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Functional Analysis of C-CAM1 Tumor Suppressor Gene by Targeted Gene Deletions

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C-CAM1 (renamed CEACAM1) is a cell adhesion molecule of the immunoglobulin supergene family. We have shown that CEACAM1 plays critical roles in prostate cancer initiation and progression. We propose to determine the functional roles of CEACAM1 in normal prostate development, prostate homeostasis and prostate tumorigenesis by using gene targeting technologies to generate CEACAM1 knockout mice. We have constructed a Ceacam1 targeting vector in which Ceacam1 gene was flanked by loxP sites to allow for generation of both straight and conditional knockout of the Ceacam1 gene. Several embryonic stem cell clones containing the recombinant gene allele were established and two were injected into blastocysts for germ line transmission of the targeting construct. In this funding period, we have succeeded in producing mice harboring the conditional Ceacam1 knockout allele. Two founder mice from two embryonic cell clones showed germ line transmission of the targeting construct containing the conditional Ceacam1 gene. Five mice with homozygous conditional Ceacam1 alleles were born from these two founder mice. These results suggest that embryonic lethality does not occur in the mice carrying conditional Ceacam1 knockout alleles, thus it is possible to pursue the function of CEACAM1 in normal prostate development, prostate homeostasis and prostate tumorigenesis in vivo.
# Table of Contents

Cover.................................................................1

SF 298...............................................................2

Table of Contents..................................................3

Introduction.........................................................4-5

Body.................................................................5-9

Key Research Accomplishments...............................9

Reportable Outcomes.............................................9

Conclusions.........................................................9-10

References........................................................10

List of Paid Personnel............................................11

Appendices.........................................................N/A
INTRODUCTION

C-CAM1 (recently renamed as CEACAM1) is a cell adhesion molecule of the immunoglobulin supergene family (1). We have shown that CEACAM1 plays critical roles in prostate cancer initiation and progression and that loss of CEACAM1 is an early event in the development of prostate cancer (2). Although tumorigenesis studies in mouse xenograft model have suggested the involvement of CEACAM1 in epithelial cell growth and differentiation, the functional roles of CEACAM1 in normal prostate development, prostate homeostasis, and prostate tumorigenesis remain unclear. Towards the aim of unraveling the roles of CEACAM1’s growth suppressive activity in prostate growth and tumorigenesis, we propose to use gene targeting and embryonic stem cell technologies to generate CEACAM1 knockout mice. Specifically, we plan (1) to determine the roles of CEACAM1’s growth suppressive function in vivo by generating mice with a targeted deletion of the CEACAM1 cytoplasmic domain; (2) to determine the roles of CEACAM1’s growth suppressive function in prostate development and tumorigenesis by generating mice with a prostate-specific knockout of the CEACAM1 cytoplasmic domain. The proposed work was divided into two Tasks to be carried out in parallel.

Task 1. Generate mice with targeted deletion of CEACAM1 cytoplasmic domain to determine the roles of CEACAM1’s growth suppressive function in vivo (months 1-30)

Task 2. Prostate-specific loss of function of CEACAM1 gene in prostate (months 7-36)

Genetic manipulation of mouse genes in vivo is a powerful approach to understanding the function of a gene, both during embryonic development and in adult tissues. This method requires full knowledge of the genomic structure of the gene of interest. Unlike humans and rats, which each have one CEACAM1 gene, two CEACAM1-like genes, Ceacam1 and Ceacam2, were identified in mice. In the previous study, we have isolated and sequenced these two closely related Ceacam genes from a mouse 129 Sv/Ev library (3). We have also examined the tissue-specific and embryonic expressions of these mouse Ceacam1 and Ceacam2 genes (3). Our sequence analysis revealed that the genes encoded nine exons and spanned approx. 16-17 kb (Ceacam1) and 25 kb (Ceacam2). The genes were highly similar (79.6%). The major
differences in the protein-coding regions were located in exons 2, 5 and 6. To determine whether functional redundancy exists between Ceacam1 and Ceacam2, we examined their expression in 16 mouse tissues by using semi-quantitative reverse transcription-PCR. As in human and rat, in mouse Ceacam1 mRNA was highly abundant in the liver, small intestine, prostate and spleen. In contrast, Ceacam2 mRNA was only detected in kidney, testis and, to a lesser extent, spleen. In a mouse embryo, Ceacam1 mRNA was detected at day 8.5, disappeared between days 9.5 and 12.5, and reappeared at day 19. On the other hand, no Ceacam2 mRNA was detected throughout embryonic development. The different tissue expression patterns and regulation during embryonic development suggest that the Ceacam1 and Ceacam2 proteins, although highly similar, may have different functions both during mouse development and in adulthood. Results from this study allow us to design a gene targeting strategy that is specific to Ceacam1 gene and also allow us to perform both straight and conditioned knockout of Ceacam1 gene in parallel.

We have designed a gene targeting strategy that is specific to Ceacam1 gene. In addition, the Ceacam1 gene in the targeting vector was flanked by loxP sites to allow for generation of both straight and conditional knockout of Ceacam1 gene. The targeting vector has been constructed and 24 embryonic stem cell clones containing the recombinant gene allele have been established. Two of the embryonic cell clones were injected into blastocysts for germ line transmission of the targeting construct toward the end of last funding period. In this funding period, we have succeeded in producing mice harboring conditional Ceacam1 gene. Two founder mice from two embryonic cell clones showed germ line transmission of the targeting construct. Five mice with homozygote conditional Ceacam1 allele were born from these two founder mice. These results suggest that embryonic lethality does not occur in mice carrying conditional Ceacam1 allele. Thus, it is possible to pursue the function of CEACAM1 in prostate in vivo.

(5) BODY (Progress report)

Because Task 1 and Task 2 are performed in parallel, we describe the progresses in these aspects together.
5.1. Gene targeting strategy

Based on genomic characterization of Ceacam1 gene, we decided to delete the cytoplasmic domain of CEACAM1. This strategy is based on the following rationales: (1) the entire Ceacam1 gene, around 20 kb, is too large to delete; and (2) the cytoplasmic domain of CEACAM1 is critical for tumor suppression. A conditional knockout construct is designed to delete exons 7–9 in a tissue-specific or non-specific manner in the mice. ES cells harboring the targeting vector are injected into blastocysts to generate mice carrying the conditional Ceacam1 allele. For straight knockout, the goal of Task 1, the conditional knockout mice will be crossed with transgenic mice carrying actin-driven Cre recombinase, which is expressed during embryonic development. For conditional knockout, the goal of Task 2, the Ceacam1 gene will be deleted in prostate specifically by crossing the conditional knockout mice with transgenic mice carrying probasin-driven Cre recombinase. Thus, Task 1 and Task 2 are being performed in parallel. The gene knockout strategy is shown in Fig. 1.

Fig. 1. Maps of the Ceacam1 genomic locus and the loxP-targeting vector designed to introduce a NeoR cassette. Exons are denoted by rectangles and loxP sites are denoted by triangles. Red rectangles indicate CEACAM coding regions. Successful targeting yields a conditional Ceacam1floxed allele. Cre-mediated recombination results in deletion of NeoR cassette and the cytoplasmic domain exons, yielding a knockout allele. Specific primers used to screen for various alleles are indicated by small arrows labeled a through c.
5.2. Targeting vector construction

A 129Sv/Ev mouse genomic library in BAC vectors was screened with a probe generated from cDNA coding for the full-length mouse CEACAM1. Three positive BAC clones were identified and their structures were determined by restriction mapping. Consistent with our previous study (3), two closely related genes, i.e. Ceacam1 and Ceacam2, were identified from restriction mapping. Based on tissue-specific and embryonic expression of Ceacam1 and Ceacam2 gene (3), we decided to delete the cytoplasmic domain of Ceacam1. A conditional knockout construct was designed to delete exons 7~9. The conditional targeting construct was designed by inserting a loxP-neo cassette at the Xmn1 site in intron 6 and a loxP fragment at the HindIII site in intron 9 of Ceacam1 (Fig. 1). The targeting vector includes 4.3 kb of homologous DNA upstream of the loxP-neo site and 3.4 kb of homologous DNA downstream of the second loxP site. The targeting vector was verified by restriction mapping.

5.3. Transfection and generation of embryonic stem cells harboring targeting vector

The targeting vector was transfected into embryonic stem cells by electroporation. A total of 270 ES cell clones were obtained from two electroporations. Genomic DNA was extracted from these ES cell clones. Half of the DNA from each sample was digested with SphI and processed for Southern blot analysis. The 5' probe used is a 0.7kb Apal/SpeI fragment from subclone BamHI-A. Using the 5' probe, a ~9.2 kb band corresponding to the endogenous Ceacam1 and a ~10 kb band corresponding to Ceacam2 were detected in most samples. Many clones (a total of 24) included an additional hybridization signal at the size (8.1 kb) predicted for the homologous recombinant allele (Fig. 2). These potential positives clones were digested with Xho I and hybridized with the 3' probe, which is a 1.2kb Sac I/Xho I fragment from subclone Apa I-4. A ~15.3 kb band corresponding to the endogenous allele of Ceacam1 and a ~17.1 kb band corresponding to recombinant allele were detected in 16 clones (Fig. 2). These 16 clones were also detected with a Neo probe, which gave a 17.1 kb band in Southern blot analysis. Taken together, a total of 16 ES cell clones containing the Ceacam1 conditional allele were generated. Three positive ES cell clones were injected into mouse blastocysts using procedures described in Chang et al. (4). Chimeric mice were obtained and they were mated with C57BL
mice. The F1 mice having germ line transmission of the loxP-targeted Ceacam1 allele were interbred to generate F2 mice.

Fig. 2. Southern blot analysis performed with a 5' and a 3' external probes to detect homologous recombination that gives rise to conditional Ceacam1flox allele.

5.4 Genotyping of the wild-type and loxP-targeted (floxed) alleles

Germ line transmission was confirmed by PCR analysis. Isolated mouse tail DNA was used for PCR identification of the genotype. Three sets of primers that detect the wild-type and the floxed allele of Ceacam1, and the Ceacam2 gene were used to genotype the mice. Primer A (5' ACACAAGGAGGCCTCCTCAGATGGCG 3') and Primer C (5'GCGCCTCCCCTACCCGAGTAATT 3'), containing sequences from Exon 6 of Ceacam1 and neomycin cassette, respectively, will produce a 488 bp PCR product from the floxed Ceacam1 allele. Primer A and Primer B (5' GACTTTGGCTTCCTGACTGGAGGA 3'), containing sequences from Intron 6, will generate a 382 bp PCR product from wild-type Ceacam1. Two founder mice from two embryonic cell clones showed germ line transmission of the targeting construct containing the conditional Ceacam1 gene. Five mice with homozygous conditional Ceacam1 alleles were born from these two founder mice. These results suggest that embryonic lethality does not occur in the mice carrying conditional Ceacam1 knockout alleles, thus it is possible to pursue the function of CEACAM1 in normal prostate development, prostate homeostasis and prostate tumorigenesis *in vivo*. 
Fig. 3. PCR analysis with mouse tail DNA to detect homologous recombination in mice.

488 b.p.  
382 b.p.  

(6) KEY RESEARCH ACCOMPLISHMENTS

- Designed a knockout strategy that allows Task1 and Task2 to be carried out in parallel.
- Constructed the targeting vector.
- Transfected the targeting vector into embryonic stem cells and selected 24 positive ES cell clones.
- Injected three ES cell clones into blastocysts.
- Bred and genotyped mice for the presence of recombinant allele.
- Obtained several mice carrying heterozygote and homozygote recombinant allele.

(7) REPORTABLE OUTCOMES

(8) CONCLUSION We propose to determine the roles of CEACAM1's growth suppressive activity in prostate growth and tumorigenesis by using gene targeting and embryonic stem cell technologies to generate knockout mice. We have designed and constructed a gene targeting vector for specific disruption of the Ceacam1 gene. This gene targeting strategy will allow us to perform both straight and conditioned knockout of Ceacam1 gene in parallel. Embryonic stem cells containing the knockout construct were generated and injected into blastocysts. We have
succeeded in generating mice carrying *Ceacam1* conditional knock out allele. These mice are being examined for *Ceacam1* message and protein levels to see whether the recombinant allele alters the endogenous *Ceacam1* expression level.

(9) REFERENCES


List of personnel receiving/received pay from the research effort:
Sue-Hwa Lin
Karen Earley
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