Generation and Characterization of a *CYP2A13/2B6/2F1*-Transgenic Mouse Model

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ABSTRACT:

CYP2A13, *CYP2B6*, and *CYP2F1*, which are encoded by neighboring cytochrome P450 genes on human chromosome 19, are active in the metabolic activation of many drugs, respiratory toxicants, and chemical carcinogens. To facilitate studies on the regulation and function of these human genes, we have generated a *CYP2A13/2B6/2F1*-transgenic (TG) mouse model (all *1 alleles). Homozygous transgenic mice are normal with respect to gross morphological features, development, and fertility. The tissue distribution of transgenic mRNA expression agreed well with the known respiratory tract-selective expression of *CYP2A13* and *CYP2F1* and hepatic expression of *CYP2B6* in humans. *CYP2A13* protein was detected through immunoblot analyses in the nasal mucosa (NM) (~100 pmol/mg of microsomal protein; similar to the

level of mouse *CYP2A5*) and the lung (~0.2 pmol/mg of microsomal protein) but not in the liver of the TG mice. *CYP2F1* protein, which could not be separated from mouse *CYP2F2* in immunoblot analyses, was readily detected in the NM and lung but not the liver of TG/*Cyp2f2*-null mice, at levels 10- and 40-fold, respectively, lower than that of mouse *CYP2F2* in the TG mice. *CYP2B6* protein was detected in the liver (~0.2 pmol/mg of microsomal protein) but not the NM or lung (with a detection limit of 0.04 pmol/mg of microsomal protein) of the TG mice. At least one transgenic protein (*CYP2A13*) seems to be active, because the NM of the TG mice had greater in vitro and in vivo activities in bioactivation of a *CYP2A13* substrate, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (a lung carcinogen), than did the NM of wild-type mice.

Introduction

The human *CYP2ABFGST* gene cluster on chromosome 19 contains several functional *CYP* genes, which encode five cytochrome P450 (P450) enzymes (*CYP2A6*, *CYP2A13*, *CYP2B6*, *CYP2F1*, and *CYP2S1*), as well as several *CYP* pseudogenes (Wang et al., 2003). The five *CYP* genes are all expressed in the respiratory tract, but their contributions to xenobiotic metabolism and target tissue bioactivation remain poorly defined. To study the in vivo function and regulation of these P450 enzymes, we have been generating transgenic mice that express the cognate human *CYP* genes. We previously reported the generation and characterization of *CYP2A6*-transgenic mice (Zhang et al., 2005a). In the present study, we prepared *CYP2A13/2B6/2F1*-transgenic (TG) mice, mainly for study of the functions of *CYP2A13*. *CYP2A13* is located ~70 kbp downstream of *CYP2B6* and immedi-

ately upstream of *CYP2F1*; all three genes are arranged in the same direction (Fig. 1A). To preserve regulatory sequences potentially important for the expression of *CYP2A13*, we selected a human genomic DNA clone encompassing all three *CYP* genes for transgenic mouse production.

CYP2A13, which is expressed preferentially in the respiratory tract, is the most efficient P450 enzyme in the in vitro metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Su et al., 2000; Jalas et al., 2005), a tobacco-specific nitrosamine and potent lung carcinogen (Hecht, 2003). *CYP2A13* is also active toward many other toxicants and carcinogens, including aflatoxin B₁ (He et al., 2006), 4-aminobiphenyl (Nakajima et al., 2006), naphthalene, styrene, and toluene (Fukami et al., 2008), and 3-methylindole (D'Agostino et al., 2009). *CYP2A13* was hypothesized to play an important role in NNK-induced lung tumorigenesis (Ding and Kaminsky, 2003). *CYP2A13*-transgenic mice would be valuable not only for directly testing the ability of this human enzyme to mediate chemical carcinogenesis but also for assessing the in vivo efficacy of chemopreventive agents that target *CYP2A13*.

CYP2B6 is expressed primarily in the liver, where it contributes 2 to 10% of the total P450 content (Wang and Tompkins, 2008). Human *CYP2B6* has also been detected, at much lower levels, in several extrahepatic tissues, including brain, kidney, intestine, lung, trachea,

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ABBREVIATIONS: P450, cytochrome P450; BAC, bacterial artificial chromosome; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; OPB, 4-oxo-4-(3-pyridyl)butanal; O⁶-mG, O⁶-methylguanine; NM, nasal mucosa; LCM, laser-capture microdissection; bp, base pair; kbp, kilo-base pair; WT, wild-type; TG, *CYP2A13/2B6/2F1*-transgenic; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

FIG. 1. Structure of the transgene and Southern blot analysis of transgenic mice. A, structure of the transgene fragment (modified from Wang et al., 2003). The ~210-kbp transgene fragment included full-length *CYP2A13*, *CYP2B6*, and *CYP2F1* genes, as well as three *CYP2* pseudogenes. B, strategy for Southern blot analysis. An 864-bp *CYP2A13* DNA probe (2A13 probe) (open box) was used. Genomic DNA was digested with HindIII. The size of the expected fragment from the *CYP2A13* transgene was 5.1 kbp. C, Southern blot analysis. Increasing amounts (0.1–5 µg) of genomic DNA from a homozygous TG mouse were analyzed; genomic DNA from a WT C57BL/6 mouse (10 µg) was used as a negative control sample, whereas human DNA (10 µg) was used as a positive control sample. The approximate sizes of the detected HindIII fragments are indicated.

nasal mucosa (NM), and skin (Hukkanen et al., 2002; Ding and Kaminsky, 2003; Wang and Tompkins, 2008). *CYP2B6* metabolizes a large number of substrates, including clinically used therapeutic agents, recreational drugs, endogenous chemicals, pesticides, and environmental chemicals (Wang and Tompkins, 2008).

CYP2F1, which is the least well characterized of the three human P450s, is expressed primarily in the respiratory tract (Carlson, 2008; Zhang and Ding, 2008; Weems et al., 2010). Little *CYP2F1* mRNA expression was detected in other tissues (Carr et al., 2003). Studies using mammalian cells containing low levels of heterologously expressed *CYP2F1* suggested that *CYP2F1* is active toward several pulmonary toxicants, including naphthalene, styrene, 3-methylindole, and benzene (Nakajima et al., 1994; Lanza et al., 1999; Powley and Carlson, 2000); however, heterologous expression of *CYP2F1* in nonmammalian systems yielded nonfunctional P450 proteins (e.g., Baldwin et al., 2005). Therefore, *CYP2F1*-transgenic mice would be useful for studying the function of *CYP2F1* in chemically induced lung toxicity.

TG mice were characterized generally with respect to viability, growth, and fertility and then were examined thoroughly regarding the tissue distribution of transgene expression, both at the mRNA level and at the protein level. Our results show that *CYP2A13* and *CYP2F1*, but not *CYP2B6*, are expressed in the lung and NM of the TG mice, whereas *CYP2B6* is expressed in the liver of the TG mice, albeit at low levels. Furthermore, metabolic studies were conducted and demonstrated that the transgenic *CYP2A13* is capable of bioactivating

NNK in vitro and in vivo, in the mouse NM. The value and limitations of this unique TG mouse model for study of the in vivo functions of the three human P450s are discussed.

Materials and Methods

Generation of TG Mice. A human bacterial artificial chromosome (BAC) clone (CTD-2535H15) containing *CYP2A13*, *CYP2B6*, and *CYP2F1* genes was obtained from Invitrogen (Carlsbad, CA). The three P450 genes in that BAC clone have all been confirmed, through sequence analysis, to be the *1 allele (<http://www.cypalleles.ki.se>). The ~210-kbp BAC DNA insert (Fig. 1A) was linearized with NotI, which removes the vector, and was isolated after pulsed-field gel electrophoresis and β -agarase digestion, according to a published method (Abe et al., 2004). Transgenic mice were produced at the Transgenic and Knockout Core Facility at the Wadsworth Center (Albany, NY), according to standard procedures (Nagy et al., 2003). Purified BAC insert was microinjected into the pronuclei of fertilized eggs from the C57BL/6J strain. The eggs either were transferred the same day or were cultured to the two-cell stage and then transferred into the oviducts of pseudopregnant B6CBAF1/J mice and were allowed to develop to term. Positive transgenic mice were identified through PCR analysis of tail DNA, with use of the following *CYP2A13*-specific PCR primers: 5'-cctggacagatgcctttaactcgc-3' (forward, starting at position +3144 of *CYP2A13*) and 5'-tgctttgacacgtcctgcact-3' (reverse, starting at position +3475) (Zhang et al., 2002). The 332-bp PCR product encompasses *CYP2A13* exon 5.

Heterozygous (+/–) TG mice were intercrossed to yield homozygotes (+/+). TG mice were also crossbred with *Cyp2f2*-null mice (Li et al., 2011); the resultant TG(+/-)/*Cyp2f2*(-/-) mice were used for detection of *CYP2F1* protein without interference from mouse *CYP2F2*. All studies with mice were approved by the Wadsworth Center Institutional Animal Care and Use Committee.

Southern Blot Analysis. Mouse genomic DNA was isolated from frozen thymus samples, whereas human genomic DNA was isolated from frozen lung tissues from an 18-year-old, black, male donor (Ling et al., 2007). HindIII-digested genomic DNA was fractionated through electrophoresis on 0.7% agarose gels, transferred to nylon membranes, and analyzed by using a ³²P-labeled, 864-bp, DNA probe corresponding to *CYP2A13* exon 2 (positions +593 to +1456). The transgene copy number was estimated through densitometric analysis of the 5.1-kbp *CYP2A13*-specific band detected. The 1 kb Plus DNA ladder (Invitrogen) was used for size determination.

Quantitative RNA-PCR Analysis. Total RNA was isolated by using a RNeasy Mini kit (QIAGEN, Valencia, CA). RNA samples were treated with DNase I (Invitrogen) before reverse transcription. Real-time RNA-PCR analysis was performed essentially as described previously (Zhang et al., 2007), with primers specific for *CYP2A13* (2A13F and 2A13R) (Zhang et al., 2004), *CYP2F1* (*CYP2F1F1* and *CYP2F1R1*) (Zhang et al., 2005b), and *CYP2B6* (5'-cattcttcgaggatagtg-3' and 5'-cctcatagtgacagagaatcg-3') (Rencurel et al., 2005). Serial dilutions of one reverse transcription product were used to generate a standard curve. A no-template control sample was used in each reaction. The levels of mouse GAPDH mRNA were also determined, as an internal standard (with primers 5'-tgtgaacgatttgccgcta-3' and 5'-tcgctcctggagatgggta-3'; product size, 120 bp).

Laser-Capture Microdissection of Lung Bronchiolar Epithelial Cells. Lung tissue samples were prepared from 2-month-old male TG mice. Fresh tissues were mounted in freezing medium and rapidly chilled on dry ice. Frozen sections (10-µm thick) were prepared with Superfrost/Plus slides (Erie Scientific Co., Portsmouth, NH). Slides were immediately placed in iced 70% ethanol for 10 min, treated with a standard hematoxylin/eosin staining protocol, and then air-dried for ~10 min before they were used for laser-capture microdissection (LCM); the LCM procedure (Pixcell Ite; Arcturus, Mountain View, CA) was completed within 4 h after slide preparation. Bronchiolar epithelial cells (from distal airways) were isolated with Arcturus Capsure caps (Molecular Devices, Sunnyvale, CA). Immediately after LCM, isolated cells were transferred into 0.5-ml LoBind microcentrifuge tubes (Eppendorf North America, New York, NY), which were preloaded with 350 µl of a lysis buffer (QIAGEN). For each lung, ~10,000 cells were captured for RNA preparation.

Immunoblot Analysis. Microsomal proteins were prepared from various tissues of 2-month-old mice, as described previously (Ding and Coon, 1990).

A monoclonal antibody against human CYP2A6 (A106; BD Gentest, Woburn, MA) was used for detection of CYP2A13 in microsomes prepared from mouse NM. For detection of CYP2A13 protein in lung microsomes, a rabbit anti-CYP2A5 polyclonal antibody (Gu et al., 1998) was used, and the CYP2A5 and CYP2A13 bands were separated with a high-resolution gel electrophoresis system, as described previously (Wong et al., 2005). Heterologously expressed CYP2A13 in a Sf9 cell microsomal preparation (Su et al., 2000) and purified recombinant CYP2A5 (Gu et al., 1998) were used as standards for immunoblot quantification.

A CYP2B6-specific monoclonal antibody (BD Gentest) was used for detection of CYP2B6 protein. Recombinant CYP2B6 in a Sf9 cell microsomal preparation (BD Gentest) was used as a standard for immunoblot quantification. A rabbit anti-peptide antibody to CYP2F1/2, which was used previously for characterization of *Cyp2f2*-null mice (Li et al., 2011), was used for detection of CYP2F1 protein in microsomes from TG(+/-)/*Cyp2f2*(-/-) mice. The sequence of the antigenic peptide [NH₂-(C)TPQEFNPEHFLD-COOH, corresponding to amino acids 404–415 of CYP2F1; the first cysteine was added for conjugation] is shared by CYP2F1 and CYP2F2. The antibody did not cross-react with recombinant CYP1A1, CYP2A6, CYP2C9, CYP2D6, CYP2E1, CYP2S1, or CYP3A4 on immunoblots (data not shown). Heterologously expressed human CYP2F1 protein contained in a Sf9 cell microsomal preparation was used as a positive control sample for immunoblot analysis. Baculoviral expression of the CYP2F1 protein in Sf9 cells was achieved by using the Bac-to-Bac baculovirus expression system (Invitrogen) and a full-length CYP2F1 cDNA entry clone purchased from GeneCopoeia (Rockville, MD). The amount of CYP2F1 protein was not determined; the recombinant CYP2F1 protein did not produce the typical reduced CO-difference P450 spectrum (Omura and Sato, 1964). For immunoblot analysis of CYP2B6 and CYP2F1, NuPAGE Bis-Tris mini-gels (10%; Invitrogen) were used. Calnexin, a marker protein for the endoplasmic reticulum, was detected by using a rabbit anti-human calnexin antibody (GenScript USA, Piscataway, NJ) and was quantified as a loading control. Prestained protein markers (Precision-Plus, dual color; Bio-Rad, Hercules, CA) were used for size estimation.

Assay for NNK Metabolism. The *in vitro* assay of NNK metabolism was performed as described previously (Su et al., 2000), with the use of 10 μ M NNK (containing 1 μ Ci of [5-³H]NNK for NM microsomes or 5 μ Ci of [5-³H]NNK for lung microsomes), 5 mM sodium bisulfite, and 0.8 mg/ml (lung) or 0.1 mg/ml (NM) microsomal protein. The rates of formation of the ketoaldehyde [4-oxo-4-(3-pyridyl)butanal (OPB)] and the ketoalcohol [4-hydroxy-1-(3-pyridyl)-1-butanone (HPB)] were determined.

Determination of O⁶-Methylguanine in Mouse Genomic DNA. Two-month-old mice were treated with a single intraperitoneal injection of NNK (Chemsyn, Lenexa, KS) dissolved in saline solution at 100 mg/kg. Mice were killed 4 h later, and the NM and lungs were obtained for isolation of genomic DNA. O⁶-mG levels were determined through liquid chromatography-mass spectrometry, as described previously (Weng et al., 2007). Female mice were studied because of their preferred use in NNK-induced lung tumorigenesis studies (e.g., Weng et al., 2007).

Results

Generation of the Transgenic Mouse Model. The structure of the 210-kbp BAC transgene insert is shown in Fig. 1A. We selected this clone on the basis of the criteria that the transgene contained the wild-type *CYP2A13**1A allele and that it retained sufficient amounts of 5'- and 3'-flanking sequences for insulation against potential integration-site effects on *CYP2A13* transgene expression. Additional genotyping and sequencing analyses indicated that the *CYP2B6* and *CYP2F1* genes were of the respective *1 alleles and the overall construct contained ~53 kbp at the 5'-end of *CYP2B6* and ~15 kbp at the 3'-end of *CYP2F1* (Fig. 1A).

We identified two transgenic founder lines (lines 349 and 864) through PCR-based detection of a 332-bp region containing the *CYP2A13* exon 5 (data not shown). Breeding records suggested that the transgene in line 864, but not that in line 349, was located on the X chromosome, a location that renders impossible the production of male and female offspring with equal transgene copy numbers. There-

fore, homozygous pups from line 349 were used for further studies. The transgenic mice are normal in terms of gross morphological features, development, and fertility (data not shown).

We estimated the transgene copy number by using Southern blot analysis, with a DNA probe complementary to *CYP2A13* exon 2 and intron 2 (Fig. 1B). As shown in Fig. 1C, the predominant band detected in DNA samples from transgenic mice and in a human lung DNA sample (positive control sample) was ~5 kbp, a size consistent with that expected (5.1 kbp) for the positive HindIII fragment (Fig. 1B). The intensity of the ~5-kbp band detected in 2 μ g of TG(+/-) genomic DNA was approximately the same as that detected in 10 μ g of the human genomic DNA. This result indicated that the transgene copy number was ~5, given the approximately same sizes of the human and mouse genomes. No bands were detected in 10 μ g of genomic DNA from a WT mouse, a result that confirmed the specificity of the DNA probe used. A second band (~8 kbp in size), weaker than the 5-kbp band, was detected in human but not TG mouse DNA samples; this band corresponds to HindIII fragments from human *CYP2A6* (8.1 kbp) and *CYP2A7* (8.2 kbp). The latter two fragments, which would not be resolved under the experimental conditions used, both contain a region that is 91% identical to the probe sequence (<http://www.ncbi.nlm.nih.gov/blast>).

An ~6-kbp band, which was ~5 times weaker than the 5-kbp band, was detected in DNA samples from TG mice but not in human lung. This band most likely represents a copy of the transgene that was truncated at a position downstream of the HindIII site in the *CYP2A13* 5'-flanking region (Fig. 1B) and was inserted downstream of a HindIII site in the mouse genome. An alternative explanation, namely, that this band corresponds to a HindIII fragment (also ~6.0 kbp) from *CYP2A18P-N* (included in the transgene construct), which contains a region that is 84% identical to the probe sequence (<http://www.ncbi.nlm.nih.gov/blast>), seems less plausible, given that the same 6-kbp band was not detected in the human lung DNA sample.

CYP2A13 Expression. CYP2A13 mRNA was detected, through RNA-PCR, in several mouse tissues, including NM, lung, liver, brain, and testis, but essentially not in small intestine, bladder, heart, or kidney; the expression level in the NM was the highest, followed by that in the lung (Fig. 2A). CYP2A13 protein can be detected with several anti-CYP2A antibodies, which vary in their reactivities with mouse CYP2A proteins. A monoclonal anti-CYP2A6 antibody (BD Gentest) was found to have much greater reactivity with CYP2A13 than with mouse CYP2A5 in pilot studies, and this antibody was used to detect CYP2A13 protein expression in the NM of TG mice. As shown in Fig. 2B, CYP2A13 protein was abundantly expressed in the NM. A densitometric analysis of the data shown in Fig. 2B (and similar data from additional experiments that are not presented) suggested that the content of CYP2A13 protein in mouse NM microsomes was ~100 pmol/mg protein, a level similar to that of CYP2A5 protein in mouse NM microsomes determined in a previous study (Gu et al., 1998) and much higher than the levels detected in human fetal or adult NM microsomes (<5 pmol/mg protein) (Chen et al., 2003; Wong et al., 2005).

We could not detect CYP2A13 protein in lung microsomes from TG mice when we used the monoclonal anti-CYP2A6 antibody (data not shown). However, CYP2A13 protein was detected in the lung (Fig. 2C) when we used a polyclonal anti-CYP2A5 antibody (Gu et al., 1998), which had a much higher titer than the monoclonal antibody did. Notably, the relative band intensities for CYP2A13 and CYP2A5 in Fig. 2C, left, could not be used directly for determination of the relative levels of the two CYP2A proteins because, as shown in Fig. 2C, right, the antibody apparently had greater reactivity toward CYP2A5 than toward CYP2A13. A quantitative analysis indicated

FIG. 2. Expression of the *CYP2A13* transgene. A, relative *CYP2A13* mRNA expression levels in various tissues of TG mice. Total RNA was isolated from tissues of 2-month-old male mice. The values presented (mean $\pm$ S.D., $n = 4$, in arbitrary units, relative to the highest level detected) were normalized with respect to the levels of mouse GAPDH transcript determined for the same samples. SI, small intestine. B, immunoblot detection of *CYP2A13* protein in the NM. NM microsomal proteins from 2-month-old WT or homozygous TG mice (male; each sample was prepared from pooled tissues from four mice), as well as a recombinant *CYP2A13* standard, were analyzed by using an anti-CYP2A6 monoclonal antibody. The position of a 50-kDa, prestained, protein size marker is indicated. Typical results are shown. C, immunoblot detection of *CYP2A13* protein in the lung. Lung microsomal proteins from 2-month-old WT or homozygous TG mice (male; each sample was prepared from pooled tissues from four mice), as well as recombinant *CYP2A5* and *CYP2A13* standards in the indicated amounts, were analyzed by using a polyclonal anti-CYP2A5 antibody. Typical results are shown.

that the content of *CYP2A13* protein in mouse lung microsomes was ~ 200 fmol/mg protein, which was ~ 10 times higher than the highest levels detected previously in microsomes from human lung biopsy samples (Zhang et al., 2007) but still ~ 25 times lower than the *CYP2A5* protein level determined in lung microsomes from homozygous TG mice (~ 5 pmol/mg) (data not shown). In experiments not shown, we also observed that the levels of *CYP2A13* protein in the lung and NM microsomes from male TG mice were $\sim 30\%$ lower than the levels in corresponding tissues from female TG mice.

We also determined the cellular distribution of *CYP2A13* mRNA in the lungs of TG mice, through RNA-PCR analysis of LCM-isolated lung bronchiolar epithelial cells. We estimated that *CYP2A13* mRNA levels (normalized to GAPDH levels) were ~ 12 -fold higher in bronchiolar epithelial cells than in the intact lung ($n = 4$) (data not shown). This result agreed well with the findings of a previous immunohisto-

chemical study of *CYP2A13* expression in human lung, where the strongest *CYP2A13* immunoreactivity was detected in bronchiolar epithelial cells (Zhu et al., 2006).

CYP2A13 protein was not detected in liver microsomes from either male or female TG mice, with a detection limit of ~ 100 fmol/mg microsomal protein (data not shown). The very low (if any) hepatic expression of *CYP2A13* protein in TG mice is concordant with the essential lack of *CYP2A13* mRNA expression in human liver (Su et al., 2000).

CYP2F1 Expression. *CYP2F1* mRNA was detected in transgenic mice at the highest levels in the NM and lung, was barely detectable in the testis, and was not detected in the other tissues examined (Fig. 3A). Efforts to resolve human *CYP2F1* and mouse *CYP2F2* proteins by using the same high-resolution gel electrophoresis system that was effective for separation of *CYP2A13* and *CYP2A5* were unsuccessful, and none of the available anti-CYP2F antibodies could distinguish between the two *CYP2F* proteins (data not shown). There-

FIG. 3. Expression of the *CYP2F1* transgene. A, relative *CYP2F1* mRNA expression levels in various tissues of TG mice. Total RNA was isolated from tissues of 2-month-old male mice. The values shown (mean $\pm$ S.D., $n = 4$, in arbitrary units, relative to the highest level detected) were normalized with respect to the levels of mouse GAPDH transcript determined for the same samples. SI, small intestine. B, immunoblot detection of *CYP2F1* and *CYP2F2* proteins. Lung, liver, and NM microsomal proteins from 2-month-old, female, WT, *Cyp2f2*-null, and TG(+/-)/*Cyp2f2*-null mice (each sample was prepared from pooled tissues from four or five mice) were analyzed by using an anti-CYP2F1/2 antibody. A recombinant *CYP2F1* protein sample (in Sf9 cell microsomes) was used as a positive control sample. Typical results are shown.

FIG. 4. Expression of the *CYP2B6* transgene. A, relative *CYP2B6* mRNA expression levels in various tissues of TG mice. Total RNA was isolated from tissues of 2-month-old male mice. The values presented (mean $\pm$ S.D., $n = 4$, in arbitrary units, relative to the highest level detected) were normalized with respect to the levels of mouse GAPDH transcript determined for the same samples. SI, small intestine. B, immunoblot detection of *CYP2B6* protein. Microsomal proteins prepared from tissues pooled from three 2-month-old, male, homozygous TG mice, as well as a recombinant *CYP2B6* standard (in Sf9 cell microsomes), were analyzed by using an anti-*CYP2B6* antibody. Typical results are shown.

fore, to confirm that *CYP2F1* protein is produced in the TG mice, we intercrossed TG mice with *Cyp2f2*-null mice (Li et al., 2011) and analyzed microsomes from the TG(+/-)/*Cyp2f2*(-/-) offspring for *CYP2F1* protein expression. As shown in Fig. 3B, *CYP2F1* protein was readily detected in microsomes from lung and NM, but not liver, of the TG/2f2-null mice. The *CYP2F1* protein level was ~ 4 times greater in the NM than in the lung (densitometric results not shown). A recombinant *CYP2F1* protein preparation (with unknown *CYP2F1* content) was used as a positive control sample. Although quantitative analysis of the actual amount of *CYP2F1* protein detected was not possible, the relative band intensities for *CYP2F1* and *CYP2F2* could be used directly for determination of the relative levels of the two *CYP2F* proteins, given the common presence of the antigenic peptide sequence in the two proteins. Comparison of the density of the *CYP2F2* band detected in WT mice with the density of the *CYP2F1* band detected in corresponding tissues of TG/*Cyp2f2*-null mice indicated that the levels of *CYP2F1* protein were ~ 10 and ~ 40 times

lower than the levels of mouse *CYP2F2* in NM and lung, respectively. In experiments not shown, we observed that there was no noticeable difference in the levels of *CYP2F1* protein in lung or NM microsomes from male and female TG/*Cyp2f2*-null mice. *CYP2F1* protein was not detected in liver microsomes from male or female TG/*Cyp2f2*-null mice (Fig. 3B and data not shown).

CYP2B6 Expression. *CYP2B6* mRNA was detected in multiple tissues. The levels were highest in NM, liver, and kidney, intermediate in small intestine and testis, and barely detectable in lung and heart (Fig. 4A). *CYP2B6* protein was detected only in liver, at an estimated level of 200 fmol/mg protein (Fig. 4B); it was not detected in NM, lung, small intestine, kidney, brain, or testis, with a detection limit of ~ 40 fmol/mg protein. There was no sex difference in *CYP2B6* protein expression in the liver of TG mice (data not shown).

NNK Bioactivation in Lung and NM of TG Mice. Microsomes prepared from lung and NM of WT and TG(+/-) mice were analyzed for their in vitro metabolic activities toward NNK, a *CYP2A13* substrate. Rates of formation of two stable metabolites of NNK were analyzed. As shown in Table 1, the rates of formation of the ketoaldehyde (OPB), which represents the α -hydroxylation pathway that leads to *O*⁶-mG DNA adduct formation (Peterson et al., 1993; Hecht, 1998), were significantly increased in both lung and NM microsomes from TG mice, compared with WT mice. Rates of formation of the ketoalcohol (HPB) were also increased but to smaller extents. These data indicated that the transgenic *CYP2A13* and/or *CYP2F1* is active in the bioactivation of NNK in vitro. The greater increase in the rates of formation of OPB than in the rates of formation of HPB is consistent with the knowledge that *CYP2A13* primarily produces OPB, whereas *CYP2A5* preferentially produces HPB (Jalas et al., 2005).

In vivo formation of *O*⁶-mG also was examined in NNK-treated WT and TG mice. At a NNK dose commonly used for lung tumor bioassays (100 mg/kg) (e.g., Weng et al., 2007), NNK-induced *O*⁶-mG formation was significantly increased (by 40%) in the NM of the TG mice, compared with WT mice (Table 1). An apparent increase (10%) was also seen in the lung, but the difference was not statistically significant. These in vivo results confirm that transgenic *CYP2A13* and/or *CYP2F1* is active in the bioactivation of NNK. Given a previous report that *CYP2F1* heterologously expressed in a mammalian cell system was a poor catalyst in NNK metabolism (Smith et al., 1992), the increase in NNK bioactivation in TG mice, compared with WT mice, seemed to be mainly (if not solely) attributable to transgenic *CYP2A13*. We conclude that the transgenic *CYP2A13* is functional in NNK bioactivation, both in vitro and in vivo.

TABLE 1
NNK metabolic activation in the lung and NM of WT and TG mice

NNK metabolism in microsomal reactions and NNK-induced *O*⁶-mG formation in vivo were assayed as described under *Materials and Methods*. Rates of ketoaldehyde and ketoalcohol formation (mean $\pm$ S.D., $n = 3$) were determined with NNK at 10 μ M. Each microsomal sample (three per group) was prepared from pooled tissues of four 3-month-old male mice. For DNA adduct analysis, tissues were obtained 4 h after NNK treatment (100 mg/kg, i.p.). The *O*⁶-mG/guanine ratios (mean $\pm$ S.D., $n = 4$) were determined for 2-month-old female mice. Values in parentheses indicate relative increases for TG mice, compared with WT mice.

Tissue	Strain	Product Formation In Vitro		DNA Adduct Formation In Vivo
		Ketoaldehyde	Ketoalcohol	
<i>pmol · min⁻¹ · mg protein⁻¹</i>				<i>O⁶-mG/10⁶ guanine</i>
Nasal mucosa	WT	275 ± 53	388 ± 80	309 ± 10
	TG	597 ± 12 ^a (220%)	575 ± 46 ^b (150%)	439 ± 40 ^a (140%)
Lung	WT	2.9 ± 0.4	16.2 ± 2.2	11.4 ± 0.8
	TG	5.7 ± 0.4 ^a (200%)	19.8 ± 4.0 (120%)	13.0 ± 0.3 (110%)

^a $P < 0.01$, compared with WT mice (Student's *t* test).

^b $P < 0.05$, compared with WT mice (Student's *t* test).

Discussion

To accomplish our main goal of achieving adequate, human-like, CYP2A13 expression in a mouse model, we considered it necessary to include *CYP2B6* and *CYP2F1* (and the neighboring *CYP* pseudogenes) in the transgene construct, given their close proximity to *CYP2A13* and the potential for the presence of long-distance and/or shared regulatory elements (such as a locus control region) in a *CYP* gene cluster. For the same reason, the human *CYP1A1* and *CYP1A2* genes had to be coexpressed in a transgenic mouse model (Jiang et al., 2005).

The preferential expression of CYP2A13 and CYP2F1 transgene mRNAs in the respiratory tract is comparable to the respiratory tract-predominant expression reported previously for CYP2A13 (Su et al., 2000; Wong et al., 2005) and CYP2F1 (Carr et al., 2003) transcripts in humans. It was reported that CYP2A13 is expressed at appreciable levels in human bladders (Nakajima et al., 2006). However, CYP2A13 mRNA levels in the bladders of TG mice were barely detectable. This discrepancy could be attributable to species differences in the expression of relevant transcription factors in the bladder.

The expression level of transgenic CYP2A13 appeared to be higher than the CYP2A13 levels found in human NM and lung microsomes. This observation may be explained by the fact that the *CYP2A13* transgene existed in multiple copies and the quality of mouse tissues was undoubtedly better than that of human biopsy or autopsy samples. Expression of *CYP2A13* may be suppressed in lung tissues from patients, because of disease-related inflammation (H. Wu and X. Ding, unpublished observations).

It is interesting to note that, although mouse CYP2A5 and CYP2F2 are both expressed in the liver as well as in the respiratory tract, human CYP2A13 and CYP2F1 are essentially not expressed in either human liver or the liver of TG mice. This observation indicated that the regulatory sequences responsible for suppressing the expression of CYP2A13 and CYP2F1 in human liver and/or for activating the expression of CYP2A13 and CYP2F1 in the respiratory tract are contained within the transgene fragment.

With the exception of CYP2A13 expression in the NM, the levels of expression of the transgenic human P450s were much lower than the levels of their orthologous mouse P450s. This fact limits the usefulness of the TG mice for direct analysis of the functions of the transgenic human P450s, because the activities of the mouse P450s would likely mask the activities of the human P450s. Even for CYP2A13 in the NM, the presence of the abundant and highly active mouse CYP2A5 (Su and Ding, 2004) could be problematic; the K_m values of recombinant CYP2A5 and CYP2A13 enzymes for OBP formation are similar ($4.3 \pm 0.8 \mu\text{M}$ for mouse CYP2A5 and $3.6 \pm 0.7 \mu\text{M}$ for human CYP2A13) (Jalas et al., 2005). However, TG mice can be cross-bred with suitable *Cyp*-knockout mice, such as *Cyp2a5*-null mice (Zhou et al., 2010), to generate a "humanized" mouse model that expresses human CYP2A13 instead of mouse CYP2A5.

The coexpression of CYP2A13 and CYP2F1 in the lung and NM of TG mice may limit the ability to identify specifically the functions for each enzyme. In that regard, efforts are underway to develop additional transgenic mouse models in which only one of the three human P450s is expressed. In the case of NNK, previous studies using recombinant human CYP2F1 indicated that, although CYP2F1 could metabolize NNK, its activity was much lower than that of CYP1A2 (Smith et al., 1992), which itself was far less active than CYP2A13 toward NNK (Jalas et al., 2005). Therefore, the transgenic CYP2F1 is unlikely to make a significant contribution to NNK metabolic activation in TG mice. The present TG mouse model should also be useful for studying compounds that are potentially metabolized by both

CYP2A13 and CYP2F1, such as 3-methylindole (Lanza et al., 1999; D'Agostino et al., 2009). For the latter compounds, we could still conclude, depending on the data, that neither enzyme contributes to metabolism/toxicity in the mouse model or that both might be important but additional studies are needed to distinguish their contributions (e.g., by using specific inhibitors). These would be valuable conclusions, considering the current paucity of data regarding the *in vivo* activity of either human P450 enzyme.

CYP2B6 protein was detected, although at a low level, in the liver of the TG mice. The preferential expression of CYP2B6 in the liver of the TG mice was similar to the expression profile for CYP2B6 in human tissues. It remains to be determined whether, and to what degree, expression of the *CYP2B6* transgene can be induced in TG mice by inducers of human *CYP2B6*, such as phenobarbital (Gervot et al., 1999). In humans, *CYP2B6* is highly inducible in the liver but not in the lung (Hukkanen et al., 2002). Efforts are underway to produce a *CYP2B6*-humanized mouse model, through crossbreeding between the TG mice described here and mice of a novel *Cyp2* gene cluster-null mouse model (Y. Wei and X. Ding, unpublished observations) in which all five mouse *Cyp2b* genes are removed.

It should be noted that there were apparent inconsistencies in the relative tissue levels of P450 proteins and P450 mRNAs for CYP2F1 and CYP2B6. Whereas CYP2F1 mRNA levels in NM and lung were not significantly different, the protein level was ~ 4 times greater in the NM than in the lung. Likewise, the mRNA expression of CYP2B6 was comparable between NM and liver; however, CYP2B6 protein was detected only in the liver and not in the NM. These observations may be explained, at least in part, on the basis of tissue- and gene-specific differences in the efficiency of post-transcriptional and/or post-translational processes that govern the levels of P450 protein expression.

In summary, we have generated a novel transgenic mouse model in which human CYP2A13 and CYP2F1 are expressed preferentially in the NM and lung, whereas CYP2B6 is expressed in the liver. We provide evidence indicating that the transgenic CYP2A13 is active toward the lung carcinogen NNK, both *in vitro* and *in vivo*. This mouse model should be valuable for a number of applications in molecular toxicology, including studies on 1) *in vivo* functions of CYP2A13 and CYP2F1 in xenobiotic metabolism and toxicity in the respiratory tract, 2) *in vivo* function and regulation of CYP2B6 in the liver, 3) mechanisms of regulation of tissue-specific expression of CYP2A13 and CYP2F1, 4) identification of potential CYP2A13 and CYP2F1 inducers, and 5) determination of *in vivo* efficacy of CYP2A13 and CYP2F1 inhibitors.

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Authorship Contributions

Participated in research design: Wei, Wu, Q. Zhang, Kluetzman, and Ding.

Conducted experiments: Wei, Wu, Li, Liu, Zhou, Q. Zhang, Weng, D'Agostino, Ling, X. Zhang, Kluetzman, and Yao.

Performed data analysis: Wei, Wu, Li, Liu, Zhou, Q. Zhang, X. Zhang, and Ding.

Wrote or contributed to the writing of the manuscript: Wei, Wu, Li, Q. Zhang, and Ding.

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