Fine Mapping and Candidate Gene Analyses of *Pulmonary Adenoma Resistance 1*, a Major Genetic Determinant of Mouse Lung Adenoma Resistance

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Abstract

*Pulmonary adenoma resistance 1 (Par1)* is a major genetic determinant of mouse lung adenoma resistance. Although Par1 was previously mapped to mouse chromosome 11 by conventional linkage analyses, its candidate region was broad and undefined. In our present study, we generated Par1 congenic mice using two mouse strains A/J (Par1−) and *Mus spretus* (Par1+/−). Analyzing these congenic mice enabled us to fine map the Par1 quantitative trait loci (QTL) into a 2.0-cM (2.2 Mb) chromosomal region between genetic marker D11Mit70 and the gene *Hoxb9*. We then conducted systematic candidate gene screening through nucleotide polymorphism and expression analyses. Genes showing differential lung tissue expression or carrying nonsynonymous single nucleotide polymorphisms and expression analyses were identified and discussed. In particular, we evaluated tumor suppressor gene *Tob1* for its Par1 candidacy. Our findings have narrowed the Par1 QTL region and will greatly facilitate the identification of the major genetic determinant of mouse lung adenoma resistance.


Introduction

Lung cancer is the leading cause of cancer death in men and women in the United States (1). Although tobacco smoking is the major risk factor, there is strong evidence for genetic susceptibility and gene-environment interactions in lung cancer development (2–5). However, genetic heterogeneity and enormous variation in exposure levels to environmental agents make it difficult to identify lung cancer susceptibility loci in humans, especially for low-penetrance cancer susceptibility genes. Inbred strains of mice exhibit substantial difference in their susceptibilities to both spontaneous and carcinogen-induced lung adenoma development, thus offering a powerful model system to study human lung cancer susceptibility (6). To date, dozens of genetic loci have been linked to mouse lung adenoma susceptibility through linkage analyses (7). The majority of these genetic loci, or quantitative trait loci (QTL), exert small dose of functions, and some of them could only be detected in specific mouse lines through advanced statistical methods (8). However, major mouse lung adenoma susceptibility loci were also detected. For instance, *pulmonary adenoma susceptibility (Pas)* 1 was mapped to distal chromosome 6 and accounts for ~40% of variances in mouse lung adenoma susceptibility (9). Other QTL, such as *Pas2* to *Pas4* and *pulmonary adenoma resistance (Par)* 1 to *Par4*, are less potent than *Pas1* but also make significant contribution to mouse lung adenoma susceptibility in individual experimental systems (10). Specifically, Par1 functions as a major modifier of Pas1 and suppresses mouse lung adenoma development (11, 12).

Par1 was previously mapped to mouse chromosome 11 in different mouse populations (A/J × *Mus spretus*) × C57BL/6 and reciprocal (SMXA24 × A/J) × A/J (11, 12), but the derived QTL regions from both studies were broad and the precise location of Par1 has not been delineated. In the present study, we generated Par1 congenic strains of mice from Par1+/+ *M. spretus* and Par1−/− A/J mice by targeting a 29-cM chromosomal region between microsatellite markers D11Mit15 and D11Mit301. Subsequent analyses on these congenic mice enabled us to narrow down the Par1 QTL into a 2.0-cM (~2.2 Mb) chromosomal region between *Hoxb9* and D11Mit70. Further analyses ensued for genes located in this region. Consequently, multiple genes were identified with nonsynonymous single nucleotide polymorphisms (SNP) or differential lung tissue expression, and tumor suppressor gene *Tob1* was studied in particular.

Materials and Methods

**Mouse breeding.** Inbred A/J and *M. spretus* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in plastic cages with hardwood bedding and dust covers, in a HEPA filtered, environmentally controlled room (24 ± 1°C, 12/12-h light/dark cycle). Animals were given Rodent Lab Chow, #5001 (Purina, St. Louis, MO) and water *ad libitum*. The breeding scheme was to transfer a ~29-cM genomic fragment of lung tumor–resistant strain *M. spretus*, encompassed by microsatellite markers D11Mit15 and D11Mit301, onto the genetic background of lung adenoma susceptible strain A/J through sequential multigenerations of backcrossing. Selection of this chromosome 11 region governed the breeding procedure until N7. At N8, 50 male congenic strains containing different chromosomal segments of Par1 QTL were generated. These individual strains were then each crossed with four A/J females to produce the N9 generation. After genotyping, N9 congenic mice with same genotypes of their parental N8 strain were selected for lung tumor bioassay.

**Genotyping and phenotyping.** Genomic DNA was isolated from mouse tail. Briefly, tail clips were homogenized and incubated overnight at 55°C in nucleic acid lysis solution (Pronase 0.4 mg/mL, 10% sodium dodecyl sulfate [w/v], 10 mmol/L Tris, 400 mmol/L NaCl, and 2 mmol/L EDTA) followed by saturated NaCl extraction, precipitation with ice-cold 100% alcohol, washing twice with 70% alcohol, and dissolving in 1× TE buffer. The following markers have been purchased from Research Genetics, Inc. (Huntsville, AL) and used for congenic mouse genotyping: D11Mit15, D11Mit117, D11Mit212, D11Mit70, D11Mit14, D11Mit223, and D11Mit301.
HoxB9 marker was based on a SNP identified in the 3′-untranslated region. The forward primer was end labeled with [γ-32P]ATP, and 30 cycles of PCR were done at 94°C for denaturation, 55°C for annealing, and 72°C for extension. Denaturing polyacrylamide gels (8%) were used for resolution of the radiolabeled PCR products followed by autoradiography.

For phenotype collection, 5-week-old N9 mice were given a single ip. injection of urethane (1 mg/g body weight) in 0.2 ml PBS. All animals were euthanized by CO2 asphyxiation 20 weeks after urethane initiation. A portion of lung tumors and normal tissue were removed and flash frozen in liquid nitrogen. The rest of lungs were fixed in Tellyesniczky’s solution and examined with the aid of a dissecting microscope for tumor multiplicity.

Data mining. Position, name, and description of each gene within the Par1 candidate region were retrieved from the latest version of Ensembl mouse genome database (version 39.36, released on June 2006).1 Information of gene expression in mouse lung tissue was retrieved from EST Profile Viewer of National Center for Biotecnology Information (NCBI) Unigene database.2 Reverse transcription-PCR expression and nucleotide polymorphism analyses. Four 6-week-old A/J and M. spretus female mice (two for each strain) were euthanized and their lung tissues were collected for RNA isolation. Briefly, 100 mg lung tissue was pulverized and total RNA was extracted using Trizol reagent according to the manufacturer’s protocol (Life Technologies, Gaithersburg, MD). The quality of the isolated RNA was assessed by absorbance at 260 nm, the A260/A280 ratio (1.7–1.9), and electrophoresis on 1% agarose/formaldehyde gels that indicated the intensity and integrity of the 28S and 18S bands. Two micrograms of total RNA were used in reverse transcription reaction to synthesize the first-strand cDNA using oligo(dT) primer. To improve reverse transcription-PCR (RT-PCR) accuracy, we have done assays at least twice for each gene. We also used DNase I-treated RNA to prepare cDNA samples and set up a genomic DNA control for possible pseudogene amplification. RT-PCR primers were designed for each gene based on their published sequences and will be available on request. For semiquantitative RT-PCR assays, the linear amplification region for each gene was predetermined by plot experiments. The reaction profile generally consisted of one cycle at 95°C for 2 min followed by 30 to 35 cycles at 94°C, 55°C, and 72°C each for 45 s with minor modifications. Twenty-two cycles of amplification was used for the β-actin control. For the Tob1 gene, a 5% DMSO was used in both PCR and sequencing reaction. Electrophoresis on 1.5% agarose/formaldehyde gels were done to resolve PCR products. Amplified PCR products were purified with QIAquick gel extraction kit (Qiagen, Valencia, CA) and subjected to direct sequencing.

Cells and cloning. Complete Tob1 open reading frames (ORF) of A/J and M. spretus mice were amplified from respective strain cDNAs by PCR with primers designed based on published 129/SvJ strain sequence (Genbank accession no. NM_009427). Sequences were confirmed in both directions. The CDNA were cloned into vector pcDNAS3.1, which carries epitopes V5 and His (Invitrogen, Carlsbad, CA). The constructs were sequenced in both directions. Protein expressions were confirmed by transient transfection in 293 cells and Western blotting with V5 antibody (Invitrogen). The constructs were sequenced in both directions. Protein expressions were confirmed by transient transfection in 293 cells and Western blotting with V5 antibody (Invitrogen).

NIH3T3 cells (CRL1658; American Type Culture Collection, Manassas, VA) were grown in DMEM with 10% fetal bovine serum. The stable transfected cells were obtained through selection with G418 (Life Technologies, Invitrogen). The expression of Tob1 was examined by Western blotting with V5 antibody.

Cell growth analysis. NIH3T3 cells expressing Tob1 transfecants (0.5 × 104 and 0.5 × 105) were seeded in 24-well plates for the growth curve assay and 60-mm dishes for colony formation assay, respectively. For the growth curve assay, cells were counted from the 2nd day (day 1) after splitting until day 5. The cells were harvested and fixed with 4% paraformaldehyde followed by staining with 0.1% crystal violet for 15 min. The cells were then washed with PBS twice, and 0.2% of Triton X-100 were added to make cells permeable. The absorption value was measured by using 96-well plate reader at 595 nm wavelength. For the colony formation assay, after 10 days, the cells were fixed with ethanol and stained with 0.1% crystal violet. The number of colonies was counted under microscopy.

Western blot. Mouse lungs from A/J and M. spretus were homogenized in lysis buffer [PBS containing 5 mmol/L MgCl2, 1 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 5 μg/mL aprotinin, 1 mmol/L benzamidine (pH7.5)] and cleared by centrifugation. NP40 was then added to 1% after centrifugation. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Western blotting followed regular procedures. Phosphorylation of Tob1 was detected using 1:100 antihuman phospho-Tob1 antibody (code no. 18911, IBL Co. Ltd., Takasaki-Shi, Gunma, Japan). This antibody has shown cross-reaction with mouse phospho-Tob1 protein and thus was used. Transient transfections of NIH3T3 cells with V5-tagged Tob1 plasmids were conducted using the manufacturer’s provided protocol (Invitrogen).

Immunohistochemistry. Tissue specimens were collected from the lung tumor bioassay. Immunohistochemical analysis with anti–phospho-Tob antibody was done by the standard method. In brief, deparaffinized 5-μm thin tissue sections were placed in microwave oven in 0.01 mol/L sodium citrate buffer (pH 6.0), for two periods of 5 min each. After tissue sections were blocked with 0.3% hydrogen peroxide in PBS for 30 min at room temperature, each section was incubated overnight at 4°C with anti–phospho-Tob antibody at 1:50. The antirabbit IgG secondary antibody was added next forming. Counterstaining was done with hematoxylin.

Statistical analysis. Two-tailed Student’s t test was used to determine P values for the lung tumor multiplicity difference between the congenic strains and control strains. The same test has also been used to compare cell growth-suppressing function among the A/J and M. spretus Tob1 isofoms.

Results

Fine map Par1 QTL into a 2.2-Mb candidate region through congenic mice. Par1 was detected previously on mouse chromosome 11 by two groups using conventional linkage analyses. Manenti et al. (11) studied (A/J × M. spretus) × C57BL/6J mice and detected a peak logarithm of odds (LOD) score of 5.3 at the Rara gene locus (Fig. 1A). The genetic difference at this locus explained 23% of the phenotypic variance when only mice carrying the highly penetrant Pas1 allele (i.e., Pas1+/−) were analyzed, whereas no QTL could be detected in Pas1−/− mice. Patera et al. (12) later worked on reciprocal (SMXA24 × A/J) × A/J mice and detected a peak LOD score of 4.35 at microsatellite marker D11Mit70 (Fig. 1B). In their system, the genetic difference at the Par1 locus explained ~15% of total phenotype variance. Both analyses delineated that Par1 acts to negatively modify the Pas1 locus. However, neither study was able to provide a defined candidate region for the Par1 QTL because of limited marker density and population size. We also noticed that based on the present mouse genome map, marker order in the peak region was mistakenly assigned by the linkage software in the Manenti et al. study (Fig. 1A). A correctly defined Par1 candidate region is imperative for positional cloning of the gene. In our previous studies, we have successfully used congenic mice to fine map two major mouse lung adenoma susceptibility loci Pas1 and Par2. Based on the initial linkage mapping results, we selected a 29-cM chromosomal region encompassed by D11Mit15 and D11Mit301 markers to generate Par1 congenic mice. Par1 congenic strains were constructed by transferring this 29-cM fragment from the lung tumor-resistant strain M. spretus (Par1+/+, donor strain) onto the genetic background of lung tumor–susceptible strain A/J (Par1−/−, recipient strain) through a total of nine generations of

1 http://www.ensembl.org/Mus_musculus/index.html

1.05 http://www.aacrjournals.org www.aacrjournals.org 2509 Cancer Res 2007; 67: (6). March 15, 2007
backcrossing. Theoretically, nine generations of backcross mating will render ~99.81% of recipient (i.e., A/J strain) genome in congenic mice (13). During the breeding, we genotyped other mouse lung adenoma susceptibility loci (e.g., Pas1–Pas4 and the marker D11Mit15 and the marker D11Mit301 substituted by the donor fragment from M. spretus. AA is the control strain in which no substitution occurs in the entire Par1 region. Substitution of one copy of A/J allele by M. spretus allele in the Par1 QTL decreased the mouse lung tumor multiplicity by ~1.6-fold. D. refinement of the Par1 QTL with congenic mice. Seven microsatellite markers and Hoxb9 SNP marker were shown to cover genotypes of the 29.0-cM region containing the Par1 QTL. Their genetic positions were based on Mouse Genome Informatics database. 

**Figure 1.** Par1 QTL and fine mapping. A, Par1 QTL detected in (AJ x M. spretus) x C57BL/6J mice. The peak LOD score 5.3 was detected at the Rara gene (11). *, the Rara and Hoxb genes were misplaced. Black bar, possible QTL candidate region based on 1-LOD score drop. B, Par1 QTL detected in reciprocal (SMXA24 x A/J) x A/J (Patau et al., 1997) backcrossing. Theoretically, nine generations of backcross mating will render ~99.81% of recipient (i.e., A/J strain) genome in congenic mice (13). During the breeding, we genotyped other mouse lung adenoma susceptibility loci (e.g., Pas1–Pas4) to assure a clean A/J background.

N9 generations of mice were carrying variant Par1 subcongenic fragments and subjected to a standard urethane-induced lung tumor bioassay (i.e., single i.p. injection at dose of 1 mg/g body weight). As shown in Fig. 1C, we observed a significantly lower lung tumor multiplicity (18.7 ± 5.0 tumors per mouse) in congenic strain AM (heterozygous A/J x M. spretus alleles for the entire 29-cM Par1 region) than in control strain AA (30.5 ± 4.5 tumors per mouse, homozygous A/J alleles for the region; P < 0.00001). Thus, the substitution of one copy of A/J allele by M. spretus in the Par1 QTL decreased the mouse lung tumor multiplicity by ~1.6-fold. Consistent with the previous study, we also observed a ~2.4-fold decrease in total tumor volume in the AM mice (data not shown). We analyzed the N9 subcongenic mice on their distinguished Par1 segment genotypes and lung tumor phenotypes. As shown in Fig. 1D, the subcongenic strains 3 and 4 exhibited comparable lung tumor multiplicities as congenic strain AM does and significantly lower lung tumor multiplicities than the control strain AA, defining the right boundary of Par1 candidate region at D11Mit70 and the left boundary of Par1 region at Hoxb9, respectively. The refined Par1 QTL region was 2.0 cM in genetic distance and 2.2 Mb in physical distance.

**Gene content in the refined Par1 candidate region.** Based on the latest Ensembl mouse genome (version 39.36), there are 49 genes located in the 2.2-Mb candidate region (Table 1). Thirty-two genes have transcript expression in mouse lungs according to the public expression profile database (EST Profile Viewer of NCBI Unigene). Their expression in lung has been confirmed by our subsequent RT-PCR assays and indicated in Table 1. Except ribosomal protein gene Mrip27 and several functionally unknown Riken transcript genes (e.g., A430060F13Rik and 9530033F24Rik), these lung organ-related genes are involved in a broad range of cellular biochemical or physiologic properties, such as cell growth and differentiation (e.g., Tob1, nerve growth factor receptor (Ngfr), I2b2p1, and Phb), embryonic development (e.g., Hoxb13), protein phosphorylation (e.g., Pdk2, Ppp1r9b, and Phosphat1), DNA replication and gene expression (e.g., Mycbbp and Myst2), drug resistance (e.g., 3300001p08Rik), neuron firing (e.g., Cacnalg), phototransduction (e.g., Gng2), energy synthesis (e.g., Atp5g1), and intercellular adherence and cell migration (e.g., Itga3 and
Abi3). Interestingly, several genes have shown association with cancer development in previous studies. For instance, deficiency of the Tob1 tumor suppressor gene led to development of multiple types of tumors in mice (14).

Differential transcript expression and nonsynonymous nucleotide polymorphism. We conducted expression analysis using cDNA samples prepared from age-matched A/J and M. spreitus lung tissues. Among the 32 genes we investigated, majority of the genes exhibited comparable transcript expression between the two mouse strains, including the bona fide tumor suppressor Tob1 (Fig. 2A). There were three genes (i.e., Itga3, Spop, and Myst2), however, seeming to be more abundant in A/J lungs than in

### Table 1. Genes in Par1 candidate region

<table>
<thead>
<tr>
<th>Start (bp)</th>
<th>End (bp)</th>
<th>Gene ID</th>
<th>Description</th>
<th>Expression in mouse lung?</th>
</tr>
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<tbody>
<tr>
<td>9402854</td>
<td>94031585</td>
<td>Tob1</td>
<td>Transducer of ErbB-2.1</td>
<td>Y</td>
</tr>
<tr>
<td>94053688</td>
<td>94058639</td>
<td>Wfikjn2</td>
<td>WAP, follistatin/kazal, immunoglobulin, Kunitz and netrin domain containing 2</td>
<td>N</td>
</tr>
<tr>
<td>94107227</td>
<td>94138001</td>
<td>3300001P08Rik</td>
<td>Cisplatin resistance-associated overexpressed protein</td>
<td>Y</td>
</tr>
<tr>
<td>9414239</td>
<td>94156011</td>
<td>Ankrd40</td>
<td>Ankyrin repeat domain 40</td>
<td>Y</td>
</tr>
<tr>
<td>9419385</td>
<td>94209066</td>
<td>Abcc3</td>
<td>ATP-binding cassette, subfamily C (CFTR/MRP), member 3</td>
<td>N</td>
</tr>
<tr>
<td>9422461</td>
<td>94289707</td>
<td>Cacna1g</td>
<td>Calcium channel, voltage-dependent, T type, αG subunit</td>
<td>Y</td>
</tr>
<tr>
<td>94295023</td>
<td>94295226</td>
<td>Spata20</td>
<td>Spermatogenesis associated 20</td>
<td>N</td>
</tr>
<tr>
<td>94307961</td>
<td>94316013</td>
<td>Epsn3</td>
<td>Epsin 3</td>
<td>Y</td>
</tr>
<tr>
<td>94317438</td>
<td>94337353</td>
<td>Mycbpap</td>
<td>Mycbp-associated protein</td>
<td>Y</td>
</tr>
<tr>
<td>94355888</td>
<td>94365299</td>
<td>Rsad1</td>
<td>Radical S-adenosyl methionine domain containing 1</td>
<td>Y</td>
</tr>
<tr>
<td>94381157</td>
<td>94385217</td>
<td>Mycbpap</td>
<td>Myc-associated protein</td>
<td>Y</td>
</tr>
</tbody>
</table>

Abbreviations: TNFR, tumor necrosis factor receptor; Y, yes; N, no; ND, not determined.
M. spretus lungs (Fig. 2A). In contrast, the Phospho1 gene transcript was more abundant in M. spretus mouse lung than in the A/J lung. The Tob1 and Phb genes do not show differential expression in mouse lung tissues. The β-actin gene was used as a control to assure equal amount of cDNA used in the reactions. DNA ladder (100 bp) was loaded before sample lanes.

On the other hand, SNP was frequently detected for the investigated genes (data not shown), which can be attributed to the far phylogenetic distance between M. spretus and A/J mouse strains. We only focused on nonsynonymous SNPs because they are the ones likely to cause alteration of protein function. Five genes (Tob1, Xylt2, Itga3, Ngfr, and Phb) were identified with nonsynonymous SNPs (Table 2). Despite encoding the same number of amino acids, the A/J and M. spretus Tob1 genes show three nonsynonymous SNPs. All of these nonsynonymous polymorphisms have been identified at its COOH terminus, making Tob1 alleles of A/J and M. spretus distinct from each another (Fig. 2B). At codon 246, the A/J Tob1 encode a proline (CCA) compared with a glutamine (CAG) in the M. spretus. In addition, codons 270 and 271 encode Pro-Gln (CCA-CAG) in the A/J Tob1, which is different from Gln-Pro (CAG-CCA) in the M. spretus Tob1. Furthermore, codon 301 encodes a glycine (GGT) in A/J but an aspartic acid (GAT) in M. spretus. The Xylt2 gene has two nonsynonymous SNPs identified at codons 370 and 544. At codon 370, the A/J allele encodes an arginine (CGT), whereas M. spretus encodes a leucine (CTT). At codon 544, the A/J allele encodes a threonine (ACG), whereas M. spretus encodes a serine (AGC).

Table 2. Nonsynonymous nucleotide polymorphisms

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description (Genbank accession no.)</th>
<th>Codon position</th>
<th>A/J</th>
<th>M. spretus</th>
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<tbody>
<tr>
<td>Tob1</td>
<td>Transducer of Erbb2 (NM_009427)</td>
<td>246</td>
<td>CCA (Pro)</td>
<td>CAG (Gln)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>270-271</td>
<td>CCA (Pro)-CAG (Gln)</td>
<td>CAG (Gln)-CCA (Pro)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>301</td>
<td>GGT (Gly)</td>
<td>Gat (Asp)</td>
</tr>
<tr>
<td>Xylt2</td>
<td>Xylotransferase II (NM_145828)</td>
<td>370</td>
<td>CCA (Pro)</td>
<td>CAG (Gln)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>544</td>
<td>GGT (Gly)</td>
<td>CTT (Leu)</td>
</tr>
<tr>
<td>Itga3</td>
<td>Integrin α3 (NM_013565)</td>
<td>511</td>
<td>CAG (Gln)</td>
<td>CGG (Arg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>537</td>
<td>TGG (Leu)</td>
<td>TCA (Ser)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>885</td>
<td>CAG (Gln)</td>
<td>CAT (His)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>895</td>
<td>GTG (Val)</td>
<td>GCC (Ala)</td>
</tr>
<tr>
<td>Ngfr</td>
<td>p75 neurotrophin receptor (NM_033217)</td>
<td>Exon 1</td>
<td>7-Leu chain</td>
<td>6-Leu chain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49</td>
<td>CCA (Pro)</td>
<td>CTT (Leu)</td>
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<td></td>
<td></td>
<td>217</td>
<td>ATC (Ile)</td>
<td>GAT (Val)</td>
</tr>
<tr>
<td>Phb</td>
<td>Prohibitin (AF105292)</td>
<td>66</td>
<td>ATC (Ile)</td>
<td>CTC (Leu)</td>
</tr>
</tbody>
</table>
Allele encodes a leucine (CTT). At codon 544, the A/J allele encodes a threonine (ACC), whereas the M. spretus allele encodes a serine (AGC; Table 2). The Itga3 gene has four nonsynonymous SNPs identified (Table 2). Codon 511 (CAG/CGG: Gln/Arg), codon 537 (TTG/TCA: Leu/Ser), and codon 895 (GTC/GCC: Val/Ala) polymorphisms were identified between A/J and A/J (Table 2). In addition, at codon 885, a glutamine (CAG) is encoded by A/J, whereas a histidine (CAT) is encoded by M. spretus. The trinucleotide CTG polymorphism in the Ngfr gene is located in exon 1 and leads to a 6-leucine chain in the Ngfr proteins of M. spretus but a 7-leucine chain in A/J. At codon 49, the A/J Ngfr encodes a proline (CCT), whereas M. spretus Ngfr encodes a leucine (CTT). The codon 217 encodes an isoleucine (ATA) in A/J but a valine (GTA) in M. spretus (Table 2). Phb has only one nonsynonymous SNP identified at codon 66, with an isoleucine (ATC) in A/J and a leucine (CTC) in M. spretus (Table 2).

**Evaluation of Tob1 tumor suppressor as Par1 candidate.** In the present study, Tob1 was evaluated in particular because of its established role as a tumor suppressor and the multiple nonsynonymous SNPs identified between A/J and M. spretus. It was shown previously that exogenously expressed human Tob1 was able to suppress growth of NIH3T3 cells, and the Tob1 growth suppression pathway is negatively regulated by the EBRB2 gene product p185 (16). Researchers observed multiple types of tumors in Tob1-deficient mice, including lung tumors (14). We wanted to know if the identified nucleotide variations would cause any change in Tob1 antitumor function. We respectively cloned A/J and M. spretus Tob1 ORFs and did functional tests using in vitro cell culture systems. Normal but immortalized NIH3T3 cell line was used in colony formation and cell growth curve assays (Fig. 3A and B). The results from these two assays suggest that the M. spretus Tob1 allele exerts stronger growth-suppressing effects than the A/J counterpart. Expression levels of the two Tob1 transfectants were confirmed to be comparable (data not shown). These results suggest that the nonsynonymous SNPs identified between the A/J and M. spretus Tob1 genes might indeed lead to some functional differences.

Because antitumor function of Tob1 is largely regulated by protein phosphorylation (17), we wondered if the three mouse Tob1 isoforms were different in their abilities to get phosphorylated by cellular kinases, which might explain the differential cell growth-suppression function observed in the cell culture studies. Indeed, one recent study showed that alteration of phosphorylation status of Tob1 is likely to contribute to early human lung tumorigenesis (18). More interestingly, in the present study, we observed positive immunohistochemical staining of phosphorylated Tob1 protein in mouse lung adenomas but not in normal mouse lung tissues (Fig. 4A and B), suggesting that phosphorylation of Tob1 might play an important role in mouse lung adenoma development. However, transient transfection of V5-tagged Tob1 into NIH3T3 cells did not show significant difference in phosphorylation levels of ectopically expressed Tob1 proteins (Fig. 3C). The result is consistent with the fact that the major phosphorylation sites of Tob1 protein are located at the NH2 terminus and remain identical for the three mouse strains (17). Therefore, the underlying mechanism for the observed functional difference between Tob1 isoforms remains to be identified.

**Discussion**

In our previous work, we conducted positional cloning for the major mouse lung adenoma susceptibility QTL Pas1 (19). Par1 has
been shown to function as a major modifier of *Pas1* to reduce mouse lung adenoma susceptibility (11, 12). Conventional linkage analyses previously mapped the *Par1* QTL into a rather large and undefined candidate region on mouse chromosome 11 (11, 12). To identify the underlying gene, refinement of the *Par1* candidate region is a prerequisite step. In the present study, we fine mapped the *Par1* QTL using a series of congenic and subcongenic mice generated from the *Par1/+* strain *M. spretus* and *Par1/−* strain A/J. Analyzing these mice confirmed that the *Par1* is a major mouse lung adenoma resistance QTL. Most importantly, our analyses have in the first time generated a defined small *Par1* candidate region, which is ~2.2 Mb in physical length and is defined by the *Hoxb9* gene and *D11Mit70*.

There are 49 genes located in the refined *Par1* candidate region and involved in a broad range of cellular activities. Thirty-two genes have shown different extents of expression in mouse lungs (Table 1). Semiquantitative RT-PCR assays revealed three genes (*Itga3*, *Spop*, and *Myst2*) more abundantly expressed in A/J versus *M. spretus* lung tissues. A decreased transcription level was observed in A/J versus *M. spretus* for the *Phospho1* gene. Nonsynonymous SNPs were identified in five genes (*Tob1*, *Xylt2*, *Itga3*, *Ngfr*, and *Phb*; Table 2). The human *SPOP* gene encodes a 374–amino acid protein with a relative molecular mass of 47,000 (20). Its amino acid sequence contains a COOH-terminal BTB/POZ domain and an NH2-terminal MATH domain (20–22). It has been shown that the TRAF homology domain in SPOP protein only interacted weakly with TRAF1 and TRAF6 and did not inhibit nuclear factor-κB (NF-κB) induction as other TRAF domains do (21), and the MATH domain has been shown to bind to the X chromosome inactivation protein macroH2A1.2 (22). The Myst2 gene (also named *Hbo1*) is a histone acetyltransferase, which interacted with the origin recognition complex 1 protein and the minichromosome maintenance protein, suggesting a role for it in control of both DNA replication and gene expression (23, 24). Another study also showed that the Myst2 protein interacted with androgen receptor (AR) and might regulate AR-dependent genes in normal and prostate cancer cells (25). The *Phospho1* gene was predicted as a putative phosphatase, but thus far, no study has been done on its function. *Pdk2* presumably inactivates pyruvate dehydrogenase through phosphorylation, therefore regulating oxidative decarboxylation of pyruvate and homeostasis of carbohydrate fuels in mammals (26). The biological function of *Xylt2* is not known. It is highly homologous to *Xylt1*, which catalyzes the transfer of UDP-xylose to serine residues within XT recognition sequences of target proteins (27). Addition of this xylose to the core protein is required for the biosynthesis of the glycosaminoglycan chains characteristic of proteoglycans. The human *PHB* is a 30-kDa intracellular, antiproliferative protein and has shown association with breast cancer susceptibility (15).

The human *NGFR* gene encodes a 75-kDa glycoprotein cell surface receptor (p75NTR), which binds with low affinity to nerve growth factor and other neurotrophins (28). The p75NTR protein shares sequence identity with other members in a receptor superfamily (such as tumor necrosis factor receptors p75TNFR, p55TNFR, and FAS) and is involved in NF-κB and apoptosis pathways. It should be noted that although NGFR is expressed in many organs outside the nervous system, recent immunohistochemistry staining studies in human lung have detected the p75NTR protein only in lung ganglionic neuron and artery smooth muscle but not in any type of pneumocytes (29). Indeed, using highly sensitive RT-PCR technology, we only detected weak

![Image of Immunohistochemistry staining of phosphorylated Tob1 protein.](https://example.com/image.png)
transcript expression (data not shown), which is likely derived from these nonpulmonary tissues. On the other hand, both differential lung expression and nonsynonymous nucleotide polymorphisms were found for the Itga3 gene. The human ITGA3 gene encodes two isoforms of the integrin α3 subunit: α3A (1,051 amino acids) and α3B (1,066 amino acids). The mouse Itga3 gene encodes a 1,053–amino acid protein, which corresponds to the human α3A isoform. Previous studies have revealed that only the α3A isoform is expressed in lung tissue (30). Functionally, the integrin α3 subunit forms a heterodimeric cell surface integrin receptor with the integrin β3 subunit, linking the extracellular matrix to structural and functional components within the cell (31). The role of Itga3 in tumorigenesis seems to be controversial and may be dependent on tumor type and tumor stage. Some studies have revealed that reduced expression of Itga3 is associated with malignant transformation and Itga3 gene expression may promote apoptosis (32–34). Clinically, it has been associated with malignant transformation and poor prognosis of patients with lung adenocarcinomas and colon cancer (35, 36). Down-regulation of the Itga3 may also contribute to the enhanced tumorigenicity of c-myc–overexpressing small cell lung cancer cells (37).

In the present study, we were particularly interested in the Tob1 gene, a tumor suppressor whose loss of function is involved in tumorigenesis (14). We found that the A/J and M. spretus encode different isoforms of the Tob1 gene. As revealed by the colony formation and growth curve studies, stronger cell growth-suppressing effects were observed for M. spretus Tob1 than for the A/J Tob1. Previous studies have shown that the TOBI growth suppression pathway is negatively regulated by the kinase-active ERBB2 gene product p185 (16). Although the molecular mechanism by which TOBI suppresses cell growth remains to be further clarified, phosphorylation has been suggested to be a crucial mechanism to regulate Tob1 functions. Phosphorylation of three regulatory serines (Ser152, Ser154, and Ser164) of Tob1 by extracellular signal-regulated kinase (Erk) 1 and Erk2 is required for Ras-mediated cell proliferation and transformation (17). The connection of Tob1 gene with the Ras signaling pathway is of large interest because the K-ras gene has been identified as one of the Pas1 candidates in our recent study (19). In the present study, we indeed observed a significantly elevated phosphorylation of Erk protein in A/J lung tissue compared with M. spretus.3 Because K-ras is one of critical upstream regulators of Erk activity, elevated phosphorylation of Erk may reflect a higher K-ras activity in A/J (Pas1/+ ) than in M. spretus (Pas1/–), as we have seen for A/J and C57BL/6 (Pas1/–) mice in the previous study (19). However, because we could not detect differential phosphorylation of ectopically expressed Tob1 protein, connection of Tob1 phosphorylation in A/J and M. spretus lungs with their differential Erk activities remains to be a question. Our identified amino acid–changing nucleotide polymorphisms are all located in the COOH-terminal region. It has been suggested that the COOH-terminal region of TOB1 controls protein stability (38). Furthermore, the identified nucleotide polymorphisms may also be able to affect intracellular distribution of Tob1 proteins, which was suggested to be important for the tumor-suppressing function of the gene (39). Clearly, more in vitro and in vivo studies will help to confirm Tob1 gene candidacy, although indisputable evidence for claiming Tob1 as the Par1 gene may not be available until Tob1 knockin mice are generated.

Human chromosomal region 17q21 shares a high degree of homology with the mouse Par1 region. Loss of heterozygosity (LOH) in the human 17q21 region has been observed in multiple types of human cancers, including lung cancer (Fig. 5; refs. 40–46). Most LOHs in cancers of organs other than lung were observed in the region near BRCA1 locus (38.4 Mb, NCBI human genome map, Build 36.1). However, in lung cancer, the “hottest” LOH spot seems not to be at BRCA1. It was previously found in Japanese lung cancer patients that the highest LOH frequency was found at D17S588 (45.6 Mb; ref. 46). In our recent LOH study in Caucasian lung cancer patients, we observed highest LOH frequency at D17S1869 (45.9 Mb; ref. 38).3 Interestingly, this marker is located at 45.9-Mb (46.3 Mb), suggesting that TOB1 gene may function as a tumor suppressor in human lung cancer development.

It is noteworthy that in the present study, we only studied gene expression and SNPs between A/J and M. spretus. The reason we did not test SM/J mice is because we could not see significant phenotype difference in congenic mice generated.

3 Unpublished data.
between A/J and SM/J strains. Therefore, it is likely that the previously identified Par1 QTL in the SMAx24 RI strain might be derived from some fixed mutations emerging in the long term of breeding process. On the other hand, although the Par1 QTL were identified in both M. spretus and SM/J at similar chromosomal positions through linkage analyses (11, 12), it may not necessarily represent the same gene. Because of the far phylogenetic distance between M. spretus and other inbred mouse strains, it is perceivable that a unique Par1 gene may function in M. spretus. In future, genetic analysis on mouse populations generated from M. spretus and SM/J may provide us a conclusive answer as to whether these two strains share the same Par1 gene.

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References

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