MOLECULAR AND CELLULAR BIOLOGY, Jan. 2003, p. 272–279 Vol. 23, No. 1
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.1.272–279.2003

Carcinoembryonic Antigen-Related Cell Adhesion Molecule 10 Expressed Specifically Early in Pregnancy in the Decidua Is Dispensable for Normal Murine Development

Daniela Finkenzeller,[1]† Beate Fischer,[1]‡ Sabine Lutz,[1]§ Heinrich Schrewe,[2]‖ Takehiko Shimizu,[2]# and Wolfgang Zimmermann[1,3]*

Institute of Molecular Medicine and Cell Research, University of Freiburg,[1] and Department of Developmental Biology, Max-Planck Institute of Immunobiology,[2] Freiburg, and Tumor Immunology Laboratory, Department of Urology, University Clinic Grosshadern, Ludwig-Maximilians-University München, D-81377 Münich,[3] Germany

Received 4 June 2002/Returned for modification 4 September 2002/Accepted 25 September 2002

The carcinoembryonic antigen (CEA) family consists of a large group of evolutionarily and structurally divergent glycoproteins. The murine CEACAM9 and CEACAM11-related proteins as well as the pregnancy-specific glycoproteins (PSG) are secreted members of the CEA family which are differentially expressed in fetal trophoblast cell populations during placental development. PSG are essential for a successful pregnancy, possibly by protecting the semiallotypic fetus from the maternal immune system. In contrast, *Ceacam10* mRNA, coding for a protein identical in structure with CEACAM11-related proteins, is expressed in the maternal decidua surrounding the implantation site of the conceptus only during early stages of gestation between day 6.5 and day 10.5 postcoitum. To determine its role during murine development, we inactivated *Ceacam10*. *Ceacam10*$^{-/-}$ mice developed, like the previously established *Ceacam9*$^{-/-}$ mice, indistinguishably from wild-type littermates with respect to sex ratio, weight gain, and fertility. However, a small but significant reduction of the litter size by 23% was observed in *Ceacam10*$^{-/-}$ matings. Furthermore, combining the *Ceacam9* and *Ceacam10* null alleles, both located on chromosome 7, by meiotic recombination and subsequent mating of heterozygotes carrying both knockout alleles on one chromosome yielded wild-type and double knockout offspring at the expected Mendelian ratio. Taken together, both *Ceacam10* and *Ceacam9*, alone or in combination, are not essential for either murine placental and embryonic development or for adult life.

The carcinoembryonic antigen (CEA) family represents one of the most divergent group of glycoproteins in mammals with respect to sequence and structure (15, 54, 59). It belongs to the populous immunoglobulin superfamily and can be subdivided into two main subgroups, based on sequence similarities and expression pattern: the CEA-related cell adhesion molecule (CEACAM) and the pregnancy-specific glycoproteins (PSG) subgroups (for the revised nomenclature of the CEA family, see reference 1). The members of the CEACAM subgroup are found in a wide variety of cell types, e.g., epithelial and endothelial cells, granulocytes, macrophages, B cells, dendritic cells, CD16$^-$ natural killer (NK) cells, and activated T cells, whereas the expression of the secreted PSG, encoded by at least 15 closely related genes in mice, is restricted predominantly to embryonic cells derived from the trophoblast cell lineages (16, 23, 37, 58).

CEA-related molecules play a role in a number of normal and pathological processes, like control of granulocyte, dendritic cell, and T-cell activation (22, 23, 29, 38), regulation of differentiation, breast duct formation, and angiogenesis (9, 20, 48). The expression of a number of CEA family members is often deregulated in tumors (21, 41, 44, 47), and in vitro and in vivo experiments suggest that they play a role in tumor genesis either by enhancement of metastasis or suppression of anoikis (17, 43, 53). CEACAM1 can act as a tumor suppressor, and its common down-regulation of expression in epithelial tumors probably causes disturbance of growth or differentiation (12, 19). On the other hand, CEACAM1 and other members of the CEA family might facilitate tumor escape from immune surveillance by interacting with CEACAM1, which can act as an inhibitory receptor on T and NK cells (24, 36, 39). Furthermore, bacterial pathogens such as *Neisseria gonorrhoeae* use the transmembrane protein CEACAM1 (and other CEACAM subfamily glycoproteins) as a receptor for their entry into host cells and appear to manipulate the immune system through inhibition of CD4$^+$ T lymphocytes via signaling through immunoreceptor tyrosine-based inhibition motifs within the cytoplasmic domain of CEACAM1 (3–5, 13, 55), whereas the mouse hepatitis virus invades host cells by using CEACAM1 and CEACAM2 as receptors (7, 40).

PSG are expressed in increasing amounts during pregnancy and represent the most abundant fetal proteins in the maternal circulation at term (28, 34). Experimental depletion of PSG

* Corresponding author. Mailing address: Tumor Immunology Laboratory, Department of Urology, University Clinic Grosshadern, Ludwig-Maximilians-University München, Marchioninistrasse 23, D-81377 Munich, Germany. Phone: 49 (0) 89 7095-4895. Fax: 49 (0) 89 7095-4864. E-mail: wolfgang.zimmermann@life.med.uni-muenchen.de.

† Present address: Genescan Europe AG, 79108 Freiburg, Germany.

‡ Present address: Division of Rheumatology and Clinical Immunology, Department of Internal Medicine, University Hospital Freiburg, Freiburg, Germany.

§ Present address: Department of Pharmaceutical Technology, Pharmaceutical Institute, University of Freiburg, Germany.

‖ Present address: School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom.

Present address: Department of Pediatric Dentistry, Nihon University, School of Dentistry at Matsudo, Chiba, Japan.

causes abortions in mice and monkeys (2, 18). PSG seem to act on monocytes via high-affinity receptors, inducing cytokines favorable to TH2 immune responses which are biased in the maternal immune system during pregnancy (45, 49, 56). The shift from a TH1- to a TH2-type immune status is thought to be important for the acceptance of the histo-incompatible fetus during pregnancy without compromising immune reactions against pathogens (57). PSG and other trophoblast members of the CEA family might thus contribute in the protection of the semiallotypic fetus from the maternal immune system. Interestingly, sole inactivation of *Ceacam9* (formerly *Cea5*), a murine CEA family member structurally related to *Psg*, which is expressed, in contrast to *Psg,* only during early stages of pregnancy in trophoblast giant cells, does not interfere with a positive outcome of allotypic and semiallotypic pregnancies (10).

Ceacam10 (formerly *Cea10*) represents an evolutionarily very recent addition to the murine *Cea* gene family. It encodes a secreted, two-immunoglobulin variable (IgV)-like domain glycoprotein and was probably formed by gene duplication of a *Ceacam1* (former *Bgp1*)-related ancestor after separation of the rat and mouse. This recent independent evolution is supported by the fact that the supposed rat counterpart *CEACAM10* (formerly *C-CAM4*) codes for a secreted, one-IgV-like domain protein also very closely related to the rat CEACAM1 (8, 59). The murine CEACAM10 exhibits the same two-IgV-like module composition as a recently discovered group of proteins encoded by *Ceacam11-Ceacam14* (25, 26; see also http://cea.klinikum.uni-muenchen.de/). However, less than 40% (37 to 40%) of the amino acids are shared between CEACAM10 and the CEACAM11-CEACAM14 proteins, which have 68 to 80% of their amino acid sequences in common. In contrast to most of the other placentally expressed *Cea* gene family transcripts, *Ceacam10* mRNA is found in a timely restricted manner in placenta only during early development (25, 26). Its cellular distribution, however, is not known. In order to clarify the in vivo functions of the *Ceacam10* gene during embryonic development, we determined the expression pattern of *Ceacam10* mRNA by in situ hybridization and inactivated *Ceacam10* by homologous recombination.

Here we report that *Ceacam10* is exclusively expressed in the maternal placenta surrounding the implantation site. *Ceacam10*$^{-/-}$ males and females are viable, fertile, and exhibit no obvious phenotype. Furthermore, double knockout mice homozygous for null mutations of both *Ceacam9* and *Ceacam10* could not be discriminated from their wild-type littermates. This indicates that members of the evolutionarily young *Cea* gene family are dispensable for mice kept under laboratory conditions and probably serve subtle functions.

MATERIALS AND METHODS

Construction of the targeting vector. The mouse genomic cosmid clone 16.2a from BALB/c liver DNA containing the *Ceacam10* gene was used to construct the targeting vector (26). Digestion with *Bam*HI yielded a 2.2-kb fragment and a 12.8-kb fragment comprising the promoter region and exon 1 as well as part of exon 2 and part of exon 3 and exon 4, respectively. For generation of the targeting vector, the 12.8-kb *Bam*HI DNA fragment was further digested with *Sst*I, and the 6.0-kb *Bam*HI/*Sst*I DNA fragment and the *Not*I-digested ptkneoXba$^-$ vector were blunt ended and ligated. The 2.2-kb *Bam*HI fragment was fitted with *Bam*HI/*Cla*I adaptors and cloned into the *Cla*I-digested targeting vector upstream of the *neo* cassette. The pMCI-HSV-TK cassette (35) 5′ of the 2.2-kb *Bam*HI *Ceacam10* fragment allowed negative selection against random integration. In comparison with the genomic sequences, the resulting targeting construct lacks a 2.7-kb *Bam*HI DNA fragment which comprises part of exon 1, intron 2, and part of exon 2. It was replaced by a 1.7-kb *neo* cassette. For electroporation, the targeting vector was linearized with *Kpn*I.

Gene targeting in ES cells and generation of mutant mice. All experiments which involved animals were registered and performed in compliance with the German Animal Protection Law. Electroporation of the targeting vector into the BALB/c-derived embryonic stem (ES) cell line BALB/c-I (42), G418–2′-fluoro-2′-deoxy-5-iodo-1-β-D-arabinofuranosyluracil (FIAU) double selection for recombinants, and ES cell propagation were performed as described before (10, 51). *Ceacam10* mutant ES cell lines were injected into 3.5-day postcoitum (dpc) C57BL/6 blastocysts. After 2 h in culture in M16 medium (Sigma), the embryos were surgically transferred into the uteri of pseudopregnant NMRI recipients at 2.5 dpc. Male chimeras were mated with BALB/c females to produce mice heterozygous for the null mutation. F_1 intercrosses of heterozygous mice finally resulted in F_2 offspring which were wild type and heterozygous or homozygous for the targeted allele, respectively. A *Ceacam10*$^{-/-}$ strain was established and maintained by breeding *Ceacam10*$^{-/-}$ males and females.

Mouse genotyping. Homologous recombination between the targeting vector and *Ceacam10* on mouse chromosome 7 was examined by Southern blotting using genomic DNA isolated from ES cells or tail biopsies after digestion in lysis buffer with 100 μg of proteinase K/ml (31). Genomic DNA was digested with *Hin*dIII, separated by electrophoresis through a 1% agarose gel, and transferred to a positively charged nylon membrane. A ^{32}P-labeled 0.2-kb *Ssp*I/*Bam*HI fragment directly upstream of the 2.2-kb *Bam*HI *Ceacam10* fragment from the targeting vector was used as probe to confirm correct homologous recombination in the 5′-flanking region. The established *Ceacam10* knockout strain was genotyped using two pairs of oligonucleotides as PCR primers in one reaction mix. The mutant allele primers (5′-TAGCGTTGGCTACCCGTG and 5′-CTCAGA AGCCATAGAGCC) bind in the neomycin expression cassette and generate a 408-bp DNA fragment, while the wild-type allele-specific primers (5′-TTAGCC TCACTTTTAACTTAC and 5′-GCACAGAAATCG-GAGTAATT), located in the N1 domain exon, gave rise to a 350-bp fragment. The *Ceacam9*$^{-/-}$/*Ceacam10*$^{-/-}$ double knockout mice were genotyped by PCR using the following primer pairs in four separate reactions: *Ceacam9* wild-type allele, 5′-CTTAAC CTGCTGGAATGCACCCGCCG and 5′-GCACTTCCAGATGCACATGTGT TAATTCG (fragment size, 349 bp; location, exon 2); *Ceacam9* mutant allele, 5′-TAGCGTTGGCTACCCGTG and 5′-ACATGTGTTAATTCGCTTTCC (fragment size, 560 bp; location, *neo* and *Ceacam9* exon 2, respectively); *Ceacam10* wild-type allele, see above; *Ceacam10* mutant allele, 5′-TTGTAAC ATCCATTAATAGGAG and 5′-GAATGTGTGCGAGGCCAG (fragment size, 197 bp; location, *Ceacam10* exon 2 and *neo*). PCRs were performed in *Taq* polymerase buffer (10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 50 mM KCl) complemented with 1.5 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, a 1 μM concentration of each primer, and 40 U of *Taq* polymerase/ml for 30 cycles (denaturation at 94°C for 20 s; annealing at 60°C for 45 s; elongation at 72°C for 3 min; and final extension at 72°C for 10 min). The resulting DNA fragments were separated by electrophoresis through 2 to 2.5% agarose gels.

In situ hybridization and RT-PCR. Unfixed placentae and embryos of various stages of development of BALB/c and BALB/c *Ceacam9*$^{-/-}$ mice were frozen in tissue freezing medium (Jung, Nussloch, Germany) diluted 1:1 with water in toto (days 6.5, 8.5, and 10.5) or after dissection (days 12.5, 14.5, and 16.5). Seven-micrometer cryosections were placed on SuperFrost/Plus microscope slides (Roth, Karlsruhe, Germany). In situ hybridization with digoxigenin-labeled sense and antisense RNA probes was performed as described before (46, 58). The gene-specific RNA probes for the detection of *Ceacam9* (11) and *Ceacam10* transcripts (location of probe, position 776 to 998 of *Ceacam10* cDNA [26]) covered most of the respective 3′-untranslated regions. The Psg18 probe contained the complete coding sequence (1.6 kb) and probably cross-reacts with all *Psg* mRNAs (58). The probes derived from the marker genes *4311* (0.75 kb), *Mash-2* (1.5 kb), and *Pl-I* (0.8 kb) are described elsewhere (6, 14, 32). Total RNA was isolated from staged placentae with embryos and colon using the RNeasy Midi kit (Qiagen, Langen, Germany). One microgram of total RNA was reverse transcribed (reverse transcription system; Promega, Mannheim, Germany) followed by PCR (25-μl total volume) with 1/20 of the reverse transcription (RT) reaction and gene-specific primers (for *Ceacam9*, 5′-CTTAACCTGCTGGAAT GCACCCGCCG and 5′-CAGCTTCTGTTACCGCGGTGCTGTCT; product size, 407 bp; annealing temperature, 68°C; for *Ceacam10*, 5′-CAGCCTCACTT TTAACTTACT and 5′-GCACAGAAATCGGAGTAATT, product size, 350 bp; annealing temperature, 62°C) using the following conditions: 30 cycles of

denaturation at 94°C for 20 s, annealing for 45 s, and extension at 72°C for 3 min; final extension was for 10 min. As a control, β-actin mRNA was analyzed using the following primers: 5′ primer, 5′-ATGGATGACGATATCGCT; 3′ primer, 5′-ATGAGGTAGTCTGTCAGGT; product size, 569 bp; annealing temperature, 58°C. To rule out amplification of processed β-actin pseudogenes from contaminating genomic DNA, PCR was also performed without prior RT. No β-actin mRNA-specific PCR product was obtained. Fifty percent of each of the PCR products was analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining.

Production of *Ceacam9*$^{-/-}$/*Ceacam10*$^{-/-}$ mice by meiotic recombination. BALB/c mice carrying two knockout alleles each for both *Ceacam9* and *Ceacam10* were generated by breeding *Ceacam9*$^{-/-}$ females with a *Ceacam10*$^{+/-}$ male. Six males and 4 females containing one *Ceacam9* and one *Ceacam10* knockout allele each on different copies of chromosome 7 were mated with BALB/c mice. One female out of 111 successfully tested offspring contained both knockout alleles. This female was mated with BALB/c males. Double knockout mice were produced by mating heterozygous offspring. Homozygous *Ceacam9*$^{-/-}$/*Ceacam10*$^{-/-}$ matings resulted in viable offspring.

Statistical analyses. To compare the mean values of the litter sizes for the various strains, a two-tailed, unpaired *t* test was performed with the MS EXEL program. The significance threshold was taken to be a P value of <0.05.

RESULTS

Expression pattern of Ceacam10 mRNA during development. *Ceacam10* has been reported to be expressed in a number of tissues in adult mice, including colon, colonic tumors, small intestine, cecum, stomach, salivary glands, and bone marrow. In addition, *Ceacam10* transcripts could be detected in a timely restricted manner (8.5 to 12.5 dpc) in placentae during early pregnancy (25, 26). It is, however, unclear in which cell type *Ceacam10* is active and which role it plays during development. In order to identify the *Ceacam10* mRNA-producing cells, we performed in situ hybridization experiments with a *Ceacam10*-specific probe on cryosectioned 6.5 to 16.5 dpc placentae and embryos. In comparison, the expression patterns of the *Cea* gene family members *Ceacam9* and *Psg18* as well as that of placental marker genes, the transcripts of which characterize trophoblast giant cells (placental lactogen-1[Pl-1]), the spongiotrophoblast (4311), and the labyrinth zone of the fetal placenta (Mash-2) (6, 14, 32), were determined. In contrast to the expression of *Ceacam9* and *Psg18*, which are exclusively expressed in trophoblast cells, a placental compartment which is of embryonic origin, *Ceacam10* transcripts are only found in maternally derived decidual cells characterized by large nuclei (Fig. 1a to c). *Ceacam10* is expressed already at 6.5 dpc in the immediate vicinity of the implantation site in the decidua capsularis and intermediate vascular region but not in the decidua basalis, a region next to the invasively growing ectoplacental cone of the embryo. At 8.5 dpc, part of the decidua basalis also expresses *Ceacam10* (Fig. 1a and b). The *Ceacam10* mRNA-positive cells represent a subpopulation of the whole decidual compartment at 6.5 dpc which can be visualized by the Mash-2 probe (Fig. 1p). *Ceacam10 e*xpression fades in decidual tissue from 10.5 dpc onward; weak signals can still be found at 12.5 dpc, and no signals can be detected at 14.5 and 16.5 dpc (Fig. 1c and data not shown). In comparison, *Ceacam9* expression starts between 6.5 and 8.5 dpc and its transcripts are found first in primary and secondary trophoblast giant cell subpopulations (Fig. 1d and e) from 10.5 dpc onward and also in spongiotrophoblast cells, diminish around 12.5 dpc and are barely detectable at 16.5 dpc (Fig. 1f and data not shown). The *Psg18* probe, which probably hybridizes with most if not all of the coordinately expressed *Psg* transcripts (reference 28 and W. Zimmermann and B. Fischer, unpublished data), detected *Psg*-related transcripts first at 6.5 dpc (not yet at 5.5 dpc [data not shown]) mostly in primary giant trophoblast cells from 10.5 dpc onward also in spongiotrophoblast cells (Fig. 1g to i). Strong expression was maintained throughout the observation interval in the spongiotrophoblast and to a lesser degree in a cell population in the decidua basalis at 14.5 dpc. The expression pattern of *Psg18* from 12.5 dpc onwards was undistinguishable from that of *4311* (data not shown).

Generation of *Ceacam10*-null mice. The *Ceacam10* gene was inactivated in the BALB/c ES cell line BALB/c-I (42). The targeting vector was constructed from isogenic genomic DNA by using the *Ceacam10* cosmid c16.2a (26). In the resulting vector, the 3′-third of exon 2, intron 1, and two thirds of exon 2 were replaced by a neomycin gene expression cassette (Fig. 2a). This construct is expected to yield a null allele after homologous recombination. An HSV-tk expression unit was added to the 5′ end of the targeting construct to allow selection against random integration events. After transfer of the linearized targeting vector by electroporation, homologous recombination was observed at a frequency of 3.4% (8 of 235) in G418-FIAU double-resistant ES cell colonies. This was inferred from hybridization of a 2.7-kb genomic *Hin*dIII DNA fragment in addition to the 6.1-kb wild-type fragment with a probe from the 5′ region of the *Ceacam10* gene (Fig. 2a and b). This confirmed that correct homologous recombination occurred at the 5′ end of the *Ceacam10* locus. Two ES cell clones (4A4 and 6B2) were used for microinjection into C57BL/6 × DBA blastocysts. Only clone 6B2 exhibited germ line transmission. Mice heterozygous for the knockout allele were identified by Southern blot analysis and were interbred. To examine the effect of the replacement mutation on *Ceacam10* expression, total RNA from placentae and embryos (8.5 dpc) from Ceacam10$^{-/-}$ mice and, as a control, from *Ceacam9*$^{-/-}$ homozygous matings was analyzed by RT-PCR using gene-specific oligonucleotides. No *Ceacam10* transcripts could be detected in colon and placenta from Ceacam10$^{-/-}$ mice. As expected, *Ceacam9* and *Ceacam10* transcripts could be identified in placentae from *Ceacam10*$^{-/-}$ and *Ceacam9*$^{-/-}$ mice, respectively (Fig. 2c). These results indicate successful disruption of the *Ceacam10* gene.

Phenotypic analyses of *Ceacam10*$^{-/-}$ mice. Genotype analyses of 184 offspring resulting from mating of *Ceacam10*$^{+/-}$ mice revealed a nearly Mendelian distribution of the various genotypes (*Ceacam10*$^{+/+}$, 22.3%; *Ceacam10*$^{-/-}$, 23.4%). A similar sex ratio (females/males) was observed for knockout (17 of 22) and wild-type (19 of 20) littermates. No obvious morphological or behavioral abnormalities could be detected in *Ceacam10*$^{-/-}$ mice. *Ceacam10* male and female knockout mice were fertile and exhibited the same weight gain as their heterozygous or wild-type littermates. We found a relatively small but statistically significant difference of 23% ($P < 0.05$) in litter size between BALB/c (mean ± standard error of the mean, 6.0 ± 0.3 pups/litter; $n = 65$) and BALB/c *Ceacam10*$^{-/-}$ mice (4.6 ± 0.6 pups/litter; $n = 22$; $P = 0.023$) which was not observed for BALB/c *Ceacam9*$^{-/-}$ mice (5.0 ± 0.4 pups/litter; $n = 33$; $P = 0.063$).

Phenotypic analyses of *Ceacam10*$^{-/-}$/*Ceacam9*$^{-/-}$ mice. In order to investigate whether the various members of the *Cea* gene family expressed during development contribute incre-

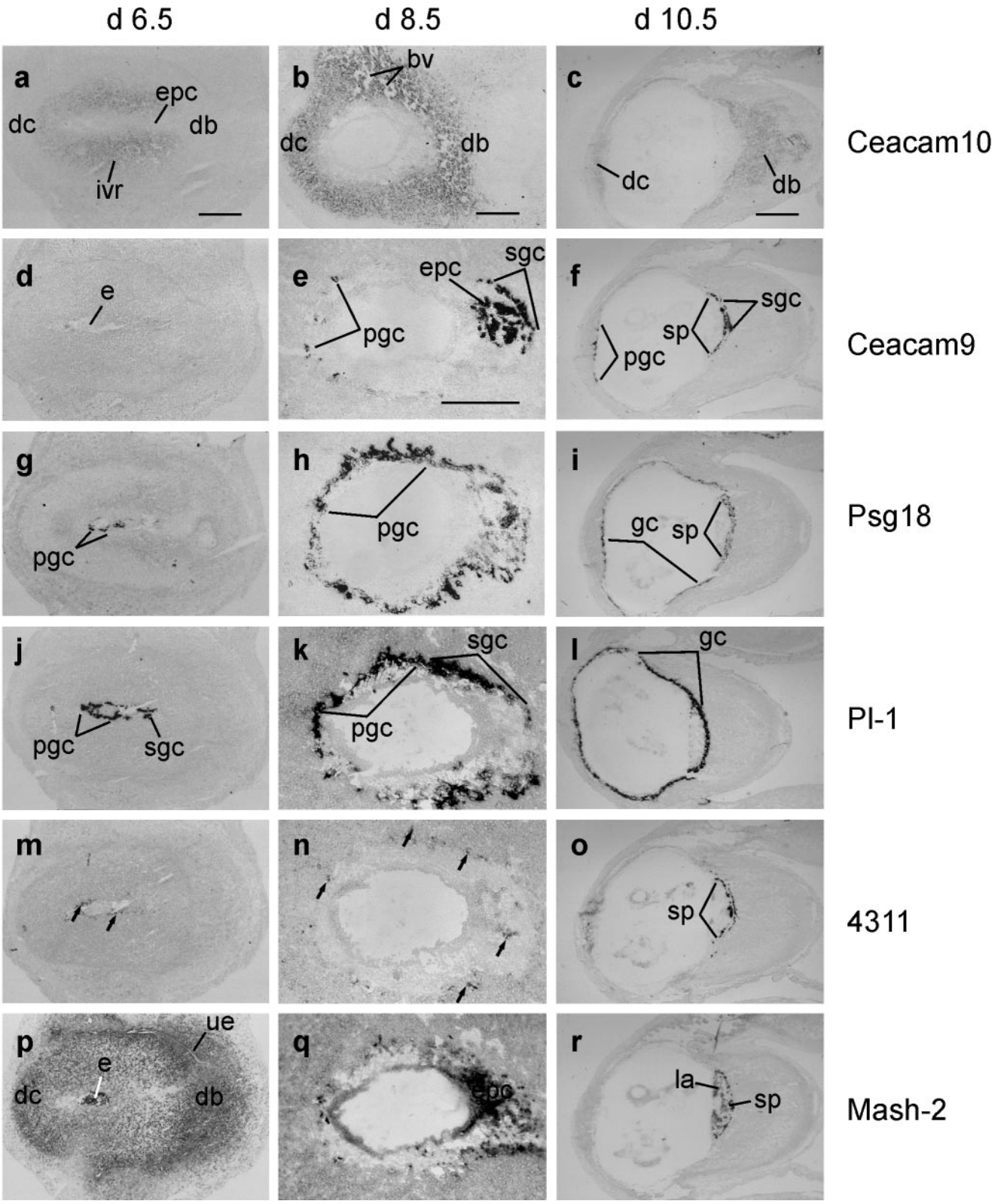

FIG. 1. Analysis of *Ceacam10* expression by in situ hybridization during placental development. Unfixed uteri from pregnant BALB/c mice at 6.5 dpc (a, d, g, j, m, and p), 8.5 dpc (b, e, h, k, n, and q) or 10.5 dpc (c, f, i, l, o, and r) were cryosectioned. In situ hybridization was performed with digoxigenin-labeled antisense Ceacam10 (a to c), Ceacam9 (d to f), Psg18 (g to i), Pl-1 (j to l), 4311 (m to o), and Mash-2 (p to r) RNA probes. The hybridized RNA was visualized by reaction with an antidigoxigenin alkaline phosphatase-conjugated antibody and subsequent incubation with the substrate nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate. The mesometrial side of the conceptus is to the right. Arrows point to individually labeled cells or cell cluster. bv, blood vessel; e, embryo; epc, ectoplacental cone; db, decidua basalis; dc, decidua capsularis; gc, giant (trophoblast) cells; ivr, intermediate vascular region; la, labyrinth zone; pgc, primary giant (trophoblast) cells; sgc, secondary giant (trophoblast) cells; sp, spongiotrophoblast; ue, uterine epithelium. Arrows in panels m and n indicate individual cells stained by the *4311* probe. Scale bars (identical within each column, except for panel b), 0.5 mm for 6.5 and 8.5 dpc placentae (1 mm in panel b); 1 mm for 10.5 dpc placentae.

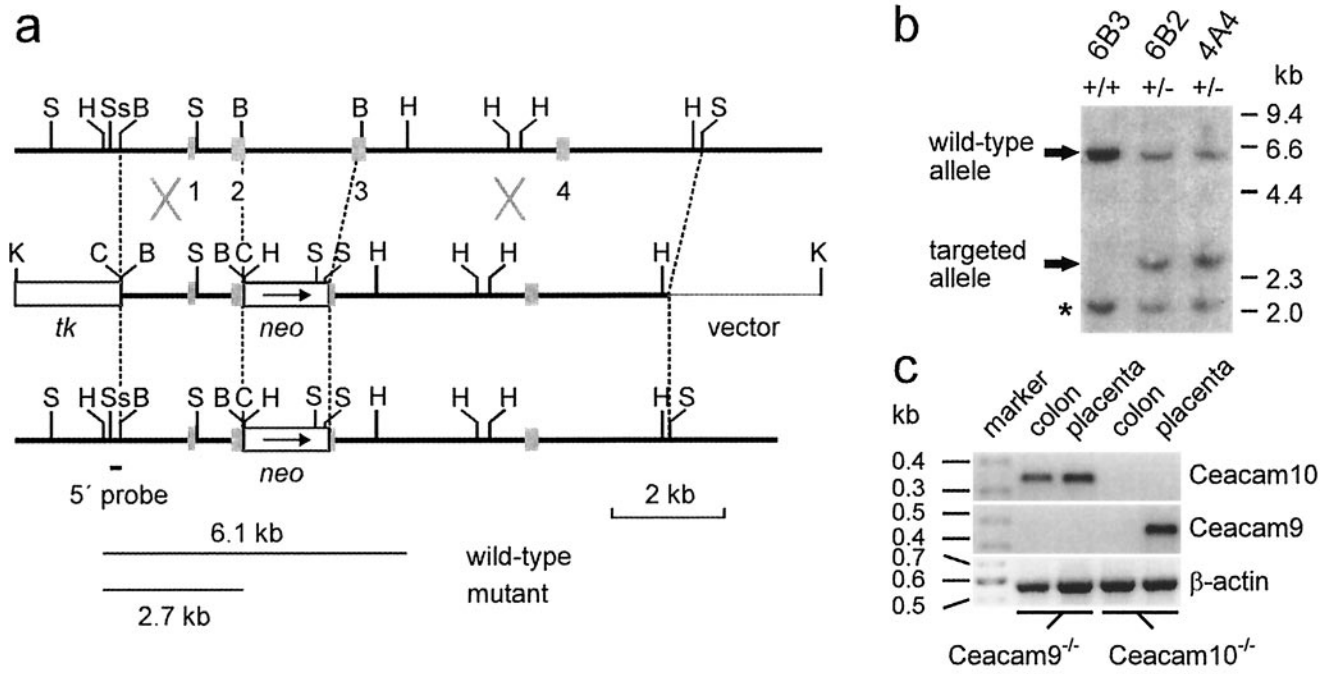

FIG. 2. Targeted disruption of the murine *Ceacam10* gene. (a) Structure of the wild-type allele (upper line), targeting construct (middle), and recombinant locus (bottom line). Gray boxes represent the four exons of *Ceacam10*. The *neo* and *tk* expression cassettes used for the selection of homologous recombinants are shown as open boxes. Arrows indicate the transcriptional direction of *neo*. The vector sequence within the targeting plasmid is shown as a thin line. The expected sizes of the DNA fragments obtained after digestion with *Hin*dIII are 6.1 kb for the wild-type allele and 2.7 kb for the correctly targeted allele, using an *Ssp*I/*Bam*HI DNA fragment located directly 5′ of the targeting construct as a probe. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sst*I; Ss, *Ssp*I. (b) Southern blot analyses of DNA isolated from G418-FIAU-resistant ES cell clones. DNA was digested with *Hin*dIII and hybridized with the ca. 250-bp *Ssp*I/*Bam*HI ^{32}P-labeled genomic 5′ probe. The sizes of marker DNA fragments are indicated in the right margin. The asterisk indicates cross-hybridizing DNA fragments. (c) RT-PCR analysis of *Ceacam9* and *Ceacam10* mRNA in total RNA isolated from 8.5-dpc placentae and embryos of *Ceacam9*$^{-/-}$ and *Ceacam10*$^{-/-}$ homozygous matings using gene-specific primers. As a control, β-actin-specific primers were used. The mobility and sizes of marker DNA fragments are shown in the left margin.

mentally to a successful pregnancy, we established *Ceacam10*$^{-/-}$/*Ceacam9*$^{-/-}$ double knockout mice. Since both genes are located on chromosome 7 about 5 centimorgans (cM) apart (50), offspring had to be analyzed for meiotic recombination events between the two knockout alleles. To this end, six male and four female BALB/c mice containing one *Ceacam9* and one *Ceacam10* knockout allele on different copies of chromosome 7 were mated with wild-type BALB/c mice. One female out of 111 successfully tested offspring contained both knockout alleles. The frequency of recombination is lower than expected from the genetic distance reported for these genes (approximately six recombination events for 111 offspring should have been observed for a genetic distance of 5 cM), which could be due to the statistical uncertainty or closer linkage of the two genes (~1 cM). The female carrying the linked knockout alleles was mated to BALB/c males. Double knockout mice were produced by mating of heterozygous offspring. The various genotypes were obtained at a Mendelian frequency (5 *Ceacam9*$^{+/+}$/*Ceacam10*$^{+/+}$, 11 *Ceacam9*$^{+/-}$/*Ceacam10*$^{+/-}$; 6 *Ceacam9*$^{-/-}$/*Ceacam10*$^{-/-}$). Homozygous *Ceacam9*$^{-/-}$/*Ceacam10*$^{-/-}$ matings resulted in viable offspring.

DISCUSSION

The studies presented here clearly demonstrate that the two secreted members of the murine CEA family, CEACAM10 and CEACAM9, which are coordinately expressed during early development in maternal and fetal placental tissues, respectively, are not essential for either a successful pregnancy or for adult life. This has been concluded from the observation that mice carrying two null alleles for *Ceacam10* and *Ceacam9*, either singly or in combination, develop normally and exhibit no obvious phenotype (this paper and reference 10).

The expression patterns of *Ceacam9*, *Ceacam10*, and *Psg18* in placental tissues during mouse development can clearly be distinguished (Fig. 1a to i and data not shown). Interestingly, *Ceacam10* and *Ceacam9* show a very similar temporal expression pattern. Their transcripts, however, are found in different compartments of the developing placenta, i.e., in the maternal decidua surrounding the implantation site and in invasively growing trophoblast populations in the ectoplacental cone of the embryo, respectively (Fig. 1a, b, and e). On the other hand, *Psg18* expression is first noticed 6.5 dpc in trophoblast giant cells surrounding the whole embryo and accumulates during pregnancy in the spongiotrophoblast (Fig. 1g to i and data not shown), a compartment of the fetal placenta with the largest proportion of maternal cells (mostly blood leukocytes). A similar temporal and possibly spatial expression can be expected for the other members of the closely related group of *Psg* genes based on mRNA quantitation of *Psg* genes 17 to 29 (reference 28 and W. Zimmermann and B. Fischer, unpublished data).

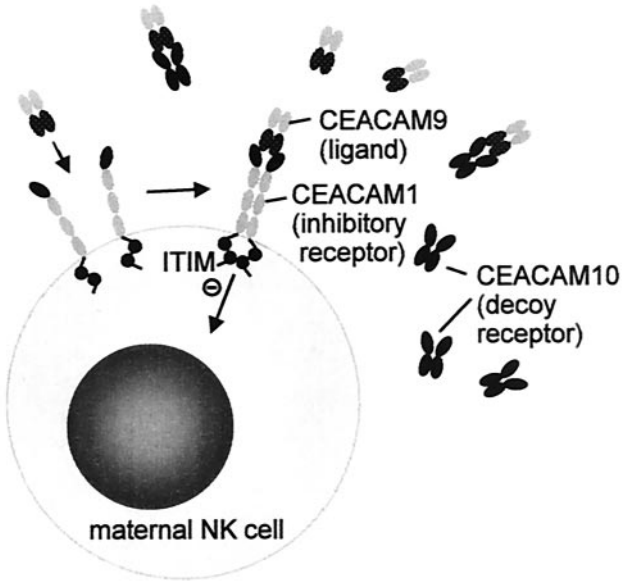

FIG. 3. Hypothetical model of immune regulatory functions of CEACAM9 and CEACAM10 during early murine pregnancy. Based on the spatiotemporal expression pattern of CEACAM1, CEACAM9, and CEACAM10 and the homo- and heterophilic properties of CEA family members, the following hypothetical functional scenario can be sketched: a dimeric, embryo-derived CEACAM9 binds to CEACAM1 on decidual NK cells of the mother. This cross-links CEACAM1, which transmits a negative signal through the immunoreceptor tyrosine-based inhibition motifs (ITIM; black dots) in its cytoplasmic tail. This reaction is counterbalanced by maternally produced CEA CAM10 dimers which function as soluble decoy receptors, thus regulating the invasively growing fetal trophoblast cells.

These findings suggest that the encoded CEACAM and PSG proteins have different functions or have similar functions during different developmental stages.

What are the functions of CEA family members during embryogenesis? It becomes more and more clear that CEACAM1 and PSG play a role in innate and adaptive immunity (22, 23, 36, 39), and they are supposed to take part in the control of the maternal immune system to avoid rejection of the semiallotypic embryo (58). It has been shown recently that engagement of CEACAM1 with CEACAM1-specific antibodies or by homotypic interaction with CEACAM1 on major histocompatibility complex class I-negative melanoma cells inhibits T-cell sensitization in a delayed type hypersensitivity mouse model (39) or cell killing by CEACAM1-positive NK cells, respectively (36).

Multiple mechanisms seem to operate in mammals to enable the maternal immune system to selectively tolerate the semiallogeneic fetus without putting the mother's alertness against pathogens at risk (52). In addition, invasion of fetal trophoblast cells into the decidua has to be correctly balanced. Human decidual $CD16^-$ $CD56^+$ NK cells, which have been shown to express CEACAM1 (37), are thought to play a role in this process (27). Assuming that CEACAM1 is also expressed on murine decidual NK cells, negative signaling through CEACAM1 could be induced by soluble members of the CEA family, most of which are known to be able to interact homo- and heterotypically with each other (30, 33). CEACAM9, which is expressed most strongly in the invasively growing ectoplacental cone at day 8.5 of pregnancy (Fig. 1e), could support invasion by holding decidual NK cells at bay. Indeed, a putative CEACAM9 receptor was identified in decidua by using a CEACAM9/human Fc-IgG1 fusion protein for immunohistological staining (D. Finkenzeller and W. Zimmermann, unpublished data). On the other hand CEACAM10, which is most closely related to CEACAM1, could counterbalance CEACAM9's action by serving as a soluble decoy which prevents CEACAM9 from binding to CEACAM1 (Fig. 3). The expression pattern of *Ceacam10* mRNA by maternal cells surrounding the implantation site reported here (Fig. 1a to c) would fit such a function.

There seems to be a driving force for the expansion of the CEA families during evolution, because it has occurred independently in primates and rodents. However, since CEACAM9 and CEACAM10, as well as double knockout mice, lack an obvious phenotype, the evolutionarily young family of *Ceacam* genes possibly arose in order to achieve optimization and fine tuning of fetal-maternal interactions.

ACKNOWLEDGMENTS

We thank Robert Kammerer for critical reading of the manuscript. The gifts of the embryonic stem cell line BALB/c-I by Birgit Ledermann and of placental marker plasmids obtained from Janet Rossant and Daniel Linzer through Reinald Fundele are gratefully acknowledged.

This work was supported by the Dr. Mildred-Scheel-Stiftung für Krebsforschung.

REFERENCES

1. **Beauchemin, N., P. Draber, G. Dveksler, P. Gold, S. Gray-Owen, F. Grunert, S. Hammarström, K. V. Holmes, A. Karlsson, M. Kuroki, S. H. Lin, L. Lucka, S. M. Najjar, M. Neumaier, B. (tm)brink, J. E. Shively, K. M. Skubitz, C. P. Stanners, P. Thomas, J. A. Thompson, M. Virji, S. von Kleist, C. Wagener, S. Watt, and W. Zimmermann.** 1999. Redefined nomenclature for members of the carcinoembryonic antigen family. Exp. Cell Res. **252:** 243–249.
2. **Bohn, H., and E. Weinmann.** 1974. Immunological disruption of implantation in monkeys with antibodies to human pregnancy specific beta 1-glycoprotein (SP1). Arch. Gynäkol. **217:**209–218. (Author's translation.)
3. **Boulton, I. C., and S. D. Gray-Owen.** 2002. Neisserial binding to CEACAM1 arrests the activation and proliferation of $CD4^+$ T lymphocytes. Nat. Immunol. **3:**229–236.
4. **Chen, T., F. Grunert, A. Medina-Marino, and E. C. Gotschlich.** 1997. Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. J. Exp. Med. **185:**1557–1564.
5. **Chen, T., W. Zimmermann, J. Parker, I. Chen, A. Maeda, and S. Bolland.** 2001. Biliary glycoprotein (BGPa, CD66a, CEACAM1) mediates inhibitory signals. J. Leukoc. Biol. **70:**335–340.
6. **Colosi, P., F. Talamantes, and D. I. Linzer.** 1987. Molecular cloning and expression of mouse placental lactogen I complementary deoxyribonucleic acid. Mol. Endocrinol. **1:**767–776.
7. **Dveksler, G. S., M. N. Pensiero, C. B. Cardellichio, R. K. Williams, G. S. Jiang, K. V. Holmes, and C. W. Dieffenbach.** 1991. Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. J. Virol. **65:**6881–6891.
8. **Earley, K., W. Luo, Y. Qiu, N. L. Thompson, J. Chou, D. C. Hixson, and S. H. Lin.** 1996. Identification of a new isoform of cell-cell adhesion molecule 105 (C-CAM), C-CAM4: a secretory protein with only one Ig domain. Biochem. J. **315:**799–806.
9. **Ergün, S., N. Kilic, G. Ziegeler, A. Hansen, P. Nollau, J. Götze, J.-H. Wurmbach, A. Horst, J. Weil, M. Fernando, and C. Wagener.** 2000. CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. Mol. Cell **5:**311–320.
10. **Finkenzeller, D., B. Fischer, J. McLaughlin, H. Schrewe, B. Ledermann, and W. Zimmermann.** 2000. Trophoblast cell-specific carcinoembryonic antigen cell adhesion molecule 9 is not required for placental development or a positive outcome of allotypic pregnancies. Mol. Cell. Biol. **20:**7140–7145.
11. **Finkenzeller, D., B. Kromer, J. Thompson, and W. Zimmermann.** 1997. *cea5*, a structurally divergent member of the murine carcinoembryonic antigen gene family, is exclusively expressed during early placental development in trophoblast giant cells. J. Biol. Chem. **272:**31369–31376.

12. **Fournes, B., S. Sadekova, C. Turbide, S. Letourneau, and N. Beauchemin.** 2001. The CEACAM1-L Ser503 residue is crucial for inhibition of colon cancer cell tumorigenicity. Oncogene **20:**219–230.
13. **Gray-Owen, S. D., C. Dehio, A. Haude, F. Grunert, and T. F. Meyer.** 1997. CD66 carcinoembryonic antigens mediate interactions between Opa-expressing Neisseria gonorrhoeae and human polymorphonuclear phagocytes. EMBO J. **16:**3435–3445.
14. **Guillemot, F., A. Nagy, A. Auerbach, J. Rossant, and A. L. Joyner.** 1994. Essential role of Mash-2 in extraembryonic development. Nature **371:**333–336.
15. **Hammarström, S.** 1999. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Semin. Cancer Biol. **9:**67–81.
16. **Hammarström, S., A. Olsen, S. Teglund, and V. Baranov.** 1998. The nature and expression of the human CEA family, p. 1–35. *In* C. P. Stanners (ed.), Cell adhesion and communication mediated by the CEA family. Harwood Academic Publishers, Amsterdam, The Netherlands.
17. **Hashino, J., Y. Fukuda, S. Oikawa, H. Nakazato, and T. Nakanishi.** 1994. Metastatic potential of human colorectal carcinoma SW1222 cells transfected with cDNA encoding carcinoembryonic antigen. Clin. Exp. Metastasis **12:**324–328.
18. **Hau, J., A. A. Gidley-Baird, J. G. Westergaard, and B. Teisner.** 1985. The effect on pregnancy of intrauterine administration of antibodies against two pregnancy-associated murine proteins: murine pregnancy-specific beta 1-glycoprotein and murine pregnancy-associated alpha 2-glycoprotein. Biomed. Biochim. Acta **44:**1255–1259.
19. **Hsieh, J. T., W. Luo, W. Song, Y. Wang, D. I. Kleinerman, N. T. Van, and S. H. Lin.** 1995. Tumor suppressive role of an androgen-regulated epithelial cell adhesion molecule (C-CAM) in prostate carcinoma cell revealed by sense and antisense approaches. Cancer Res. **55:**190–197.
20. **Huang, J., J. D. Hardy, Y. Sun, and J. E. Shively.** 1999. Essential role of biliary glycoprotein (CD66a) in morphogenesis of the human mammary epithelial cell line MCF10F. J. Cell Sci. **112:**4193–4205.
21. **Ilantzis, C., S. Jothy, L. C. Alpert, P. Draber, and C. P. Stanners.** 1997. Cell-surface levels of human carcinoembryonic antigen are inversely correlated with colonocyte differentiation in colon carcinogenesis. Lab. Investig. **76:**703–716.
22. **Kammerer, R., S. Hahn, B. B. Singer, J. S. Luo, and S. von Kleist.** 1998. Biliary glycoprotein (CD66a), a cell adhesion molecule of the immunoglobulin superfamily, on human lymphocytes: structure, expression and involvement in T cell activation. Eur. J. Immunol. **28:**3664–3674.
23. **Kammerer, R., D. Stober, B. B. Singer, B. Öbrink, and J. Reimann.** 2001. Carcinoembryonic antigen-related cell adhesion molecule 1 on murine dendritic cells is a potent regulator of T cell stimulation. J. Immunol. **166:**6537–6544.
24. **Kammerer, R., and S. von Kleist.** 1994. CEA expression of colorectal adenocarcinomas is correlated with their resistance against LAK-cell lysis. Int. J. Cancer **57:**341–347.
25. **Kataoka, K., Y. Takata, A. Nakajima, S. Saito, and N. Huh.** 2000. A carcinoembryonic antigen family cDNA from mouse placenta encoding a protein with a rare domain composition. Placenta **21:**610–614.
26. **Keck, U., P. Nédellec, N. Beauchemin, J. Thompson, and W. Zimmermann.** 1995. The cea10 gene encodes a secreted member of the murine carcinoembryonic antigen family and is expressed in the placenta, gastrointestinal tract and bone marrow. Eur. J. Biochem. **229:**455–464.
27. **King, A., S. E. Hiby, L. Gardner, S. Joseph, J. M. Bowen, S. Verma, T. D. Burrows, and Y. W. Loke.** 2000. Recognition of trophoblast HLA class I molecules by decidual NK cell receptors. A review. Placenta **21**(Suppl. A)**:** S81–S85.
28. **Kromer, B., D. Finkenzeller, J. Wessels, G. Dveksler, J. Thompson, and W. Zimmermann.** 1996. Coordinate expression of splice variants of the murine pregnancy-specific glycoprotein (PSG) gene family during placental development. Eur. J. Biochem. **242:**280–287.
29. **Kuijpers, T. W., M. Hoogerwerf, L. J. van der Laan, G. Nagel, C. E. van der Schoot, F. Grunert, and D. Roos.** 1992. CD66 nonspecific cross-reacting antigens are involved in neutrophil adherence to cytokine-activated endothelial cells. J. Cell Biol. **118:**457–466.
30. **Kuroki, M., H. Abe, T. Imakiirei, S. Liao, H. Uchida, Y. Yamauchi, S. Oikawa, and M. Kuroki.** 2001. Identification and comparison of residues critical for cell-adhesion activities of two neutrophil CD66 antigens, CEACAM6 and CEACAM8. J. Leukoc. Biol. **70:**543–550.
31. **Laird, P. W., A. Zijderveld, K. Linders, M. A. Rudnicki, R. Jaenisch, and A. Berns.** 1991. Simplified mammalian DNA isolation procedure. Nucleic Acids Res. **19:**4293.
32. **Lescisin, K. R., S. Varmuza, and J. Rossant.** 1988. Isolation and characterization of a novel trophoblast-specific cDNA in the mouse. Genes Dev. **2:**1639–1646.
33. **Lin, S. H., H. Cheng, K. Earley, W. Luo, and J. Chou.** 1998. Demonstration of adhesion activity of the soluble Ig-domain protein C-CAM4 by attachment to the plasma membrane. Biochem. Biophys. Res. Commun. **245:**472–477.
34. **Lin, T. M., S. P. Halbert, and W. N. Spellacy.** 1974. Measurement of pregnancy-associated plasma proteins during human gestation. J. Clin. Investig. **54:**576–582.
35. **Mansour, S. L., K. R. Thomas, and M. R. Capecchi.** 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature **336:**348–352.
36. **Markel, G., N. Lieberman, G. Katz, T. I. Arnon, M. Lotem, O. Drize, R. S. Blumberg, E. Bar-Haim, R. Mader, L. Eisenbach, and O. Mandelboim.** 2002. CD66a interactions between human melanoma and NK cells: a novel class I MHC-independent inhibitory mechanism of cytotoxicity. J. Immunol. **168:** 2803–2810.
37. **Möller, M. J., R. Kammerer, F. Grunert, and S. von Kleist.** 1996. Biliary glycoprotein (BGP) expression on T cells and on a natural-killer-cell subpopulation. Int. J. Cancer **65:**740–745.
38. **Morales, V. M., A. Christ, S. M. Watt, H. S. Kim, K. W. Johnson, N. Utku, A. M. Texieira, A. Mizoguchi, E. Mizoguchi, G. J. Russell, S. E. Russell, A. K. Bhan, G. J. Freeman, and R. S. Blumberg.** 1999. Regulation of human intestinal intraepithelial lymphocyte cytolytic function by biliary glycoprotein (CD66a). J. Immunol. **163:**1363–1370.
39. **Nakajima, A., H. Iijima, M. F. Neurath, T. Nagaishi, E. E. Nieuwenhuis, R. Raychowdhury, J. Glickman, D. M. Blau, S. Russell, K. V. Holmes, and R. S. Blumberg.** 2002. Activation-induced expression of carcinoembryonic antigen-cell adhesion molecule 1 regulates mouse T lymphocyte function. J. Immunol. **168:**1028–1035.
40. **Nédellec, P., G. S. Dveksler, E. Daniels, C. Turbide, B. Chow, A. A. Basile, K. V. Holmes, and N. Beauchemin.** 1994. Bgp2, a new member of the carcinoembryonic antigen-related gene family, encodes an alternative receptor for mouse hepatitis viruses. J. Virol. **68:**4525–4537.
41. **Neumaier, M., S. Paululat, A. Chan, P. Matthaes, and C. Wagener.** 1993. Biliary glycoprotein, a potential human cell adhesion molecule, is down-regulated in colorectal carcinomas. Proc. Natl. Acad. Sci. USA **90:**10744–10748.
42. **Noben-Trauth, N., G. Kohler, K. Burki, and B. Ledermann.** 1996. Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line. Transgenic Res. **5:**487–491.
43. **Ordonez, C., R. A. Screaton, C. Ilantzis, and C. P. Stanners.** 2000. Human carcinoembryonic antigen functions as a general inhibitor of anoikis. Cancer Res. **60:**3419–3424.
44. **Rosenberg, M., P. Nedellec, S. Jothy, D. Fleiszer, C. Turbide, and N. Beauchemin.** 1993. The expression of mouse biliary glycoprotein, a carcinoembryonic antigen-related gene, is down-regulated in malignant mouse tissues. Cancer Res. **53:**4938–4945.
45. **Rutherfurd, K. J., J. Y. Chou, and B. C. Mansfield.** 1995. A motif in PSG11s mediates binding to a receptor on the surface of the promonocyte cell line THP-1. Mol. Endocrinol. **9:**1297–1305.
46. **Schaeren-Wiemers, N., and A. Gerfin-Moser.** 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry **100:**431–440.
47. **Schölzel, S., W. Zimmermann, G. Schwarzkopf, F. Grunert, B. Rogaczewski, and J. Thompson.** 2000. Carcinoembryonic antigen family members CEACAM6 and CEACAM7 are differentially expressed in normal tissues and oppositely deregulated in hyperplastic colorectal polyps and early adenomas. Am. J. Pathol. **156:**595–605.
48. **Screaton, R. A., L. DeMarte, P. Draber, and C. P. Stanners.** 2000. The specificity for the differentiation blocking activity of carcinoembryonic antigen resides in its glycophosphatidyl-inositol anchor. J. Cell Biol. **150:**613–626.
49. **Snyder, S. K., D. H. Wessner, J. L. Wessells, R. M. Waterhouse, L. M. Wahl, W. Zimmermann, and G. S. Dveksler.** 2001. Pregnancy-specific glycoproteins function as immunomodulators by inducing secretion of IL-10, IL-6 and TGF-β1 by human monocytes. Am. J. Reprod. Immunol. **45:**205–216.
50. **Stubbs, L., E. A. Carver, M. E. Shannon, J. Kim, J. Geisler, E. E. Generoso, B. G. Stanford, W. C. Dunn, H. Mohrenweiser, W. Zimmermann, S. M. Watt, and L. K. Ashworth.** 1996. Detailed comparative map of human chromosome 19q and related regions of the mouse genome. Genomics **35:**499–508.
51. **Swiatek, P. J., and T. Gridley.** 1993. Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. Genes Dev. **7:**2071–2084.
52. **Thellin, O., B. Coumans, W. Zorzi, A. Igout, and E. Heinen.** 2000. Tolerance to the foeto-placental "graft": ten ways to support a child for nine months. Curr. Opin. Immunol. **12:**731–737.
53. **Thomas, P., A. Gangopadhyay, G. Steele, Jr., C. Andrews, H. Nakazato, S. Oikawa, and J. M. Jessup.** 1995. The effect of transfection of the CEA gene on the metastatic behavior of the human colorectal cancer cell line MIP-101. Cancer Lett. **92:**59–66.
54. **Thompson, J. A., F. Grunert, and W. Zimmermann.** 1991. Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. J. Clin. Lab. Anal. **5:**344–366.
55. **Virji, M., S. M. Watt, S. Barker, K. Makepeace, and R. Doyonnas.** 1996. The N-domain of the human CD66a adhesion molecule is a target for Opa

proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Mol. Microbiol. **22:**929–939.
56. **Waterhouse, R., C. Ha, and G. S. Dveksler.** 2002. Murine CD9 is the receptor for pregnancy-specific glycoprotein 17. J. Exp. Med. **195:**277–282.
57. **Wegmann, T. G., H. Lin, L. Guilbert, and T. R. Mosmann.** 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol. Today **14:**353–356.
58. **Wessells, J., D. Wessner, R. Parsells, K. White, D. Finkenzeller, W. Zimmermann, and G. Dveksler.** 2000. Pregnancy specific glycoprotein 18 induces IL-10 expression in murine macrophages. Eur. J. Immunol. **30:**1830–1840.
59. **Zimmermann, W.** 1998. The nature and expression of the rodent CEA families: evolutionary considerations, p. 31–55. *In* C. P. Stanners (ed.), Cell adhesion and communication mediated by the CEA family. Harwood Academic Publishers, Amsterdam, The Netherlands.