

# Trophoblast Cell-Specific Carcinoembryonic Antigen Cell Adhesion Molecule 9 Is Not Required for Placental Development or a Positive Outcome of Allotypic Pregnancies

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**The carcinoembryonic antigen (CEA) family consists of a large group of evolutionarily divergent glycoproteins. The secreted pregnancy-specific glycoproteins constitute a subgroup within the CEA family. They are predominantly expressed in trophoblast cells throughout placental development and are essential for a positive outcome of pregnancy, possibly by protecting the semiallotypic fetus from the maternal immune system. The murine CEA gene family member *CEA cell adhesion molecule 9* (*Ceacam9*) also exhibits a trophoblast-specific expression pattern. However, its mRNA is found only in certain populations of trophoblast giant cells during early stages of placental development. It is exceptionally well conserved in the rat (over 90% identity on the amino acid level) but is absent from humans. To determine its role during murine development, *Ceacam9* was inactivated by homologous recombination. *Ceacam9*<sup>-/-</sup> mice on both BALB/c and 129/Sv backgrounds developed indistinguishably from heterozygous or wild-type littermates with respect to sex ratio, weight gain, and fertility. Furthermore, the placental morphology and the expression pattern of trophoblast marker genes in the placentae of *Ceacam9*<sup>-/-</sup> females exhibited no differences. Both backcross analyses and transfer of BALB/c *Ceacam9*<sup>-/-</sup> blastocysts into pseudopregnant C57BL/6 foster mothers indicated that *Ceacam9* is not needed for the protection of the embryo in a semiallogeneic or allogeneic situation. Taken together, *Ceacam9* is dispensable for murine placental and embryonic development despite being highly conserved within rodents.**

The carcinoembryonic antigen (CEA) family is a branch of the immunoglobulin superfamily and consists of a large group of evolutionarily highly divergent glycoproteins (11, 55, 60). Based on sequence similarities and expression pattern, they can be subdivided into two subgroups: the CEA and the pregnancy-specific glycoprotein (PSG) subgroups. The members of the CEA subgroup are mostly membrane anchored and are found in a wide variety of cell types, e.g., epithelial and endothelial cells, granulocytes, macrophages, B cells, and activated T cells, whereas the expression of the PSGs is restricted predominantly to trophoblast cell lineages (12).

CEA-related molecules have been implicated in a number of normal and pathological processes. CEA-related glycoproteins seem to play a role in the control of granulocyte and T-cell activation (20, 23, 49), as well as in the regulation of differentiation and tissue remodeling, such as the supposed involvement of CEA cell adhesion molecule 1 (CEACAM1) in breast duct formation and angiogenesis (6, 7, 16). The expression of a number of CEA family members is often deregulated in tumors (18, 34, 42, 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 (13, 36, 54). CEACAM1 (for the recently revised nomenclature of the CEA

family, see reference 1) can act as a tumor suppressor, and its common loss of expression in epithelial tumors probably causes deregulation of growth or differentiation (15, 24). Furthermore, most CEA subfamily glycoproteins serve as receptors for bacterial pathogens, such as *Neisseria gonorrhoeae* (3, 9, 56), whereas murine CEACAM1 and CEACAM2 are responsible for mouse hepatitis virus entry into cells (5, 33).

Although PSGs represent the most abundant fetal protein in the maternal circulation at term (27), little is known about their function. Low levels of PSG correlate with poor outcomes of pregnancies, and anti-PSG antibodies have been demonstrated to induce abortions in mice and monkeys (2, 14, 28, 30, 31, 53). Recent findings indicate that PSGs might act via receptors on monocytes by inducing cytokines favorable to T<sub>H</sub>2 immune responses which are biased in the maternal immune system during pregnancy (44, 58). The shift from a T<sub>H</sub>1 to a T<sub>H</sub>2-type immune status together with a preference of the innate over the adaptive immune system in the placenta is thought to be important for the acceptance of the histoincompatible fetal tissue during pregnancies without compromising strong immune reactions against pathogens (45, 57, 59). PSGs and other trophoblast members of the CEA family might thus be involved in protecting the semiallotypic fetus from the maternal immune system.

The murine *Ceacam9* gene encodes a secreted two-immunoglobulin (Ig)-domain glycoprotein which cannot be clearly assigned to either subgroup. Although it shares a higher degree of sequence similarity with CEA subgroup members, it is exclusively expressed in trophoblast cells reminiscent of PSGs. However, its spatio-temporal expression pattern differs from that of the PSGs. *Ceacam9* mRNA is found during early stages of placental development mainly in specific subpopulations of

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primary and secondary trophoblast giant cells, whereas PSG transcripts accumulate in the mature placenta, predominantly in the spongiotrophoblast (8, 22). Due to the rapid evolution of the CEA families, orthologous genes are difficult to assign even between closely related mammals, such as rat and mouse. CEACAM9 represents an exception in exhibiting a sequence identity of 91% (Finkenzeller and Zimmermann, unpublished results). In contrast, the only other identifiable homologous gene product, CEACAM1, shares 74% of its amino acid sequence between the rat and mouse. This suggests that CEACAM9 plays an important function during placental development in rodents.

In order to clarify the *in vivo* functions of *Ceacam9*, gene ablation experiments were performed. Here we report for the first time on the inactivation by homologous recombination of a member of the murine *Cea* gene family. *Ceacam9*<sup>-/-</sup> males and females are viable and fertile and exhibit no obvious phenotype. This indicates that even highly conserved members of the evolutionarily young *Cea* gene family serve subtle functions.

## MATERIALS AND METHODS

**Construction of the targeting vector.** The mouse genomic cosmid clone cosC3 from BALB/c liver DNA containing the *Ceacam9* gene was used to construct the targeting vector (8, 43). Digestion with *Bam*HI yielded a 7.5-kb fragment and a 3.5-kb fragment comprising the promoter region and part of exons 2 and 3, respectively. To generate the targeting vector, the 7.5-kb *Bam*HI DNA fragment was ligated with a *Bam*HI-*Hind*III adapter which destroyed the *Bam*HI sites and subcloned into the *Hind*III-digested pPGKneo vector (48) upstream of the neomycin expression cassette. After digestion of the 3.5-kb *Bam*HI DNA fragment with *Eco*RI, the 1.7-kb *Bam*HI-*Eco*RI fragment was cloned after ligation of an *Eco*RI-*Bam*HI adapter into the *Bam*HI-digested targeting vector downstream of the *neo* cassette. To allow negative selection against random integration, a pMCI-HSV-TK cassette (29) was introduced 3' of the 1.7-kb *Bam*HI-*Eco*RI *Ceacam9* fragment. The resulting targeting construct lacks, in comparison with the genomic sequences, a 1.5-kb *Bam*HI DNA fragment which comprises 340 bp of the 5'-flanking region, exon 1, intron 1, 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 embryonic stem cells and generation of mutant mice.** The BALB/c-derived embryonic stem (ES) cell line BALB/c-1 (35) was maintained on a layer of mitomycin C-treated G418-resistant embryonic mouse fibroblasts used as feeder cells in Dulbecco's modified Eagle medium (Life Technologies) with 15% heat-inactivated fetal calf serum (FCS) (Life Technologies) containing 4.5 mg of glucose/ml, 2 mM glutamine, 50 U of penicillin per ml, 50 µg of streptomycin per ml, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1,000 U of leukemia inhibitory factor/ml, and 0.8 mg of adenosine 0.73 mg of cytidine, 0.85 mg of guanosine, 0.24 mg of thymidine, and 0.73 mg of uridine per 100 ml (all supplements were from Life Technologies). A sample of 10<sup>7</sup> BALB/c-1 ES cells was electroporated with 25 µg of the linearized targeting vector DNA at 240 V and 500 µF in phosphate-buffered saline. The electroporated cells were placed under selection and replica plated as previously described (51).

*Ceacam9* mutant ES cell lines were injected into day 3.5 postcoitum (p.c.) (C57BL/6 × DBA/2)F<sub>1</sub> blastocysts. After 2 h in culture in M16 medium (Sigma), the embryos were surgically transferred into the uteri of pseudopregnant NMRI recipients at day 2.5 p.c. Male chimeras were mated with BALB/c females to produce mice heterozygous for the null mutation. F<sub>1</sub> intercrosses of heterozygous mice finally resulted in F<sub>2</sub> offspring which were wild type and heterozygous or homozygous for the targeted allele. Heterozygous mice were backcrossed to BALB/c and 129/Sv mice for four generations. Then, *Ceacam9*<sup>-/-</sup> strains were established by crossing heterozygous animals and maintained by breeding *Ceacam9*<sup>-/-</sup> males and females.

**Mouse genotyping.** Homologous recombination between the targeting vector and *Ceacam9* on mouse chromosome 7 was examined by PCR and Southern blotting using genomic DNA isolated from ES cells or tail biopsies after digestion in lysis buffer with 100 µg of proteinase K/ml (25). PCR was performed using three primers in two separate reaction tubes. One pair of primers included the mutant allele-specific bpA primer (5'-TGGGAAGACAATAGCAGGCATGC), which binds in the 3' region of the neomycin cassette, and a second pair of primers included the wild-type allele-specific wild-type oligonucleotide (5'-TCA GCACAGTGATAGGAAAACCG), which binds in the N-terminal domain exon. Both primers were combined with the common M8-ko oligonucleotide (5'-ATCCTGCTGACTGGAGTTTACC), which binds in the 3' untranslated region of *Ceacam9* downstream of the 1.7-kb *Bam*HI-*Eco*RI fragment also present in the targeting construct. The mutant allele gave rise to a 2,012-bp fragment, and the wild-type allele gave rise to a 2,089-bp fragment. Both PCRs

were performed in *Taq* polymerase buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) complemented with 2.5 mM MgCl<sub>2</sub>, 0.2 mM (each) deoxynucleoside triphosphates, 1 pmol of each primer/µl, and 1 U of *Taq* polymerase for 35 cycles (denaturation, 94°C, 15 s; annealing, 65°C, 30 s; elongation, 72°C, 3 min).

The established *Ceacam9* knockout strain was genotyped using two different pairs of oligonucleotides as PCR primers in one reaction mixture. The mutant allele-specific primers (5'-ATGCGGCGGCTGCATACGCTTGATCC and 5'-C GTCAAGAAGGCGGATAGAAGGCGATGC-3' [32]) bind in the neomycin gene and generate a 427-bp DNA fragment, while the wild-type allele-specific primers (5'-CTTAACCTGCTGGAATGCACCCGCCG and 5'-GCACTTCCA GATGCACATGTG-TTAATTCG), located in the N-terminal domain exon, gave rise to a 349-bp fragment. The PCR was performed as described above using modified cycle conditions (denaturation, 94°C, 45 s; annealing, 60°C, 45 s; elongation, 74°C, 1 min).

**Southern and Northern blot analyses.** Genomic DNA isolated from ES cells or tail tips was digested with *Hind*III, separated by electrophoresis through a 1% agarose gel overnight, and transferred to a positively charged nylon membrane. <sup>32</sup>P-labeled 0.5- and 1.8-kb *Eco*RI-*Bam*HI fragments were used as probes to confirm correct homologous recombination in the 5'- and 3'-flanking regions, respectively (Fig. 1A). Northern blot analysis of *Ceacam9* and β-actin transcripts was performed as described before (8).

**In situ hybridization.** Placentae and embryos collected on day 8.5 of development of *Ceacam9*<sup>-/-</sup> mice and wild-type controls were frozen in Tissue Freezing medium (Jung) diluted 1:1 with water. Seven-micrometer cryosections were placed on SuperFrost/Plus microscope slides (Roth). In situ hybridization with digoxigenin-labeled sense and antisense RNA probes was performed as described before (46, 58). The RNA probes for the detection of transcripts of the marker genes *4311* (750 bp) and *P<sub>L</sub>-I* (800 bp) are described elsewhere (4, 26).

**Blastocyst transfer and genetic backcross to C57BL/6 mice.** BALB/c *Ceacam9*<sup>-/-</sup> mice and, as a control, *Ceacam9*<sup>+/+</sup> mice were mated overnight, and the day of the vaginal plug was designated day 0.5 of gestation. Mice were anesthetized by inhalation of isoflurane (Forene; Abbott) and sacrificed by cervical dislocation on day 3.5 p.c. Blastocysts were flushed out from the uterus by using M2 medium (Sigma) with 10% FCS. Compacted morulae were collected, washed in M2-10% FCS, and cultured at 37°C in the presence of 5% CO<sub>2</sub> in M16 medium until they were transferred into day 2.5 p.c. pseudopregnant C57BL/6 foster mothers. The mice were killed by cervical dislocation 13 days after blastocyst transfer, and fetuses were dissected from the uteri and inspected for the presence of abnormalities.

Alternatively, male BALB/c *Ceacam9*<sup>-/-</sup> mice (H-2<sup>d/d</sup>) were mated with female C57BL/6 *Ceacam9*<sup>+/+</sup> mice (H-2<sup>b/b</sup>). Resulting double heterozygous (*Ceacam9*<sup>+/+</sup>-H-2<sup>b/d</sup>) male mice were backcrossed with BALB/c *Ceacam9*<sup>-/-</sup> females. The haplotype of the littermates was determined by PCR and restriction endonuclease digestion of the resulting product (37).

## RESULTS

**Generation of CEACAM9-null mice.** A targeting vector was constructed using isogenic DNA from the *Ceacam9* cosmid cosC3 (8) for inactivation of the *Ceacam9* gene in the BALB/c ES cell line BALB/c-1. In the resulting vector, exon 1 (which comprises the start codon), intron 1, and two-thirds of exon 2 were replaced by a neomycin gene expression cassette (Fig. 1A). This construct should yield a null allele after homologous recombination. An HSV-tk expression unit was added to the 3' end of the 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 12% (16 of 130) in G418 and FIAU double-resistant ES cell colonies. This was inferred from hybridization of a 13-kb genomic *Hind*III DNA fragment in addition to the 23-kb wild-type fragment with probes from the 5' and 3' regions of the *Ceacam9* gene (Fig. 1A; data not shown). Two ES cell clones (2C3, 2D7) were used for microinjection into C57BL/6 × DBA blastocysts. Only clone 2D7 exhibited germ line transmission. Mice heterozygous for the knockout allele were identified by allele-specific PCR and were interbred. The offspring were analyzed by Southern blot hybridization with probes from the 5'- and 3'-flanking regions of *Ceacam9* (Fig. 1B). The results obtained with both probes confirmed that correct homologous recombination occurred at the *Ceacam9* locus. No additional integration events of the targeting construct could be detected using a *neo* probe (Fig. 1B). To examine the effect of the replacement mutation on *Ceacam9* expression, total RNA from day 10.5 p.c. placentae of

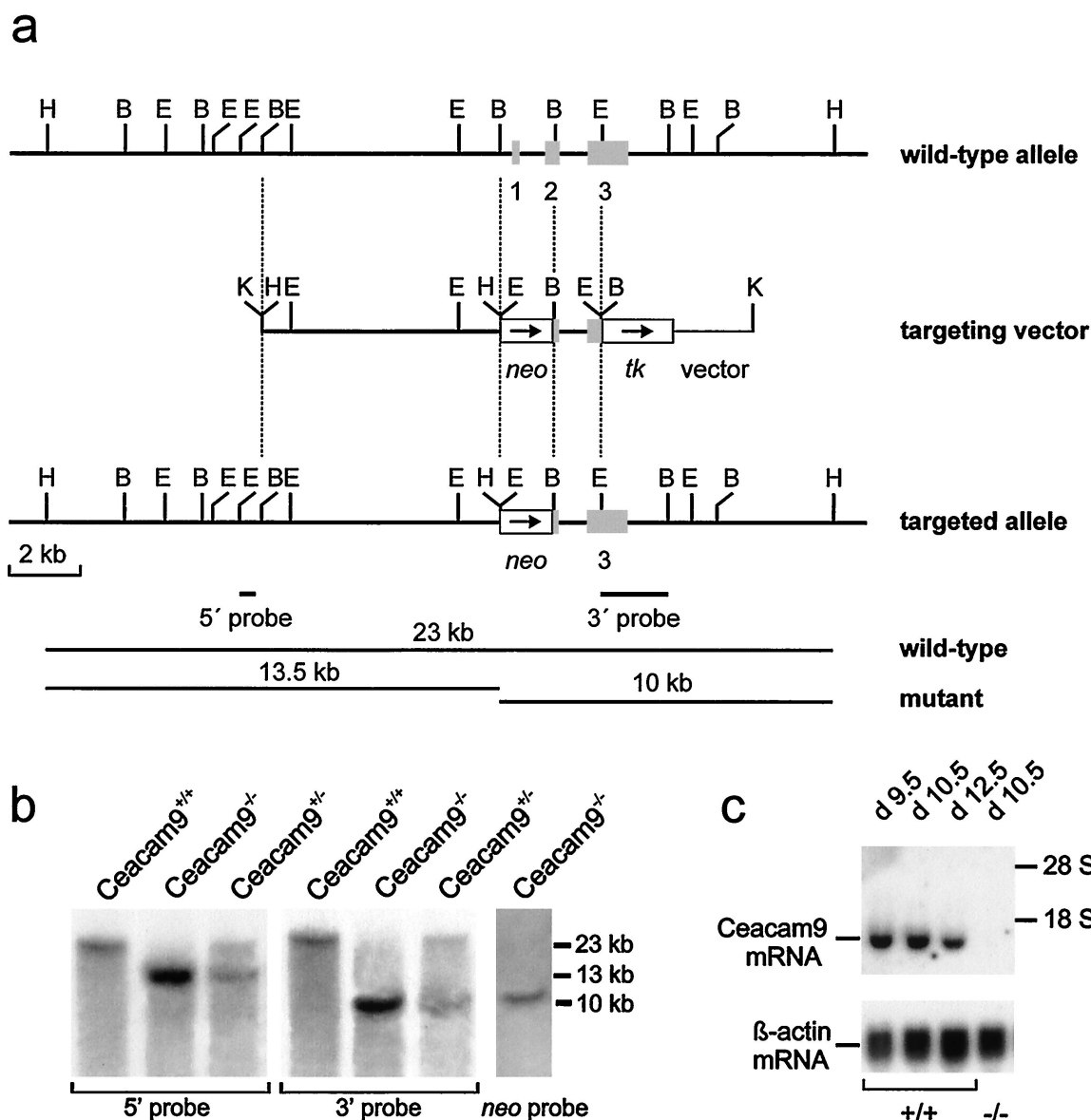


FIG. 1. Targeted disruption of the murine *Ceacam9* gene. (a) Structure of the wild-type allele, targeting construct, and recombinant locus. Gray boxes represent the three exons of *Ceacam9*. The *neo* and *tk* expression cassettes used for the selection of homologous recombinants are shown as open boxes. Arrows indicate their transcriptional directions. The vector sequence within the targeting plasmid is shown as a thin line at the 3' end of the *tk* gene. The expected sizes of the DNA fragments obtained were 23 kb after digestion with *Hind*III for the wild-type allele and 13.5 and 10 kb after digestion with both probes, which are located 5' and 3' of the targeting construct, respectively, for the correctly targeted allele. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I. (b) Southern blot analyses of DNA isolated from tail biopsies obtained from the progeny of a mating of *Ceacam9*<sup>+/-</sup> parents. The DNAs were digested with *Hind*III and hybridized with a genomic probe 5' of the *Ceacam9* sequence present in the targeting vector. The blot was rehybridized with a probe from the 3'-flanking region. To exclude additional nonhomologous integration events of the targeting construct, the blot was hybridized with the whole *Hind*III-linearized <sup>32</sup>P-labeled pGK-neotk vector as a probe, which did not reveal additional hybridizing fragments apart from the 10-kb *Hind*III DNA fragment. The sizes of the hybridizing DNA fragments are in the right margin. (c) Northern blot analysis of total RNA isolated from days 9.5, 10.5, and 12.5 p.c. placentae of wild-type (+/+) and day 10.5 p.c. placentae of *Ceacam9*<sup>-/-</sup> mice (-/-). The same filter was sequentially hybridized with <sup>32</sup>P-labeled *Ceacam9* and  $\beta$ -actin cDNA probes. The mobilities of the 28S and 18S ribosomal RNAs are shown.

*Ceacam9*<sup>-/-</sup> mice was analyzed by Northern blotting. No full-length or truncated *Ceacam9* transcripts could be detected (Fig. 1C). It is known that *Ceacam9* expression is restricted to a subset of primary and secondary trophoblast giant cells of the placenta (Fig. 2b) (8). In situ hybridizations were performed with day 8.5 p.c. placentae from *Ceacam9*<sup>-/-</sup> mice and wild-type littermates to rule out loss of this cell population during tissue preparation for Northern blot analyses. Again, no *Ceacam9* mRNA was found in placentae of *Ceacam9*<sup>-/-</sup> mice (Fig. 2a), while transcripts of the trophoblast marker genes

*P<sub>L</sub>-I* and *4311* could be identified (Fig. 2c and d). These results indicated successful disruption of *Ceacam9*.

**Phenotypic analyses of *Ceacam9*<sup>-/-</sup> mice.** Genotype analyses of 200 offspring resulting from the mating of *Ceacam9*<sup>+/-</sup> mice revealed a nearly Mendelian distribution of the various genotypes (*Ceacam9*<sup>+/+</sup>, 29%; *Ceacam9*<sup>-/-</sup>, 23%). No obvious morphological or behavioral abnormalities were observed in *Ceacam9*<sup>-/-</sup> mice. They exhibited the same weight gain and fertility as their heterozygous or wild-type littermates on both BALB/c and 129/Sv backgrounds (data not shown). Further-

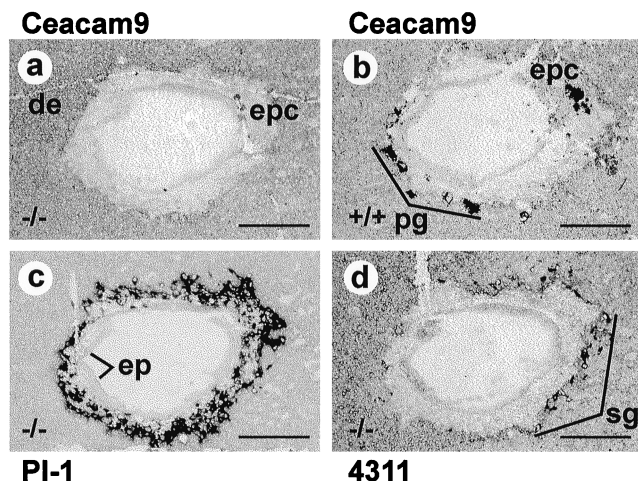


FIG. 2. Analysis of trophoblast marker gene expression in placentae from wild-type and *Ceacam9*<sup>-/-</sup> mice by in situ hybridization. Placentae from wild-type (+/+) and homozygous mutant (-/-) mice were isolated day 8.5 p.c., cryo-sectioned, and hybridized with digoxigenin-labeled antisense RNA probes. Specifically binding RNA was visualized after incubation with anti-digoxigenin antibodies coupled with alkaline phosphatase using NBT-BCIP (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate) as a chromogenic substrate. No labeling was observed with sense probes (data not shown). The probes used are indicated above and below the panels. Bars, 0.5 mm. De, decidua; ep, embryo proper; epc, ectoplacental cone; pg, primary giant trophoblast cells; sg, secondary giant trophoblast cells.

more, histological analyses of the placentae and fetuses between days 10.5 and 16.5 p.c. using paraffin-embedded sections did not reveal morphological differences. In addition, the expression pattern of the placental marker genes *P<sub>L</sub>-I* and *4311*, which are expressed in primary and secondary trophoblast giant cells and spongiotrophoblast cells and their precursors, respectively, was unchanged (Fig. 2c and d and data not shown).

***Ceacam9* is not essential for allogeneic pregnancies.** Since members of the CEA family which are specifically expressed in trophoblast cells are suspected of being involved in the protection of the allotypic fetus from the maternal immune system, the survival of *Ceacam9*<sup>-/-</sup> embryos was analyzed in allogeneic and semiallogeneic mothers. BALB/c *Ceacam9*<sup>-/-</sup> or *Ceacam9*<sup>+/+</sup> blastocysts (H-2<sup>d</sup>) were transferred into C57BL/6 foster mothers (H-2<sup>b</sup>) with a wild-type *Ceacam9* genotype. Both transfers yielded normal embryos with comparable efficiencies (Table 1), which indicates that fetal expression of CEACAM9 is dispensable for the maintenance of allotypic pregnancies. In order to evaluate whether maternally expressed CEACAM9 contributes to the survival of semiallogeneic embryos or *Ceacam9*<sup>-/-</sup> embryos suffer from competition by *Ceacam9*-positive embryos, *Ceacam9*<sup>-/-</sup> BALB/c females (H-2<sup>d/d</sup>) were first mated with *Ceacam9*<sup>+/+</sup> C57BL/6 males (H-2<sup>b/b</sup>). Resulting *Ceacam9*<sup>+/-</sup> males (H-2<sup>b/d</sup>) (C57BL/6 × BALB/c *Ceacam9*<sup>-/-</sup>) were crossed with *Ceacam9*<sup>-/-</sup>/H-2<sup>d/d</sup> BALB/c females, and offspring were genotyped 3 weeks after birth. Mice with all possible genotypes were born at similar frequencies (H-2<sup>d/d</sup>/*Ceacam9*<sup>+/-</sup>, five offspring; H-2<sup>b/d</sup>/*Ceacam9*<sup>+/-</sup>, five offspring; H-2<sup>d/d</sup>/*Ceacam9*<sup>-/-</sup>, seven offspring; H-2<sup>b/d</sup>/*Ceacam9*<sup>-/-</sup>, eight offspring), which excludes a major role of CEACAM in the tolerance of semiallogeneic embryos.

## DISCUSSION

The results presented here clearly demonstrate that *Ceacam9* is dispensable for the development, adult life, and reproduction of mice on a BALB/c and BALB-c-129/Sv mixed

background kept under non-pathogen-free laboratory conditions despite its high degree of conservation among rodents. Furthermore, transfer experiments with allotypic *Ceacam9*<sup>-/-</sup> embryos as well as backcrosses leading to semiallogeneic pregnancies did not reveal an essential function of *Ceacam9* in the protection of the fetal allograft from the maternal immune system. Multiple mechanisms have been suggested to explain the phenomenon of maternal tolerance toward the semiallogeneic fetus in mammals. Transient specific T-cell tolerance to paternal alloantigens and a biased expression of T<sub>H</sub>2 cytokines during pregnancy are thought to play a role in this process (52, 57). Furthermore, the lack of classical major histocompatibility complex class I molecules on fetal trophoblast cells which are in intimate contact with maternal blood has been implicated in the ignorance of paternal antigens expressed by the fetus (21, 39). In addition, it has been proposed that Fas ligand molecules present on trophoblast cells contribute to the immune privilege of the placenta by inducing apoptosis via Fas receptors on maternal lymphocytes reactive against fetal antigens (17, 19). It has been demonstrated recently by forced expression of major histocompatibility complex class I in placenta and genetic disruption of Fas-mediated cell death, however, that neither of the latter two mechanisms or a combination of the two is sufficient to abolish maternal tolerance in mice (41). Taken together, these results imply that several nonessential mechanisms contribute either incrementally to materno-fetal tolerance or highly redundant processes secure the positive outcome of mammalian pregnancies.

Preliminary experiments using CEACAM9-human IgG-Fc fusion proteins indicate the presence of putative CEACAM9 receptors in the decidua (Finkenzeller and Zimmermann, unpublished results). The maternal part of the placenta, therefore, probably represents the target tissue of secreted CEACAM9. This could imply that CEACAM9 directly influences the maternal system to achieve favorable physiological conditions for the fetus. On the other hand, CEACAM9 and its putative receptor could be players in the maternal-fetal and paternal conflict postulated by Haig (10), in which the CEACAM9 receptor could function as a molecular sink to reduce the amounts of the fetally expressed CEACAM9, which possibly promotes fetal growth at the mother's expense. This would prevent excessive drainage of maternal resources. Knocking out one of the redundant arms produced during the materno-fetal "arms race" in evolution would, therefore, show no or only a marginal phenotype. If this assumption were true, one would expect that overexpression of CEACAM9 rather than loss of function would exhibit an effect on fetal development.

Alternatively, the viability and fertility of *Ceacam9* null mutants could be due to compensatory effects exerted by the PSGs, which are also predominantly expressed by trophoblast cells. This appears unlikely, however, because of the unique structure and spatio-temporal expression pattern of *Ceacam9*. In addition, the uniqueness of *Ceacam9* is supported by the fact that it is separated by 2 centimorgans from the *Psg* gene cluster on chromosome 7, which consists of about 15 tightly

TABLE 1. Survival of allogeneic BALB/c *Ceacam9*<sup>-/-</sup> embryos (H-2<sup>d</sup>) in C57BL/6 *Ceacam9*<sup>+/+</sup> foster mothers (H-2<sup>b</sup>)

Genotype of transferred blastocysts	No. of transferred blastocysts (no. of foster mothers)	No. of viable embryos 13 days after transfer	No. of resorbed embryos
<i>Ceacam9</i> <sup>-/-</sup>	20 (2)	12 (60%)	2
<i>Ceacam9</i> <sup>+/+</sup>	12 (1)	4 (30%)	3

linked and coordinately expressed genes (22, 40, 43, 50; W. Zimmermann, unpublished results). However, functional replacement of *Ceacam9* by *Psg* genes can only be rigorously proven or excluded by deletion of the closely related *Psg* genes, e.g., by chromosomal engineering (38).

In conclusion, the lack of an obvious phenotype suggests that the evolutionarily young family of *Ceacam* genes does not serve an essential role but arose in order to achieve optimization of a function which is important for competitiveness under natural conditions.

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