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TITLE: ROLE OF STAT-3 IN ER+ BREAST TUMORS

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Role of Stat-3 in ER- Breast Tumors

The hypothesis to be tested in this application is whether c-myb and A-myb genes regulate ductal cell proliferation and whether abnormalities in the expression of these genes results in breast cancer. During the first year of this study, we constructed targeting vectors that can be used for the deletion of exons 6 and 9 of mouse A-myb and c-myb genes. These vectors were transfected into embryonic stem cells and G418 resistant clones were screened for homologous recombination. Following selection, the cell lines were subjected to transient Cre expression and selection for ganciclovir-resistant clones. The ES cell clones that have undergone homologous recombination with our targeting vectors were injected into blastocysts to generate chimeric mice. To validate the results obtained from the mouse models in the human system, we have conducted parallel studies with human breast tumor cell lines. Analysis of human breast tumor cell lines showed that c-myb is expressed in a majority of ER+ human breast carcinomas, while A-myb expression is seen predominantly in ER- cell lines. Our results also shows that blockage of the biochemical function of c-myb results in a complete block to ER+ breast tumor cell proliferation.
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A. INTRODUCTION

The myb gene family currently consists of three members, named A, B and c-myb, all of which encode nuclear proteins that bind DNA in a sequence-specific manner and function as regulators of transcription. Of these, c-myb has been studied extensively in cell culture systems of differentiation and transformation and during mouse development. These studies have provided evidence for the crucial function of the c-myb gene in the development, proliferation and oncogenesis of the hematopoietic system. A series of recent experiments have implicated a role for this gene family in breast development and breast cancer. The first evidence that implicated a role for c-myb in breast tumors came from the observation that this gene is highly expressed in all estrogen receptor positive (ER+) breast tumors. Our in situ hybridization studies also show that this gene is highly expressed in proliferating epithelial cells of the ductal epithelium, in virgin as well as pregnant mice, suggesting that this gene, like in the hematopoietic cell system, might play a critical role in the proliferation of these cells. Our in situ hybridization studies also show that A-myb is not expressed, or is expressed at very low levels, in ductal cells derived from virgin mice. These levels, however, increase dramatically during the cell proliferation that accompanies pregnancy, resulting in ductal branching and alveolar development. Since this phase of ductal branching is mainly induced by the combined action of estrogen and progesterone, these results suggest that A-myb might play an important role in this phase of ductal cell proliferation and morphogenesis into alveolar structures. This view was further supported by our studies where we developed A-myb null mutant mice, which were unable to produce milk and showed defects in mammary tissue proliferation following pregnancy. The loss of A-myb expression seems to result in a loss or diminution of progesterone-induced proliferative events associated with the pregnancy-induced morphogenesis of breast tissue.

Based on these studies, we hypothesized that c-myb and A-myb genes play a pivotal role in the proliferation of breast epithelial cells in the adult mice. It is our hypothesis that proliferative events associated with ductal cell proliferation of virgin mice is mediated by c-myb and that proliferative events associated with ductal branching and alveolar development that occur following pregnancy are mediated by the combined action of c-myb and A-myb. It is also our contention that proper down regulation of A-myb and c-myb are essential for apoptotic events associated with involution. Events that lead to deregulation of expression of c-myb and A-myb might represent a point at which the onset of neoplasia, which, in combination with other mutations and deletions in oncogenes and growth suppressor genes, results in a metastatic disease. A-myb null mutant mice have been extremely useful to our understanding of the role of A-myb in mammary gland development. Unfortunately, however, c-myb null mutant mice have not been very useful to study the role of this gene in mammary gland development since these
mice die in utero due to failure of fetal hepatic hematopoiesis. To address the role of c-myb in mammary development, we proposed to develop c-myb mutant mice where the expression of this gene is interrupted specifically in the mammary gland using the Cre-lox system.

Task 1: Months 1-12: During this period we proposed to construct targeting vectors that can be used for the deletion of exons 6 and 9 of mouse c-myb genes. These vectors were to be transfected into embryonic stem cells and G418 resistant clones were to be screened for homologous recombination. Following the selection of the clones, the cell lines were to be subjected to transient Cre expression and selection for ganciclovir-resistant clones.

B. PROGRESS REPORT

To generate breast-specific c-myb null genotype in mice, we have isolated a Bam H1 genomic clone of c-myb encoding exons 2 through 8, which was used to produce a gene targeting vector (Fig. 1). A 0.9 kb fragment containing exon 6 was generated by PCR amplification and cloned into the BamHI site of the pfax vector. The flanking 6.0 kbp fragment on the 5' end containing exons 2-5 was generated by a combination of PCR and restriction enzyme digestion and cloned into the Sall site. Similarly, the flanking 2.5 kb fragment on the 3' end containing exons 7-8 was generated by a combination of PCR and restriction enzyme digestion and cloned into the XhoI site. Following the construction of the targeting vector, the entire clone was sequenced to ascertain that PCR technique did not produce any mutations or deletions.

The targeting construct, linearized by NotI digestion, was introduced into ES cells via electroporation. ES cells were plated on gelatin coated culture plates and selected for 10 days with medium containing 150 µg/ml of active G418. This procedure yielded approximately 600 clones. Genomic DNA from these ES clones was digested with Stu1 and homologous recombinants identified by Southern blotting using c-myb specific probe downstream of exon 9. Homologous recombinants (Fig. 2) revealed two bands: 11.5 kb (wt) and 12.6 kb (recombinant). In order to verify that the recombinants retained all the three loxP sites, DNA digested with EcoRI was analyzed by Southern blotting using loxP specific probe. All the positive recombinants revealed three bands of sizes 0.3 kb, 1.7 kb and 2.1 kb indicating the presence of 5' loxP, internal loxP and 3' loxP sites respectively.

To produce ES cell subclones that contained the Type 1 (systemic) and Type II (conditional) c-myb mutations, ES clones were subjected to transient Cre expression by electroporation of supercoiled pPGK-Cre-bpA and subsequent selection in the presence of ganciclovir. Subclones resistant to ganciclovir were isolated and analyzed by genomic Southern blotting. Using the genomic probe on DNA digested with BamHI, Type II deletions were verified by identifying a band of 6.0 kbp. Type I deletions would reveal a band
Fig 1. Mouse c-myb genomic structure and targeting vector production. (A) Restriction map of the mouse genomic clone is shown. The pflox vector is used as depicted to generate a c-myb targeting vector and contains two selectable markers tk and neo (black arrows). Thirty-four bp loxP sites are depicted as black arrowheads. (B) Homologous recombination between the wild-type (WT) c-myb allele in embryonic stem (ES) cells will generate the c-mybF[tkneo] allele to be used as a substrate for subsequent recombination by Cre recombinase. Positions of the probe used to determine homologous recombination and for additional genomic Southern blotting studies of targeted c-myb allele structure are depicted. (C) Production of ES cells bearing Type I and Type II deletions in the c-mybF[tkneo] allele will be generated following Cre expression and ganciclovir selection. Restriction enzyme sites: B, BamHI; E, EcoRI; H, HindIII; S, SalI; X, XhoI. Deletion of exon 6 results in the disruption of c-Myb DNA-binding domain, which renders the protein inactive.

Fig.2. Homologous recombination at the c-myb locus in embryonic stem (ES) cells. Southern blot analysis was performed on ES cell DNA digested with StuI and hybridized with c-myb specific probe downstream of exon 9. Out of a total of 384 ES cell clones (G418 resistant) analyzed, 7 clones revealed the presence of conditionally targeted c-myb allele. Homologous recombinants revealed two bands: 11.5 kb (wt) and 12.6 kb (recombinant). The results shown for clones numbered 90, 116 and 158 are representative.
of 11.5 kb, close to the wild-type band. To further confirm the deletions, DNA digested with BamHI was probed with loxP to identify two bands of sizes 6.0 kb and 2.5 kb (Type II) or a single band of size 11.5 kb (Type I).

We have microinjected the $c$-$myb^F$ (Type II) ES cell clones into C57BL/6 blastocyst-stage embryos. To date several chimeric mice have been produced and are currently being used to generate heterozygous mice for the $c$-$myb^F$ allele by crossing chimeric mice with C57/B6 mates. Germline transmission is being assessed by digesting tail DNA with BamHI and Southern blotting using the genomic probe to reveal two bands: 11.5 kb (wt) and 6.0 kb (recombinant). We plan to investigate deletion of $c$-$myb$ gene in breast tissue using transgenic mice expressing Cre recombinase specifically in breast tissue. Dr. Lothar Hennighausen of NIH, under a collaborative agreement has agreed to provide us with two sets of transgenic mice, with the Cre recombinase under the control of Whey Acidic Protein (WAP) promoter and MMTV promoter. In these mice, the Cre recombinase is not expressed in any tissue other than the breast. Even in the breast tissue, the recombinase is not expressed in mammary tissue of virgin mice and the first expression of the recombinase was seen at day 14 of pregnancy and increased during lactation. Interestingly, the recombinase activity could be detected 30 days after weaning, suggesting that the transgene has been active in putative stem cells. These mice would be ideal to determine whether $c$-$myb$ presence is necessary for breast epithelial cell proliferation during pregnancy and alveolar development and if the expression of this gene is necessary for ductal morphogenesis following involution.

To validate the results obtained from the mouse models in the human system, we have conducted parallel studies with human breast tumor cell lines. Analysis of several human breast tumor cell lines showed that the $c$-$myb$ is expressed in a vast majority of ER+ human breast carcinoma cell lines, while $A$-$myb$ expression is seen predominantly in ER- cell lines. To establish the role of $c$-$myb$ in ER+ cell proliferation, we have constructed dominant negative mutants of c-Myb, which effectively block $c$-$myb$-mediated transactivation of target genes. Expression of these dominant negative mutants in ER+ breast tumor cell lines resulted in complete block to their proliferative potential. In addition, these tumor cell lines lost their tumorigenic activity as evidenced by their inability to grow in soft agar. These results firmly establish that $c$-$myb$ gene plays an essential role in the proliferation of human breast carcinomas. We are currently in the process of assessing the role of $A$-$myb$ in the proliferation of ER- breast carcinomas.