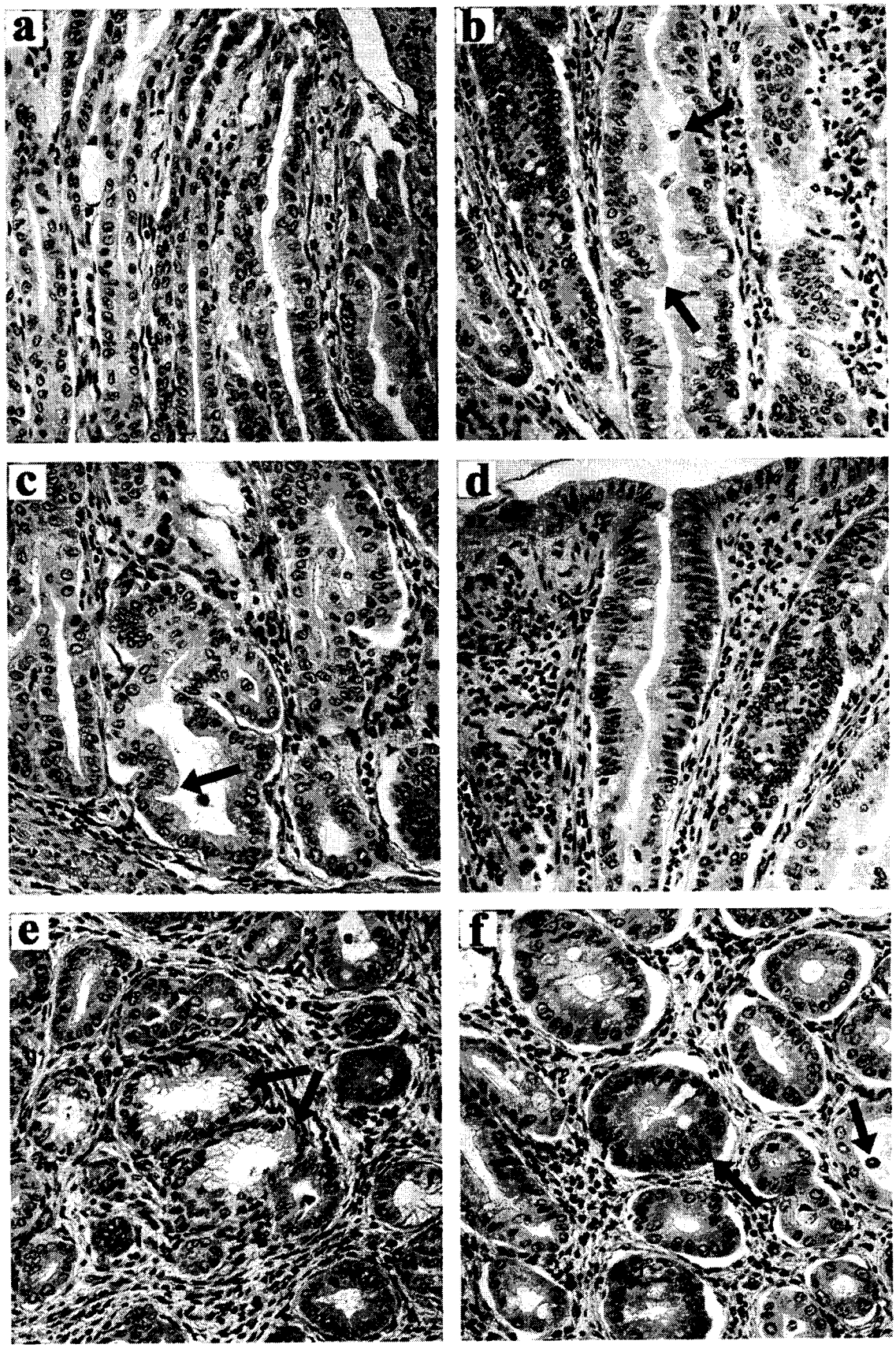
a
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Figure 6: Histopathological findings, including serrated adenomatous and dysplastic morphology, in CEABAC20 tumors. a-d) longitudinal sections of colonic crypts. **e-f)** cross sections of colonic crypts. **a)** upper third of elongated crypts, showing typical morphology for human serrated adenomas. **b**, **c)** serrated crypts with sawteeth like in-folding (blue arrows) at the mid-crypt (**b**) and crypt base (**c**). Note marked basal crypt branching in **c**. **d)** dysplastic crypt with elongated nuclei and nuclear stratification. **e)** hyperplastic crypts showing abundant mucinous vacuoles (red arrows) surrounded by dysplastic crypts. **f)** dysplastic crypt with nuclear stratification (green arrow). Note the presence of anchorless colonocytes in the crypt lumen (black arrows) in **b** and **f**. There is also evidence of myofibroblastic (spindle cell) proliferation in the lamina propria in **a**, **b** and **d**. Original magnification: × 400. Staining: hematoxylin and eosin.

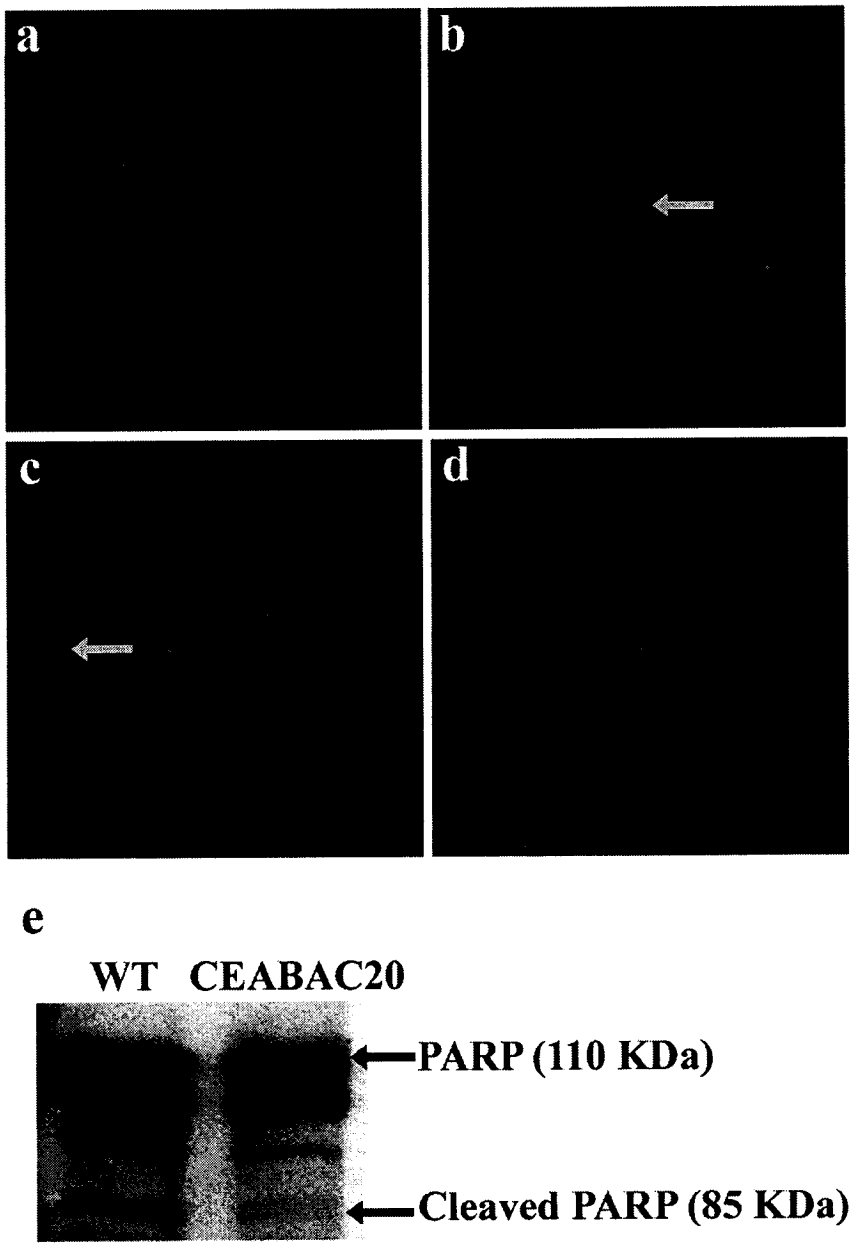

Figure 7: Inhibition of apoptosis in CEABAC20 mice. a-d) TUNEL assays on wild-type (**a**) and CEABAC20 (**b-d**) colons. Cyan: apoptotic nuclei; Blue: non-apoptotic nuclei. Original magnification: × 400. Staining: DAPI. Note that less apoptotic cells are present in CEABAC20 colon and the presence of non-apoptotic anchorless cells in the crypt lumen (yellow arrows). **e)** immunoblot of colon protein extracts for PARP showed reduced cleavage product of PARP indicating an overall reduction of apoptosis. WT denotes wild-type mice.

Wnt-signal perturbation is not associated with tumors in CEABAC20 mice

Perturbation of the Wnt signaling pathway (i.e., APC or β-catenin mutation) is commonly believed to be the initial step of colon tumorigenesis (Kinzler and Vogelstein 1996; Giles et al. 2003). The common feature of this pathway is nuclear accumulation of β-catenin due to its reduced degradation (Kinzler and Vogelstein 1996; Giles et al. 2003). However, recent studies have suggested the existence of alternative pathways of colon tumor initiation that are Wnt-independent (Takayama et al. 2001; Smith et al. 2002). For instance, the serrated pathway seen in human serrated adenomas, which are also present in the CEABAC20 mice, is a pertinent example (Jass et al. 2002; Jass 2004). A Western blot for β-catenin showed that steady state levels of β-catenin were similar in WT and CEABAC20 mice (Fig. 8a). Immunohistochemical investigation showed no nuclear β-catenin staining (Fig. 8b), indicating that the tumors with serrated features in CEABAC20 mice were not initiated through perturbation of the Wnt signaling pathway, as in human serrated adenomas. Involvement of other signaling pathways is currently under investigation.

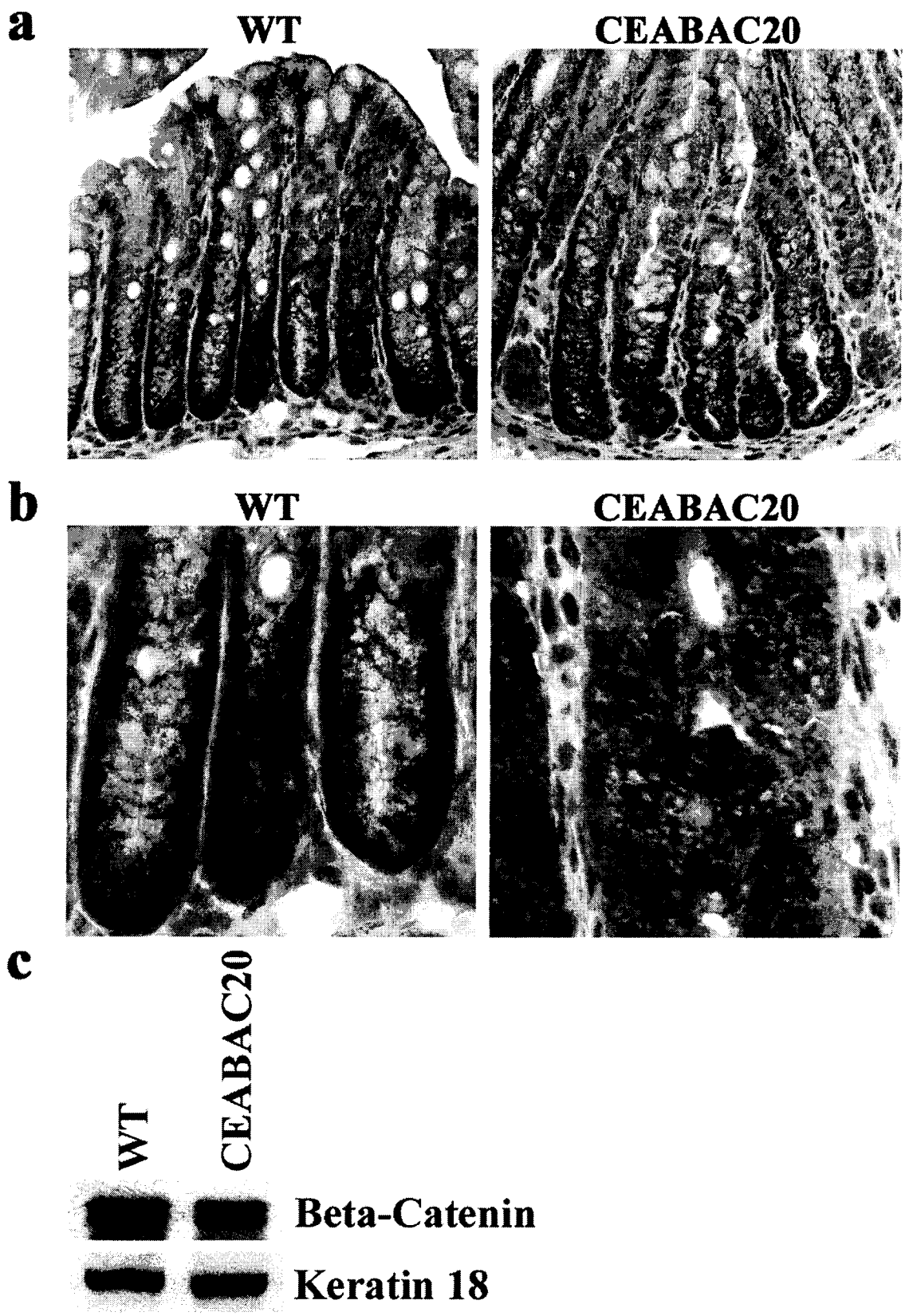
a
WT
CEABAC20
b
WT
CEABAC20
c
WT
CEABAC20
Beta-Catenin
Keratin 18

Figure 8: β-catenin expression and localization in CEABAC20 mice. Original magnification: ×400; staining: hematoxylin (**a** and **b**). WT denotes wild-type mice. **a)** expression level and pattern of β-catenin (brown staining) are similar between 3 week-old WT and CEABAC20 mice. **b)** β-catenin (brown staining) remained in the cytoplasm of colonocytes (with blue nuclei) of dysplastic crypts of 3 month-old CEABAC20 mice as in WT mice. **c)** expression level of β-catenin is slightly reduced in CEABAC20 relative to WT mice. Keratin 18 is used as loading control for epithelial cells and is approximately the same, indicating that the proportions of epithelial cells of the colonic tissue (estimated to be about 70-80%) are similar.

DISCUSSION

Human CEA and CEACAM6 are expressed in many normal tissues (Hammarstrom 1999), but are expressed at a much higher level in as many as 70% of human cancers, including those from colon, lung and breast (Chevinsky 1991; Allard et al. 1994; Ballesta et al. 1995; Sugita et al. 1999; Carrasco et al. 2000; Scholzel et al. 2000; Jantscheff et al. 2003). The mechanism for such an over-expression is presently unclear. Rearrangement, amplification and mutations of the CEA and CEACAM6 genes leading to their over-expression have never been identified (Tran et al. 1988; Boucher et al. 1989; Toribara et al. 1989; Cao et al. 1998); promoter hypomethylation of the CEA gene leading to transcriptional derepression is commonly found in human colorectal cancers (Tran et al. 1988; Boucher et al. 1989). Regardless of the mechanism, CEA/CEACAM6 over-expression can block cell differentiation, inhibit anoikis/apoptosis, disrupt cell polarization and tissue architecture, enhance metastasis, and increase chemoresistance *in vitro* and in xenograft mouse models (Eidelman et al. 1993; Gangopadhyay et al. 1996; Rojas et al. 1996; Leconte et al. 1999; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002; Wirth et al. 2002; Duxbury et al. 2004ab). Recently, we have introduced part of the human CEACAM family gene locus into mice (Chan and Stanners 2004) and have shown that this human gene locus is a susceptibility locus for developing lung and colon tumors (See Chapters 3 and 4). Thus, the presence of these genes and their de-regulated over-expression could play an important role in cancer development *in vivo*.

During investigation of the mechanisms for this increased susceptibility, we observed that the CEABAC colons were less differentiated and more proliferative in a gene dosage (expression level) dependent manner (Figs. 1 and 2). A common view is that lack of cell differentiation is a consequence of hyperproliferation. Alternatively, a block of cell differentiation maintains more cells with proliferative potential, which would lead to hyperproliferation. It is challenging to separate these two alternatives without mechanistic information at the molecular level. Nonetheless, CEA and CEACAM6 have been shown to block cell differentiation *in vitro* (Eidelman et al. 1993; Rojas et al. 1996; Ilantzis et al. 2002) but have no influence on cell proliferation in cells cultured in monolayer (Screaton et al. 1997). Hence, lack of cell differentiation in CEABAC colons

was likely a direct effect mediated by the CEA/CEACAM6 over-expression. It is not clear, however, whether hyperproliferation was a secondary effect of the differentiation block or an independent one in the *in vivo* context. In any case, over-expression of human CEA/CEACAM6 appears to result in a differentiation block and hyperproliferation in the mouse colon.

Depending on the expression level, CEA/CEACAM6 causes different degrees of hyperproliferation and leads to different outcomes. At mild to moderate expression levels (in CEABAC2 and CEABAC10 mice), CEA/CEACAM6 causes hyperproliferation and a mild to moderate differentiation block. Since proliferating cells are more susceptible to the acquisition of mutations (Bielas and Heddle 2000), it is possible that hyperproliferation caused by CEA/CEACAM6 expression could lead to a higher rate of tumor initiation and progression. This speculation is currently under investigation. However, at high or human tumor-like expression levels (in CEABAC20 mice), CEA/CEACAM6 causes a complete differentiation block (Fig. 1), an extreme hyperproliferation (Fig. 2) and an inhibition of apoptosis (Fig. 7) that progressed to severe hyperplasia and dysplasia by only 3 months of age (Figs. 5 and 6). Thus, only when CEA/CEACAM6 levels are above a critical threshold as in the CEABAC20 mice do they lead to progressive neoplasia. Since this neoplastic progression was observed only in CEABAC20 mice, the question arises as to whether these effects could have been due to random insertional mutation of an endogenous gene rather than to direct effects of the elevated expression level of CEA/CEACAM6. The former is unlikely, however, for the following reasons. Firstly, random mutation of endogenous genes by transgenesis is an uncommon event (Rijkers et al. 1994). Secondly, the probability of mutation of a gene resulting specifically in colorectal tumors is even lower. Thirdly, to our knowledge, such dramatic tumorigenic effects have never been reported in any knockout models. Lastly and most importantly, both independently produced transgenic lines, which would have different sites of integration, showed similar tumorigenic changes directly related quantitatively to expression level. It is not clear from this work, however, which of the CEACAMs present in the CEABAC are responsible for these effects. Since, as in humans, CEACAM7 is expressed only at very low levels in these transgenics' colons (Chan and Stanners 2004) and under-expressed rather than over-expressed in human

colorectal cancers (Hammarstrom 1999), and CEACAM3 is not at all expressed in the colon (Hammarstrom 1999; Chan and Stanners 2004), it seems likely that CEA and/or CEACAM6 are responsible and, in fact, both of the latter genes have been shown to elicit tumorigenic effects in various model systems *in vitro* and in xenograft mouse models (Eidelman et al. 1993; Gangopadhyay et al. 1996; Rojas et al. 1996; Leconte et al. 1999; Ordonez et al. 2000; Soeth et al. 2001; Ilantzis et al. 2002; Wirth et al. 2002; Duxbury et al. 2004ab).

The most striking and unexpected feature of the present results is first, the magnitude of the effects, which are arguably greater than those seen for any single oncogene or inactivated tumor suppressor gene expressed in animals and second, the fact that this tumorigenic phenotype involves the entire colon in a diffuse mosaic pattern of changes (Figs. 5 and 6). CEA over-expression has been shown to be mutagenic (Screaton et al. 1997) but, if random mutations were required, the neoplastic changes observed would be focal, as is the case for all other published mouse models with tumorigenic genetic alterations. The present case may bear some resemblance to that of mucin Muc2 deletion in mice (Velcich et al. 2002), however, in which relatively small non-focal increases in proliferation and decreases in apoptosis were observed in the entire gastrointestinal tract; in this case, focal carcinomas appeared later in life, presumably due to secondary mutations. In the CEABAC20 mice, the primary changes are so dramatic that any focal carcinomas would likely have little selective growth advantage and, in any case, the animals tend to die from digestive complications before such outgrowths could occur. Hence, this non-focal feature suggests that human CEA and/or CEACAM6 alone are sufficient to initiate this tumorigenic phenotype. Since human colonic tumorigenesis is in fact focal, we suggest that, unlike the situation here where every cell has multiple copies of CEA and CEACAM6 genes, their over-expression in the adult human colon could occur by focal up-regulation in expression as observed in the carcinogen-induced colon tumors in CEABAC mice (See Chapter 3).

Another interesting feature of these tumors was their resemblance to mixed hyperplastic polyps, serrated polyps and serrated adenomas in human patients (Jass et al. 2002; Jass 2004), which are subtypes of pre-cancerous lesions (Jass 2004) that show increased expression of CEA (Jass et al. 1984; Baker et al. 2004). In contrast to

conventional adenomas in which perturbation of the Wnt-signaling pathway is commonly believed as the initiating event (Kinzler and Vogelstein 1996; Giles et al. 2003), this serration is a consequence of alteration in cell proliferation and apoptosis leading to inactivation of DNA repair genes by promoter methylation and secondary mutations that cause tumor progression (Jass et al. 2002; Jass 2004). Thus, any gene product that increases cell proliferation and inhibits apoptosis could initiate a series of neoplastic events, called the *serrated pathway* (Jass et al. 2002; Jass 2004), without the involvement of the Wnt-signaling pathway. Based on the proliferative and anti-apoptotic effects of CEA/CEACAM6 shown in the CEABAC mice, it is not surprising that the over-expression of CEA/CEACAM6 could be a tumor initiating event (Fig. 8). These mice represent the first animal model for this type of human colorectal cancers.

However, the non-focal concomitant transition from hyperplasia to dysplasia in the CEABAC20 mice is very controversial. From a traditional standpoint, hyperplasia is believed to be *dead-end* and does not progress to dysplasia. Although the serrated pathway allows such a transition, only a small proportion of hyperplastic lesions transform to dysplastic serrated adenomas after acquiring other gene mutations, such as hMLH1 or MGMT (Jass et al. 2002; Jass 2004). The frequent transition to dysplasia observed in CEABAC20 mice suggests that over-expression of CEA and/or CEACAM6 to a critical threshold level alone is sufficient to transform a normal epithelium to a state of dysplasia. Further studies, including mutational analysis of many oncogenes, tumor suppressor genes and DNA repair genes are required to confirm this speculation.

In summary, the CEABAC mouse model has shown that human CEA and CEACAM6, in an expression level dependent manner, can have tumorigenic effects *in vivo*. Moderate expression levels cause an imbalance of tissue homeostasis leading to increased tumor susceptibility. High or tumor-like expression levels produce a severe imbalance and are sufficient to initiate colon tumor formation, at least the serrated sub-types, without the involvement of the Wnt-signaling pathway. Overall, these results represent a significant advance in our understanding of the relationship between CEA/CEACAM6 over-expression and cancer development. Moreover, they suggest a new paradigm for the use of CEA/CEACAM6 levels in cancer clinics and cancer therapies. Firstly, a higher basal tissue level of CEA and CEACM6 may lead to a higher

chance to develop sporadic cancer. Secondly, cancer therapies should aim to reduce their expression levels, and should not further up-regulate them to enhance tumor targeting. Lastly, reduction of their expression may reverse some tumorigenic phenotypes, as shown in some studies (Soeth et al. 2001; Wirth et al. 2002; Duxbury et al. 2004a; Ilantzis et al. unpublished data), and lead to higher sensitivity to chemotherapy (Duxbury et al. 2004b; Ordonez et al. unpublished data). Thus, development of agents that inhibit expression and function of these proteins could prove beneficial to clinical practice.

Chapter 6

Thesis Discussion and Conclusion

The GPI-anchored CEACAM family members, CEA and CEACAM6, are over-expressed in as many as 70% of all human cancers. This correlation suggests a possible role for them in tumor progression. Although their tumorigenic role was supported by extensive results obtained with several *in vitro* model systems and xenograft mouse models, their contribution and relevance to tumorigenesis *in vivo* has been unclear and somewhat controversial among researchers in the CEACAM field worldwide. In this thesis, we have constructed a novel mouse model for the CEA and CEACAM6 genes and clearly demonstrated their functional contribution to tumorigenesis *in vivo*. Moreover, our CEABAC mice will serve as animal model for the research on CEACAM-related diseases and for the development of CEACAM-based therapeutics. However, new controversies and paradigms have arisen from our studies that may affect both clinical and basic cancer research communities. A summary of our major findings, discussion of new controversies/paradigms and suggestions for future work are presented below.

The CEABAC Mouse Model

Transgenic mice harboring a 187 kb portion of the human CEACAM family gene locus contained in a bacterial artificial chromosome (CEABAC) that includes CEA, CEACAM3, CEACAM6, and CEACAM7 genes were successfully constructed in this study (Chapter 2). Two independent transgenic lines (CEABAC2 and CEABAC10, with 2 and 10 transgene copies, respectively) were established. The spatiotemporal expression pattern in both transgenic lines was strikingly similar to that of humans. In brief, CEA was found to have a more restricted expression pattern, with the highest levels in colon, stomach and vagina, than CEACAM6, which was shown to have a broader expression pattern with the highest levels in bone marrow (primarily immature myelocytes) and vagina. Expression of CEACAM7 was shown to be highly restricted to colorectum. The presence of CEACAM3 in mature neutrophils remained inconclusive. As for other published transgenics with large DNA constructs, the expression level of the CEABAC transgenes is solely dependent on transgene copy number. Based on these properties, this mouse model can have multiple applications, as exemplified in some of the following sections.

Conservation of Transcriptional Control between Humans and Mice

Conservation of transcriptional control of human genes in mice was observed in many published transgenic mouse models. Mouse homologous genes are usually present for those human genes tested; therefore, conservation of transcriptional regulation can easily be conceived. In this study, however, we have introduced four *novel* genes into mice (i.e., no homologous genes are present) and three of them, at least, are expressed very similarly to humans (Chapter 2). Since a similar observation was previously made in transgenic mice for the CEA gene (Eades-Perner et al. 1994; Clarke et al. 1998) and the CEACAM8 gene (Eades-Perner et al. 1998), this is no new finding for this large gene family; but now, we know that at least 4 of the 7 human CEACAM family genes and all four human GPI-anchored CEACAM family genes can be expressed the same way in mice as in humans.

As mentioned earlier, the GPI-anchored CEACAM family genes evolved after the primate radiation and have different spatiotemporal expression patterns than the transmembrane mouse CEACAMs. The question arises as to how the transcriptional regulation of these *novel* genes evolved in such a way that it is still conserved in mice. Two alternative hypotheses can be postulated. First, transcriptional regulation may have evolved between gene duplication of the ancestral gene and the primate radiation (i.e., in the common ancestor). However, this simple hypothesis has many problems: 1) if genes were duplicated before the evolution of the GPI-anchors, all GPI-anchors of the human GPI-anchored CEACAMs would have to be evolved independently (i.e., a very unlikely event); and 2) it is believed that some GPI-anchored CEACAM family genes were duplicated after the primate radiation (Zimmermann 1998), but yet they are expressed differently from each other. Second, assuming a conservation of *trans*-activating factors between mice and humans, *cis*-regulatory elements could have been added or deleted from the ancestral CEACAM1-like gene after gene duplication and evolution in the primate radiation. This hypothesis sounds plausible, but may not be exact based on the facts that: 1) *trans*-activating factors may not be completely conserved; and 2) the origin of added *cis*-elements is presently undefined. They could have been added by random nucleotide mutations, insertion of transposons or chromosomal exchange/rearrangements.

Thus far, only small portions of the human CEA, CEACAM6 and CEACAM1 promoter sequences have been studied and only a limited number of *cis*-regulatory elements were identified in these genes. Hence, it is important to identify *cis*-regulatory elements further upstream and downstream of the human CEA, CEACAM6 and CEACAM1 genes, and those present in other human and primate CEACAM family genes. As shown in Figure 2 of Chapter 1, each CEACAM family gene has a distinct set of *cis*-regulatory elements, some of which are shared and some of which are not. By combining these elements with the previously postulated phylogenetic tree of these genes, we can approximate how these *cis*-regulatory elements evolved during evolution.

Evolution of the GPI-anchored CEACAMs in Primates

Although the sequence of evolutionary events of this multigene family had been postulated (Zimmermann 1998), the exact selective pressure, especially on evolution of the tumorigenic GPI-anchored members, is debatable. Host-pathogen co-evolution models suggest the advantage of evolution of the GPI-anchored CEACAMs based on pathogen interactions. Since some pathogens had evolved to bind CEACAM expressed on the cell surface for infection, GPI-anchored CEACAMs, which can easily be shed off from the cell surface, were suggested to be subsequently selected for to act as molecular decoy for pathogen binding (Kammerer et al. 2004b). This model, however, did not consider the fact that soluble or secreted CEACAMs, which can serve the same purpose, can perhaps act even better than the GPI-anchored CEACAMs and that the GPI-anchored CEACAMs bearing tumorigenic functions may be important during embryonic morphogenesis.

In the CEABAC mouse model, we have clearly demonstrated the tumorigenic role of GPI-anchored family members CEA and CEACAM6 (Chapters 3-5). Also, although not present in this thesis, expression of CEA and CEACAM6 were detected in the developing gut and other endoderm-derived organs of the CEABAC mouse embryos/fetuses. Preliminary results showed a delay in colonic differentiation in the developing gut of CEABAC mouse fetuses when compared to the WT littermates (data not shown). While CEABAC2 and CEABAC10 mouse colons resumed normal development probably due to a *normal down-regulation* of CEA/CEACAM6 expression

in maturing gut (data not shown), CEABAC20 mouse colons progressed to neoplasia. Perhaps, the expression level of CEA/CEACAM6 was still above a critical threshold after the *normal down-regulation* in the CEABAC20 mice and certain embryonic features could have been retained in the maturing gut. If this is the case, the neoplastic phenotype should be reversible by down-regulating CEA/CEACAM6 expression. In fact, some tumorigenic effects were shown to be reversed in human colon and pancreatic cancer cell lines by knocking down CEA and CEACAM6 expression (Soeth et al. 2001; Duxbury et al. 2004a). Moreover, these tumorigenic effects could be important for the evolution of endodermal morphogenesis in higher order mammals and advantageous for their survival in later life. Hence, more detailed studies should be performed on embryogenesis of the CEABAC mice in order to gain more insight on possible selective advantages for the evolution of tumorigenic GPI-anchored CEACAM family genes in primates.

Tumorigenic Role of CEA and CEACAM6 in vivo

Previous results showed that over-expression of CEA and CEACAM6 can block cell differentiation, inhibit anoikis/apoptosis, and disrupt cell polarization and tissue architecture *in vitro* and in xenograft mouse models (see Chapter 1). In this thesis, these tumorigenic properties of CEA and CEACAM6 received a remarkable confirmation *in vivo* (Chapter 5). Due to these tumorigenic effects, at low to moderate expression levels of CEA and CEACAM6 (in CEABAC2 and CEABAC10 mice), the CEABAC mice are more susceptible to develop spontaneous lung tumors (Chapter 4) and carcinogen-induced colon tumors (Chapter 3). At higher or human colorectal tumor-like expression levels (in CEABAC20 mice), spontaneous colon tumor formation with dysplastic and serrated adenomatous morphology were present in the CEABAC mice (Chapter 5). Altogether, these results provide direct evidence for an important role of CEA/CEACAM6 in cancer development *in vivo*. However, the non-focal nature of the lesions, the concomitant transition from hyperplastic to dysplastic lesions, the Wnt-signal independence of tumor initiation, and the pleiotropic effects in the CEABAC20 colons require more attention and further investigation.

What does non-focal neoplasia mean?

Cancer is known to be a clonal disease in which various genetic changes are accumulated in a given cell and which drive its expansion to a focal lesion. In contrast, the spontaneous tumors developed in the CEABAC20 colons were non-focal, i.e., the tumorigenic phenotype involved the entire colon in a diffuse mosaic pattern of changes. To our knowledge, this type of drastic phenotype has never been reported in any human cases of cancers and any genetically altered animal models. This disparity, however, does not bring down the value of this finding in relation to cancer development. Unlike those typical cases of cancers, every cell here has the same inherited genetic alteration (i.e., the presence of 20 copies of CEA and CEACAM6 genes). Hence, it simply suggests that over-expression of human CEA and/or CEACAM6 alone is sufficient to initiate tumor formation to a certain extent without the acquisition of other mutations.

Progression to dysplasia from hyperplasia

Progression from hyperplasia to dysplasia raises another major concern. From a traditional standpoint, hyperplasia is believed to be a *dead-end* state and does not progress to dysplasia. Although the *serrated pathway* allows such a transition, only a small proportion of hyperplastic lesions have been found to transform to dysplastic serrated adenomas after acquiring other gene mutations, such as hMLH1 or MGMT (Jass et al. 2002; Jass 2004). The extensive transition to dyplasia from hyperplasia here leads to a hypothesis that over-expression of CEA/CEACAM6 drive the colons to an extreme mutator phenotype. In fact, CEA over-expression has been shown to be mutagenic (Screaton et al. 1997); but, if random mutations were required, the neoplastic progression observed would be focal, as is the case for all other published mouse models with tumorigenic genetic alterations. However, the non-focal concomitant transition from hyperplasia to dysplasia in the CEABAC20 mice may indicate that over-expression of CEA and/or CEACAM6 to a critical threshold level alone is sufficient to transform a normal epithelium to dysplastic epithelium. Alternatively, the CEA/CEACAM6-induced random mutation rate could be high enough to convert most hyperplastic structures to focal dysplastic lesions extensively admixed within a hyperplastic background, so that it would not be possible to clearly separate them individually. In any case, mutational

analysis on many oncogenes, tumor suppressor genes and DNA repair genes in the dysplastic lesions present in the CEABAC20 mice are required to confirm either speculation. Moreover, mutational analysis of known oncogenes or tumor suppressor genes in the carcinogen-induced colon tumors of CEABAC2 and CEABAC10 mice (i.e., to test if mutation frequency changes) could help in determining mutagenic properties of CEA and CEACAM6, if any.

Why don't the CEABAC20 mice have cancer?

Another concern is a lack of a complete spectrum of malignant features, i.e., the presence of adenocarcinoma, local invasion and metastasis. This shortcoming is mainly due to the apparent gastrointestinal complications (obstruction, malabsorption, chronic diarrhea, and rectal prolapse) of the CEABAC20 mice, which give them a very short lifespan (1 week to 6 months, mostly 2-4 weeks). Hence, they do not have a chance to develop advanced cancers. If the CEABAC20 mice could live longer, a full spectrum of cancer would likely have been observed. To eliminate this limitation in future studies, implantation of CEABAC20 colon tumor tissues into normal mice could test their ability to develop advanced cancers.

Neoplastic pathways and molecular mechanisms

Perturbation of the Wnt signaling pathway (i.e., loss of functional APC or activating mutations of β-catenin) is commonly believed to be the initial step in malignant transformation by most clinicians/researchers. The common feature of these tumors is the nuclear accumulation of β-catenin due to its improper degradation or uncontrolled activation. However, the spontaneous tumors (both hyperplastic and dysplastic regions) of CEABAC20 mice do not present with nuclear β-catenin accumulation. Hence, their tumorigenic progression is unlikely initiated by the perturbation of Wnt-signaling pathway. Alternatively, the *serrated pathway*, which is independent of the Wnt-signaling pathway, is started by a general inhibition of anoikis (causing hyperplasia) and followed by the acquisition of other gene mutations, such as hMLH1 or MGMT (causing dysplasia). Since the histological features of these CEABAC20 tumors resembled those of mixed hyperplastic polyps, serrated polyps and serrated adenomas in human patients,

CEA/CEACAM6 over-expression could act through this *serrated pathway*. Once again, the non-focal concomitant transition from hyperplasia to dysplasia could question the validity of the latter speculation. The integrin signaling pathway is currently believed to play a major part in the CEA/CEACAM6 mediated effects. Preliminary results have shown that integrin α_5 is up-regulated in the CEABAC mice and its downstream elements are subsequently more activated (Camacho-Leal et al., unpublished data). More detailed studies should be performed in order to construct a complete molecular mechanism for the pleotropic effects mediated by the over-expression of CEA/CEACAM6. Moreover, the CEABAC mice can be crossed with the *floxed* (conditional knockout) β_1-integrin mice and locally (rectum in our case) infected with adenoviral Cre recombinase to remove β_1-integrin expression (Raghavan et al. 2000). If $\alpha_5\beta_1$ integrin is definitively involved in the CEA/CEACAM6-mediated neoplastic phenotype, a marked reduction in tumorigenicity should be observed. In any case, over-expression of CEA and/or CEACAM6 could represent an alternative initiating event in colon cancers.

Can we separate the effects of CEA from that of CEACAM6?

Although the tumorigenic role of CEA and CEACAM6 in colorectal cancer was well established and confirmed in this study, the present study failed to distinguish the individual contributions of CEA and CEACAM6 in the tumorigenic phenotype in the CEABAC mice. However, based on previous *in vitro* studies, they both perform similar functions and give similar effects (Eidelman et al. 1993; Rojas et al. 1996; Ordonez et al. 2000; Ilantzis et al. 2002; Duxbury et al. 2004a); nevertheless, we cannot rule out the contrary without direct evidence, since they may act differently in the *in vivo* context and they may not have the same efficiency in driving tumorigenesis. This can be done by two approaches: 1) construction of two other CEABAC transgenic mice, one with CEA knocked out and one with CEACAM6 knocked out; and 2) systemic knockdown of either CEA or CEACAM6 by siRNA technology. In either case, their tumorigenic effects *in vivo* could be individually assessed; however, this does not guarantee any tumorigenic phenotype since both CEA and CEACAM6 may be required for the observed effects in this study.

Is there synergy with other gene mutations?

The APC mutants and activated K-ras were shown to have an important role in the initiation and progression of colon cancer (Kinzler and Vogelstein 1996). Likewise, an important tumorigenic role of CEA and CEACAM6 is presented in this thesis and in other studies (see Introduction). As mentioned in the introduction, mutations in the APC gene (or alternatively, the β-catenin gene), mutations in the K-ras gene and the over-expression of CEA/CEACAM6 can be found in the early lesions of colon cancers. Hence, it will be interesting to investigate the synergism between these neoplastic events, if any, in future studies using the CEABAC mouse model.

Mice harbouring APC mutations, such as the $APC^{Min/+}$ mice (Su et al. 1992), $APC^{\Delta 716}$ mice (Oshima et al. 1995) and APC^{1638N} mice (Fodde et al. 1994), give focal intestinal, but rarely colonic, polyps that eventually progress to adenomas and carcinomas due to inactivation of the wild-type APC allele and/or acquisition of other gene mutations (Fodde et al. 2001). By crossing these mice with the CEABAC mice, we can determine any synergism between them through the observation of phenotypic changes, such as changes in tumor load, tumor localization (i.e., intestinal vs colonic) and latency of tumor initiation and progression. Similarly, CEABAC mice can be crossed with mice transgenic for the activated K-ras, such as the $K\text{-}ras^{V12G}$ transgenic mice (Janssen et al. 2002), or mice with other gene mutations.

Role of CEA and CEACAM6 in other cancers

The expression of the human CEACAM family genes in gastrointestinal, breast, respiratory, urogenital and hematopoietic systems in the CEABAC mice could facilitate studies of many CEA/CEACAM6-overexpressing cancers. Since dramatic tumorigenic effects of CEA/CEACAM6 were documented for colorectal cancer in this study, they could play an important role in other cancers, such as lung cancer, breast cancer and leukemia, as well. In fact, the over-expression of CEA and CEACAM6 in the CEABAC mice was shown to increase the incidence of spontaneous lung tumors (see Chapter 4). Further studies on the cellular and molecular mechanism in the lung system may provide more insight as to how the expression of CEA and CEACAM6 predisposes mice to spontaneous lung tumors.

Since CEA and CEACAM6 were shown to be over-expressed in breast cancer and CEACAM6 in leukemia (Chevinsky 1991; Allard et al. 1994; Kuroki et al. 1994; Ballesta et al. 1995; Boccuni et al. 1998; Hansen et al. 1998; Carrasco et al. 2000), one would expect a higher susceptibility to these two types of cancers as well in the CEABAC mice. However, until now, no breast cancer or leukemia was found in the CEABAC mice. This may be due to the aforementioned sub-threshold expression levels of CEA/CEACAM6 or the absence of other gene mutations or over-expression, such as the recombinant BCR-ABL protein for leukemia (Daley 1993; Van Etten 2001; Wong and Witte 2001) and the over-expression of Neu/ErbB-2 for breast cancer (Muller et al. 1998). Hence, studies on their possible role in other cancers, in which our CEABAC mouse model could be used for crosses with other mutant mice [for example, the BCR-ABL transgenic mice for leukemia (Daley 1993; Van Etten 2001; Wong and Witte 2001) and the Neu/ErbB-2 transgenic mice for breast cancer (Muller et al. 1998)], may result in a significant contribution to the understanding of these multi-faceted diseases.

The CEABAC Mice in Studies of Other Human Diseases

The expression of the CEACAM family genes in gastrointestinal, breast, respiratory, urogenital and hematopoietic systems in the CEABAC mice could facilitate the search for therapeutic agents against other diseases associated with the human CEACAM family and also provides an adequate model for basic research. Examples include: 1) *Neisseria Gonorrhoeae* infection, a sexually transmitted disease, uses various human CEACAM family members as major binding receptors (Dehio et al. 1998). Since mice do not possess human CEACAM family genes, mice are not susceptible to gonococci infection. With the presence of these human CEACAMs, our CEABAC mice should be susceptible to infection. Thus agents that inhibit this interaction *in vivo* may be useful for prevention and their effectiveness could be assessed in the CEABAC mouse model. 2) Human CEACAM family members can activate neutrophils and increase their adhesion to endothelial cells and extracellular matrices (Kuijpers et al. 1992; Nair and Zingde 2001; Skubitz et al. 2001). They can also induce cytokine release from immune cells (Gangopadhyay et al. 1996; Filella et al. 2001). These properties suggest their role in inflammatory reactions and their potential use as targets for anti-inflammatory drugs.

Significance in Clinics and Therapeutics

Overall, the results of this thesis provide significant advances in understanding the relationship between CEA/CEACAM6 over-expression and cancer development. However, these results create a new paradigm for CEA/CEACAM6 in cancer clinics. First, higher a basal tissue level (which may not be well represented by the serum level) of CEA and CEACM6 may lead to a higher chance of developing sporadic cancer. Second, cancer therapies should aim to reduce their expression levels, and not further up-regulate them to enhance tumor targeting. Last, reduction of their expression may reverse some tumorigenic phenotypes and lead to higher sensitivity to chemotherapy. Thus, development of agents that inhibit expression and function of these proteins could prove beneficial to clinical practice.

As mentioned in the thesis introduction, CEA and CEACAM6 represent popular targets for novel cancer therapies, including cancer vaccines, cellular immunotherapy, radioimmunotherapy and antibody therapy. Although pre-clinical animal data can show promising effects, clinical outcomes have commonly been unfavorable, such as low therapeutic response or the presence of associated toxicity. This discrepancy suggests improper or insufficient assessment by present animal models. Although previously constructed transgenic mice bearing the CEA gene alone can improve the validity of pre-clinical tests (Mizobata et al. 2000; Xu et al. 2000; Wilkinson et al. 2002), a transgenic model with a closer human approximation is necessary to ensure valid pharmacokinetics of test agents and to avoid possible treatment toxicity and/or reduced efficacy from cross-reaction with other highly homologous human CEACAM family members. Thus, our CEABAC mouse model should provide a better test model, but this requires confirmation.

Concluding Statement

The contents of this thesis have clearly demonstrated an important role of the human GPI-anchored CEACAM family members CEA and CEACAM6 in tumorigenesis *in vivo*. However, follow-up research of the type discussed here should be considered to advance our knowledge on this devastating disease and other diseases related to the CEACAM family genes.

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APPENDICES