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SYMPOSIUM OVERVIEW

Drug Metabolic Enzymes in Developmental Toxicology[1]

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Although much is known about the metabolism of environmental toxicants in adult organisms, little information exists on the role of cytochrome P450 (CYP) enzymes during development. The developing organism is remarkably dynamic, presenting a constantly changing metabolic profile as various enzyme systems are activated or repressed. This may explain the markedly different sensitivities to various toxicants that are exhibited throughout the developmental period. The application of molecular biological methods has provided important information on the roles of these enzymes in modulating the response of the developing organism to toxicological exposures. The first talk will focus on the identification and role of CYPs during early organogenesis, particularly on how these enzymes influence the response of the conceptus and early embryo to toxic chemicals. The second presentation will discuss the identification of CYPs expressed during human development, as many of the enzymes present in adults are not expressed in the fetus. The third speaker will discuss the developmental consequences of loss of expression of particular metabolic enzymes, focusing on recent studies employing knockout mice to examine the role of drug metabolic enzymes during development. The last two talks will discuss some of the short- and long-term consequences of *in utero* exposures to toxic chemicals and the role of CYP in modulating the toxic response of the developing organism. The first of these will focus on the role of CYP2E1 in human fetuses during late gestation and the response of this enzyme to inducing agents such as alcohol. The last talk will discuss the role of CYP1A1 in the activation of the Ki-*ras* oncogene following *in utero* exposure to carcinogens as a mechanism for lung tumor formation in a pharmacogenetic mouse model.

The cytochrome P450 (CYP) gene family consists of a group of heme-containing enzymes responsible for the metabolic oxidation of a wide variety of endogenous and exogenous substrates, including steroids, fatty acids, drugs, and environmental toxicants such as chemical carcinogens. Although a great deal of information has been acquired relating to the identification of different CYP forms and the characteristics of these enzymes in adult organisms, the role of these enzymes in the developing embryo and fetus has received relatively little attention. Studies on the identification and characterization of CYP during gestation have been hampered by the low levels of CYP present in the developing organism, making purification difficult, as well as the overlapping substrate specificity of these enzymes, which precludes the identification of specific CYP forms by standard biochemical techniques.

Previous studies by several laboratories have provided strong evidence for the presence of multiple forms of CYP in the developing organism (see the following review articles: Pelkonen, 1985; Anderson *et al.*, 1989; Juchau *et al.*, 1989, 1992; Raucy and Carpenter, 1993; Miller, 1994). Recent advances in molecular biological techniques have now made it possible to determine the role of CYP both in normal gestational development and in the toxification/detoxification of environmental contaminants. The first two presentations will discuss the identification of specific forms of CYP present in the developing embryo and fetus. In particular, these talks will focus on the observations that the developing organism lacks many of the forms of CYP present in the adult, contains some forms seen in adult animals, and also contains certain forms that are expressed only during various times of gestation. These alterations in CYP content may help explain the markedly different sensitivities of the developing organism to various drugs and environmental chemicals. The CYP content apparently varies with the developmental age, presenting a constantly changing metabolic profile as various CYP forms are either expressed or repressed.

The third presentation will outline the use of knockout mouse models to determine the functional consequences of a loss of expression of particular forms of CYP, perhaps

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aiding in the identification of the role of these enzymes in normal development. The last two presentations will focus on the toxicological consequences of fetal exposures to environmental toxicants. The role of CYP2E1 in mediating the effects of *in utero* exposure to alcohol will be discussed. In addition, a pharmacogenetic mouse model will be presented that demonstrates the role of fetal CYP1A1 in determining the susceptibility of individual fetuses to lung cancer induction by the polycyclic aromatic hydrocarbon, 3-methylcholanthrene. It should be clear from these presentations that the developing organism is not a "little adult" and contains its own unique repertoire of drug metabolic enzymes. Future studies on the role of metabolic enzymes in development will need to take into account these gestational age- and tissue-specific factors when examining embryonic and fetal tissues.

CYTOCHROME P450-DEPENDENT MONOOXYGENATION OF EMBRYOTOXIC/TERATOGENIC CHEMICALS IN HUMAN EMBRYONIC TISSUES (M. R. JUCHAU AND H. L. YANG)

The period of organogenesis, which extends roughly from Day 18 to Day 60 of gestation in humans, is widely regarded as the period during which the developing conceptus is, in general, most sensitive to the deleterious effects of exposure to drugs and other chemicals. It is also generally recognized that serious toxic effects elicited by most foreign organic chemicals are to a large extent dependent upon their conversion via biotransformation from relatively nontoxic entities to metabolites with considerably higher capacity to elicit toxic damage. These conversions, commonly referred to as bioactivation, tend to produce the greatest damage in the tissues/cells in which they are generated because the toxic metabolites are frequently too unstable to be capable of producing damage at sites distant to their generation due to further degradation to nontoxic metabolites prior to reaching such distant sites. These basic toxicologic principles, well recognized for more than 30 years, have been applied only minimally to embryotoxicity and teratogenesis, i.e., bioactivation in human embryonic tissues during organogenesis has thus far received only minimal investigative attention.

Because the P450 heme–thiolate proteins are currently regarded as the most important tissue catalysts of bioactivating reactions (Nelson *et al.,* 1996), we have embarked upon an investigation of the expression of these important proteins in human embryonic tissues during the period of organogenesis. During this period, the conceptus is referred to as an embryo; following organogenesis, the prenatal conceptus is referred to as a fetus. Several studies of conceptal xenobiotic biotransformation have been reported during the fetal period (reviewed by Raucy and Carpenter, 1993) but only a very few have been reported during the embryonic period. Our studies of P450-dependent biotransformation in organogenesis-stage embryonic tissues of humans were motivated by earlier observations (reviewed by Juchau *et al.,* 1992) that extremely low levels of P450 isoforms in rodent embryos were still sufficient to catalyze bioactivation to the extent that grossly observable and profound abnormalities resulted from the bioactivation process.

Our investigations of human embryonic xenobiotic biotransformation/bioactivation were initiated (Lee *et al.,* 1991) with studies of the capacity of several human embryonic tissues (Days 50–60 of gestation) to catalyze the *O*-dealkylation and *O*-debenzylation of a series of phenoxazone ethers, also frequently referred to as resorufin ethers. With human embryonic hepatic, adrenal, renal, pulmonary, and cardiac homogenates as enzyme sources and four phenoxazone ethers (methoxy, ethoxy, pentoxy, and benzyloxy) as probe substrates, the data generated provided some intriguing results. Each of the human embryonic tissues under study exhibited the capacity to catalyze easily measurable *O*-dealkylation/debenzylation of each of the four phenoxazone ethers. The reactions were strongly inhibited by such general P450 inhibitors as carbon monoxide, ketoconazole, electron sinks, and hypoxic incubation conditions but not by a series of other, more specific P450 isoform inhibitors or by inhibitory antibodies raised against P450 subfamilies. A tentative conclusion was that a number of unique, unidentified, and possibly conceptus-specific P450 heme–thiolate proteins would be necessary to account for the observed monooxygenation reactions. Later investigations with additional probe substrate–inhibitor combinations, however (Namkung *et al.,* 1994; Yang *et al.,* 1994, 1995), yielded results that appeared to be in conflict with those of the initial investigations.

In studies with 2-acetylaminofluorene (Namkung *et al.,* 1994), it was found that human embryonic hepatic and adrenal tissue homogenates catalyzed readily measurable, 7,8-benzoflavone-inhibited hydroxylation of this arylamide substrate at the 7 and 5 positions. Embryonic renal and pulmonary tissue preparations catalyzed the same reactions but at very low rates. Activities with embryonic cardiac tissues were on the borderline of the limits of detectability and may probably be regarded as negligible. *N*-hydroxylation was borderline/negligible for all tissues studied except for very low activities observed in certain embryonic adrenal preparations. The results strongly suggested that at least one functional member of the Family 1 P450 isoforms was expressed at readily detectable levels in human embryonic tissues, particularly in the liver and adrenal gland. P4501A1 was regarded as a highly likely major contributor in view of its capacity to catalyze such reactions and of its susceptibility to selective inhibition by 7,8-benzoflavone. P4501A2 was regarded as a much less likely significant contributor in view of the lack of observable *N*-hydroxylating activity. P4501B1

was not considered at that point. More detailed investigations (Yang *et al.*, 1995) into the possibility that functional P4501A1 might be expressed in human embryonic hepatic tissues during organogenesis provided a body of positive evidence for the concept. It was found that human embryonic hepatic microsomal fractions (obtained from reportedly nonsmoking donors) contained readily measurable ethoxyphenoxazone *O*-deethylase activity that was strongly inhibited by both 7,8-benzoflavone and anti-P4501A antibodies. The previous lack of inhibitory effects with embryonic tissue homogenates as enzyme source (Lee *et al.*, 1991) was resolved by showing (Yang *et al.*, 1995) that a preponderance of the *O*-deethylase activity observable in conceptal tissue homogenates was localized in the cytosolic (104,000*g* 1-hr supernatant) fraction and that the cytosolic activity did not depend on P4501A isoforms. In the microsomal fraction, methoxyphenoxazone *O*-demethylase activity was below the limits of detectability, suggesting that expression of P4501A2 in embryonic hepatic tissues was extremely low or negligible. This result agreed well with previous experimental data indicating a lack of *N*-hydroxylation of 2-acetylaminofluorene in human embryonic hepatic tissues. Very strong inhibition of conceptal hepatic microsomal ethoxyphenoxazone *O*-deethylase activity by inhibitory antibodies raised against P4501A1/2 and by 7,8-benzoflavone thus suggested that most of the microsomal *O*-deethylase activity could be accounted for by functional P4501A1, although a possible participation of P4501B1 was not ruled out.

During investigations with *R*- and *S*-warfarin as probe substrates (Yang *et al.*, 1994), we found that homogenates of human embryonic hepatic tissues would catalyze relatively rapid hydroxylation (particularly of the *R*-enantiomer) at the 10-position. This activity is often regarded as diagnostic for the P4503A subfamily but appeared to conflict with earlier results (Lee *et al.*, 1991) in which we observed no statistically significant inhibition by triacetyloleandomycin (TAO) of the *O*-debenzylation of benzyloxyphenoxazone. We had previously shown (Hulla and Juchau, 1989) that the TAO-inhibited *O*-debenzylation of benzyloxyphenoxazone was an excellent enzymatic marker for the P4503A subfamily. Subsequent investigations revealed that human conceptal hepatic microsomal *O*-debenzylase activity was strongly inhibited by TAO as well as by inhibitory anti-P4503A antibodies and, again, that lack of such inhibition in experiments in which conceptal homogenates were utilized as enzyme source was explicable in terms of relatively high *O*-debenzylase activity in the cytosolic fraction. The cytosolic activity was not inhibited at all by TAO. Strong inhibition of *O*-debenzylase activity by TAO and antibodies in the microsomal fraction suggested that nearly all of the microsomal *O*-debenzylase activity could be accounted for by functional P4503A isoforms. P4503A(s) protein was readily detectable in human embryonic hepatic microsomal fractions not only with the probe substrate–inhibitor combinations mentioned above, but also with immunoblots (Western blots). The corresponding mRNA(s) was also readily detectable with reverse-transcription PCR (RT-PCR) analyses and Northern blots. Experiments with cloning and sequencing (Yang *et al.*, 1994) indicated that by far the major P4503A message expressed in human embryonic hepatic tissues was P4503A7. P4503A5 appeared to be detectable in very minor quantities but 3A4 and 3A3 were not detected.

During the course of the above-described investigations, we utilized RT-PCR extensively as a highly sensitive and specific probe for the expression of P450 genes in human embryonic tissues during organogenesis (45–60 days of gestation). The data obtained from the experiments with RT-PCR have provided some intriguing information which appears to merit further, more detailed investigations. The RT-PCR data, together with other pertinent data, are summarized in Table 1. In general, the results obtained thus far with RT-PCR are in very good agreement with the data generated from studies with substrate probes, chemical inhibitor probes, immunoprobes (immunodetection, immunoinhibition), and other molecular probes such as Northern and Southern blotting, cloning, and sequencing. In addition,

TABLE 1
Xenobiotic-Biotransforming P450 Hemoproteins Detected/ Assessed in Human Embryonic Tissues during Organogenesis

P450 isoform	Method of detection/analysis			Enzymatic assay
	RT-PCR	Northern	Western	
1A1	Pos.[a]	Neg.	Neg.	Pos.
1A2	Neg.	Neg.	Neg.	Neg.
1B1	Pos.[b]	NA	NA	NA
2E1	Pos.[c]	NA	NA	NA
3A4	Neg.	Neg.	Neg.	NA
3A5	Pos.[d]	NA	NA	NA
3A7	Pos[d]	Pos.	Pos.	Pos

Note. In the table, "Pos." indicates that positive results were obtained with the method under investigation at Days 45–60 of gestation; "Neg." indicates that negative results were obtained, and "NA" indicates that a specific assessment has not yet been undertaken. The possible expressions of other xenobiotic biotransforming, human P450 isoforms (e.g., 2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2F1, 2J2, 4B1) have not yet been rigorously investigated in embryonic tissues.

[a] Evidence for 1A1 expression has been obtained with RT-PCR in hepatic and cephalic (but not adrenal) tissues and with enzymatic analyses in hepatic tissues.

[b] Evidence for 1B1 expression has been obtained with RT-PCR in hepatic, adrenal, cephalic, pulmonary, renal, and cardiac tissues.

[c] Evidence for 2E1 expression has been obtained with RT-PCR in cephalic but not hepatic or adrenal tissues.

[d] Evidence indicates that 3A3/3A4 was not detectable in human embryonic hepatic tissues, 3A5 was minimally detectable, and 3A7 accounted for nearly all of the embryonic 3A detected. 3A isoforms were not detected in human embryonic extrahepatic tissues.

however, they indicate that more detailed studies are merited pertaining to human embryonic P4501B1 (detected in all human embryonic tissues examined thus far by RT-PCR) and to P4502E1. A relatively strong and consistent signal for P4502E1 mRNA has been observed with RT-PCR in repeated experiments with human embryonic cephalic but not hepatic tissues. The observations pertaining to embryonic cephalic P4502E1 would seem to have very important implications because the prenatal brain is an exceptionally important target for the deleterious effects of P4502E1 substrates. The most notable example is provided by the permanent/semipermanent neurotoxic effects produced prenatally by ethanol. We feel that this observation should be probed in much greater depth. The observations pertaining to P4501B1 also are very exciting and merit further in-depth studies. The P4501B1 heme–thiolate protein is a relatively newly discovered isoform that may function importantly in developmental processes.

COMPARISON OF CYTOCHROME P450 ENZYMES AND THEIR ACTIVITIES IN HUMAN FETAL AND ADULT LIVER (F. P. GUENGERICH, T. SHIMADA, H. YAMAZAKI, AND M. MIMURA)

Levels and catalytic activities of cytochrome P450 (P450) enzymes involved in the oxidation of drugs and carcinogens were determined in human adult and fetal livers. P450s immunoreactive with anti-human P450 1A1 and anti-human P450 3A antibodies were detected in fetal liver microsomes by immunoblotting analysis. Drug oxidation activities with the substrates ethoxyresorufin, coumarin, 7-ethoxycoumarin, bufuralol, and testosterone were determined in these microsomes; none of the activities were higher in fetal livers than in adult livers. Activation of procarcinogens to reactive metabolites that induce *umu* gene expression in *Salmonella typhimurium* TA1535/pSK1002 or NM2009 was also examined and it was found that activities with (+)- and (−)-enantiomers of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene were higher in fetal liver microsomes than in adult liver microsomes. α-Naphthoflavone, a known inhibitor of P4501A-related activities, did not affect procarcinogen activation in fetal liver microsomes. Fetal liver microsomes catalyzed activation of aflatoxin B_1 and sterigmatocystin, two procarcinogens known to be activated by P4503A4/7 in humans, although activation of carcinogenic arylamines that are good substrates for P4501A2 was much lower in microsomes of fetal livers and adult lungs than in those of adult livers. These results suggest that in human fetal livers at least two P450 enzymes, forms of P450 that are immunoreactive to P4501A1 and P4503A7, are expressed.

Analysis of fetal mRNA samples showed expression of P4501B1 in the kidney and heart and, to a lesser extent, brain and liver. P4501B1 cDNA was expressed in *Saccharomyces cerevisiae* and the microsomes were used to examine the selectivity of this enzyme in the activation of a variety of environmental carcinogens and mutagens in the *typhimurium* TA1535/pSK1002 and NM2009 tester strains, using the SOS response as an endpoint of DNA damage. We also determined and compared these activities of P4501B1 with those catalyzed by recombinant human P4501A1 and P4501A2, which were purified from membranes of *Escherichia coli.* The carcinogenic chemicals tested included 27 polycyclic aromatic hydrocarbons and their dihydrodiol derivatives, 17 heterocyclic and arylamines and aminoazo dyes, three mycotoxins, two nitroaromatic hydrocarbons, *N*-nitrosodimethylamine, vinyl carbamate, and acrylonitrile. Among the three P450 enzymes examined here, P4501B1 was found to have the highest catalytic activities for the activation of 11,12-dihydroxy-11,12-dihydrodibenzo[*a,l*]pyrene, 1,2-dihydroxy-1,2-dihydro-5-methylchrysene, (+)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, 11,12-dihydroxy-11,12-dihydrobenzo[*g*]chrysene, 3,4-dihydroxy-3,4-dihydrobenzo[*c*]phenanthrene, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 2-aminoanthracene, 3-methoxy-4-aminoazobenzene, and 2-nitropyrene. P4501B1 also catalyzed the activation of 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-aminofluorene, 6-aminochrysene and its 1,2-dihydrodiol, (−)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, 1,2-dihydroxy-1,2-dihydrochrysene, 1,2-dihydroxy-1,2-dihydro-5,6-dimethylchrysene, 2,3-dihydroxy-2,3-dihydrofluoranthene, 3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[*a*]anthracene, and 6-nitrochrysene to appreciable extents. However, P4501B1 did not produce genotoxic products from benzo[*a*]pyrene, *trans*-3,4-dihydroxy-3,4-dihydrobenzo[*a*]anthracene, *trans*-8,9-dihydroxy-8,9-dihydrobenz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene and its *cis*-5,6-dihydrodiol, 5-methylchrysene, 11,12-dihydroxy-11,12-dihydro-3-methylcholanthrene, 1,2-dihydroxy-1,2-dihydro-6-methylchrysene, benzo[*c*]phenanthrene, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-acetylaminofluorene, benzidine, 2-naphthylamine, aflatoxin B_1, aflatoxin G_1, sterigmatocystin, *N*-nitrosodimethylamine, vinyl carbamate, or acrylonitrile in this assay system. P4501B1 is expressed constitutively in fetal tissue samples, and is highly inducible in various organs by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds in experimental animal models. Thus, activation of procarcinogens by P4501B1 may contribute to fetal toxicities, as well as to extrahepatic tumors in adults.

USE OF KNOCKOUT MOUSE LINES TO STUDY THE ROLE OF DRUG-METABOLIZING ENZYMES DURING REPRODUCTION AND DEVELOPMENT (D. W. NEBERT AND J. J. DUFFY)

The late seventies ushered in the explosion of molecular biology and recombinant DNA technologies. In the past sev-

eral years these advances, which have occurred at increasingly astounding rates, are now leading to the generation of conventional plus inducible "knockout" mouse lines in which one can characterize the phenotype of intact animals—*in utero* or later in life—carrying the homozygous disruption of the gene of your choice. At the beginning of these studies, it was expected that knocking out a gene in a critical signal transduction pathway would probably be lethal to the animal. To everyone's surprise, few knockout mouse lines are embryonically lethal, underscoring the biologic and evolutionary importance of redundancy of the components in almost every signal transduction pathway.

For more than two decades this laboratory has studied the dioxin-inducible mouse [*Ah*] gene battery. This battery comprises at least six genes that "cross-talk" with one another; these genes are believed to play important roles in reproduction, development, toxicity, cancer, and oxidative stress (reviewed in Nebert, 1989, 1994; Nebert *et al.*, 1993). In addition to two P450 genes—*Cyp1a1*[2] and *Cyp1a2*—we have shown that four Phase II [*Ah*] genes include *Nmo1, Ahd4, Ugt1*06,* and *Gsta1*. The AHR-mediated coordinate induction is controlled positively in all six [*Ah*] battery genes. Oxidative stress up-regulates all four Phase II [*Ah*] genes, and binding of the AHR to the electrophile response element has been implicated in this process (Vasiliou *et al.*, 1995).

Presently this laboratory is generating conventional, plus inducible, knockout mouse lines having homozygous disruptions in the above-mentioned genes; below I describe this novel methodology. If the conventional knockout is lethal during development, this model will provide important information about this gene during embryogenesis or fetal development. Yet, the inducible knockout can still be used—at selected ages and even in selected tissues or cell types—for studies involving the mechanisms of environmental toxicity and cancer. Such inducible knockout lines might elucidate for the first time the interplay between tissues or organs, e.g., aberrant liver and immune system development in *Ahr(−/−)* mice (Fernandez *et al.*, 1995), or might help us to understand secondary effects that have little or nothing to do with the disrupted gene, e.g., multifocal inflammation in *TGFβ1 (−/−)* mice (Diebold *et al.*, 1995). Inducible knockouts, thus, can circumvent these secondary effects and reveal truly primary effects.

What is Cre recombinase and loxP? The Cre recombinase and *loxP* are an enzyme and a DNA recognition site, respectively, used by bacteriophage P1 (Gu *et al.*, 1994). It was discovered that two *loxP* sites—as far apart as 200 kb (Li *et al.*, 1996)—will be recognized by the Cre recombinase, which will then excise all the DNA between these two *loxP* sites. Intriguingly, Cre recombinase has even been shown to mediate recombination and excision between nonhomologous chromosomes (van Deursen *et al.*, 1996).

How to introduce the loxP sites. Site-specific recombination using the Cre–*loxP* system has been shown to work both in mouse embryonic stem (ES) cells (Gu *et al.*, 1993; reviewed in Bronson and Smithies, 1994) and in transgenic mice (Lakso *et al.*, 1992; Orban *et al.*, 1992; reviewed in Schwenk *et al.*, 1995; reviewed in Spencer, 1996). The two-step strategy is being used in many laboratories. In cultured ES cells, one can generate in parallel (a) the *loxP*-flanked ("floxed") gene (or gene segment) and (b) a deletion of the same piece of DNA.

In the first step, three *loxP* sites—in addition, such selection marker genes as *neo, HSV-tk,* or *hprt* (Thomas and Capecchi, 1987; Mortensen *et al.*, 1992; reviewed in Capecchi, 1989)—are introduced by way of homologous recombination into (presumably) nonfunctional regions of the target gene. There are reports of one selection marker being preferred over another. For example, the *HSV-1/tk* is being used less because of possibly causing male sterility in some transgenic lines (Al-Shawi *et al.*, 1991). Typically, an intron, exonic nontranslated segment, or 5′ or 3′ flanking regions of the gene are used for the locations of *loxP* sites. The *HSV-tk* gene is placed at one end of the construct for counter-selection against cells in which the vector has integrated randomly. The targeting construct is then electroporated into *hprt*$^-$ ES cells, and the transfected cells are grown on a mitomycin C-treated mouse embryo fibroblast feeder layer. Individual HAT-resistant ganciclovir-sensitive clones are later isolated. PCR and Southern blot hybridization analyses are then used to confirm that homologous recombination has succeeded within the gene in the targeted ES cells and that the targeted *hprt* minigene and the single distal *loxP* site have successfully been transferred into ES cell lines.

In the second step, the *Cre* gene encoding the CRE enzyme is expressed in the genetically modified ES cells, via transient transfection or injected with small amounts of protein. Three different possible deletions can be generated. The *Type I* deletion results in excision of the target gene from the genome of the ES cells, i.e., this will become the conventional knockout. The *Type II* deletion, on the other hand, results in a floxed gene at the targeted locus. This will be-

[2] Abbreviations used: *Cyp1a1* and CYP1A1, mouse cytochrome P450 1A1 gene and enzyme; *Cyp1a2* and CYP1A2, mouse cytochrome P450 1A2 gene and enzyme; *Nmo1* and NMOI, mouse NAD(P)H:menadione oxidoreductase (also called quinone reductase, DT-diaphorase) gene and enzyme; *Ahd4* and AHD4, mouse aldehyde dehydrogenase-3c gene and enzyme; *Ugt1a6* and UGT1A6, mouse UDP glucuronosyltransferase-1A6 gene and enzyme; *Gsta1* and GSTA1, glutathione transferase (Ya or class α) gene and enzyme; AHR, Ah receptor; HAT, medium containing hypoxanthine, aminopterin, and thymidine; *neo,* neomycin phosphotransferase gene conferring resistance to G418; HSV-*tk,* herpes simplex virus thymidine kinase gene conferring sensitivity to ganciclovir; *hprt,* hypoxanthine phosphoribosyltransferase gene conferring resistance to HAT medium and sensitivity to 6-thioguanine; "floxed," insertion of *loxP* sites to flank a gene or gene segment which Cre recombinase can then act upon; CMV, cytomegalovirus; IE, immediate early.

come the inducible knockout. The third possible type of deletion, which deletes the target gene but leaves the *hprt* gene in the genome, will not be observed, because ES cells carrying such a deletion will die after 6-thioguanine treatment during selection of the Type I and Type II deletion mutants.

Generation of transgenic mouse lines carrying the Type I and Type II targeted alleles. The protocol for the generation of chimeric mice—in which the coat color phenotype is used for selection—is well established (Bradley *et al.,* 1984). Briefly described, targeted ES cells (derived from 129/SV mice, *agouti*) are injected into the blastocoele cavity of 3.5-day embryos bearing the *nonagouti* phenotype. The resulting chimeric blastocysts are then transferred to a pseudopregnant female's uterus. Identification of chimeric pups can be determined by presence of the agouti coat color at 7–10 days of age. After subsequent crosses, a completely agouti coat color usually but not always (discussed in Liang *et al.,* 1996) denotes there has been germline transmission by the father.

Generation of the conventional knockout mouse line. Mouse lines homozygous for the Type I (conventional knockout) gene disruption can then be generated by breeding the F_1 heterozygotes described above. If the knockout is viable and fertile [e.g., as this laboratory found for its *Cyp1a2(−/−)* mouse (Liang *et al.,* 1996)], this line should be valuable for environmental toxicity and cancer studies. If the conventional knockout is nonviable, with death occurring at some gestational age, this line can suggest an important role of the homozygously disrupted gene during that stage of embryogenesis; however, death *in utero* would not provide the researcher with a suitable model system for environmental toxicology or carcinogenesis.

Generation of the inducible, or "conditional," knockout mouse line. This mouse is typically produced from two mouse lines. The Type I heterozygote, described above, is bred to the Type II heterozygote—to give progeny which carry one allele as a deletion and the second allele as the floxed gene. Such a mouse will (a) require less Cre recombinase needed for excision and (b) decrease the chance of translocation (and other unanticipated events) that may alter or scramble the chromosome. This, then, is the first mouse line.

The second mouse is a transgenic line in which the *Cre* gene is expressed under the control of a tetracycline-responsive promoter. In the original system with the wild-type Tet repressor (Hillen and Berens, 1994) or with a fusion tetracycline-controlled *trans*-activator protein (tTA) (Gossen and Bujard, 1992; Efrat *et al.,* 1995; Kitamura, 1996), the cell line or animal is kept on tetracycline to keep gene activity from occurring, and removal of tetracycline causes activation of the gene. In a recent modification of this system, termed the "reverse tet system," a transactivator has been developed that reverses the DNA-binding properties, i.e., the *trans*-activator requires the presence of tetracyclines for binding to *tet* operator sequences. One of the new *trans*-activators (Gossen *et al.,* 1995; Deuschle *et al.,* 1995) fuses the activating domain of viral protein VP16 of herpes simplex virus with a mutant *tet* repressor from *E. coli;* this transactivator thus requires tetracycline analogues for specific DNA binding. Doxycyclin has been shown to be the most potent activator (Gossen *et al.,* 1995). Also, an interferon-responsive promoter has recently been shown to control Cre recombinase expression in the intact mouse (Kühn *et al.,* 1995); one might be wary of using this promoter if one's gene being studied is associated with such cellular processes as cytokine production, inflammation, or the acute phase response.

Mouse lines having the *Cre* gene under the control of a minimal promoter (as well as tissue-specific promoters) fused to the *tet* operator sequences, plus the rtTA *trans*-activator, are being produced (Gu *et al.,* 1994; reviewed in Schwenk *et al.,* 1995; reviewed in Spencer, 1996) and will undoubtedly be sold commercially very soon. The advantages of such mouse lines would be the possibility of breeding such a mouse with any other mouse line carrying the floxed gene of your choice. Inducible knockout lines specific for CRE activity in the liver, lung, brain, and heart are all expected to be available within the year.

By breeding the mouse carrying the Type I deletion allele and the Type II (floxed) targeted allele with the mouse containing the inducible *Cre,* you now have one copy of the "gene of your choice" functioning—yet awaiting the signal for disruption. Oral or subcutaneous treatment of the mouse with doxycycline then turns on the *Cre* gene, thereby disrupting the gene of your choice at a specified age. Hence, you could study the function of your gene at, for example, Gestational Day 14, 5 weeks postpartum, or 12 months of age. Moreover, the conditional gene knockout can be induced globally (in all tissues of the mouse) or in selected tissue or cell types, dependent on tissue- and cell type-specific promoters currently being designed and studied.

The "neighborhood effect." It has become increasingly appreciated that (a) just where a transgene is inserted, (b) how much of the gene segment is removed, or (c) heterogeneity of the genetic background of the knockout line can result in dramatically different phenotypes (Sibilia and Wagner, 1995; Threadgill *et al.,* 1995; reviewed in Bedell *et al.,* 1996; reviewed in Milot *et al.,* 1996; reviewed in Olson *et al.,* 1996). It should be appreciated that, for example, a C57BL/6J (from Jackson Laboratory) and a C57BL/6N (from NIH) have diverged from one another for more than 45 years and therefore should not be considered genetically identical. This neighborhood effect might explain the major reported differences in phenotype between two *Cyp1a2(−/−)* lines (Pineau *et al.,* 1995; Liang *et al.,* 1996)

and between two *Ahr(−/−)* lines (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). Given the same two mouse substrains used (e.g., injecting 129/SV-derived ES cells into the blastocoele cavity of C57BL/6J embryos), the resulting knockout mouse lines will contain some unknown admixture of these two genetic backgrounds, which of course will vary from one mouse to the next within the knockout line. Each knockout line generated, therefore, must be further bred if one wishes to approach homogeneity of genetic background (and, thus, decreases in interindividual variability during any experiment). Given >95% homogeneity of (e.g., C57BL/6J) background still does not guarantee identical phenotypes, if different targeting strategies had removed different numbers of base pairs from the same gene; for example, it is feasible that one but not the other strategy might excise an exon from an unknown activator or inhibitor gene on the antisense DNA strand, somehow affecting the phenotype of the knockout mouse.

Conclusions. In summary, just as the *Ahr(−/−)* mouse line has been found to exhibit diminished viability and fertility (Fernandez-Salguero *et al.*, 1995), we anticipate that *Cyp1a1(−/−)* and *Nmo1(−/−)* conventional knockout lines might be similarly affected. We therefore have begun to generate conventional plus inducible knockout lines for these and other above-mentioned genes that participate in the [*Ah*] battery and appear to play important roles in reproduction, development, toxicity, cancer, and oxidative stress. Other systems in the planning stages include (a) insertion of human alleles in place of the knocked-out mouse orthologous gene, and (b) generation of tissue- and cell type-specific conventional plus inducible knockout mouse lines.

IDENTIFICATION AND CHARACTERIZATION OF HUMAN FETAL CYP2E1 (J. L. RAUCY AND S. P. CARPENTER)

The human fetus metabolizes numerous xenobiotics, many of which are catalyzed by the cytochrome P450 enzymes. As in the adult, metabolism of certain substances by fetal P450 enzymes may result in the formation of toxic metabolites. Also similar to the adult, expression of each fetal P450 enzyme may vary among individual embryos, the result being interindividual differences in rates of xenobiotic metabolism. Altered P450 content may be due to genetic factors or to xenobiotic exposure. Indeed, transplacental exposure to P450 inducers may cause enhanced expression of enzymes in fetal liver, thereby altering the metabolism of certain compounds. The focus of this presentation is on the presence of a single P450, namely CYP2E1, in human fetal liver.

CYP2E1 is involved in the catalysis of mainly low-molecular-weight volatile compounds such as halothane, ether, and acetone (Koop, 1992). The enzyme also has the ability to convert certain procarcinogens, such as *N*-nitrosodimethylamine, to reactive intermediates that can elicit tumorigenesis (Yang *et al.*, 1990). Furthermore, several therapeutic agents can be metabolized by CYP2E1, including chlorzoxazone and acetaminophen (Peter *et al.*, 1990; Raucy *et al.*, 1989). The most widely ingested xenobiotic by humans is also oxidized by CYP2E1, namely ethanol. For the most part, catalysis of most substrates by this P450 results in the formation of toxic intermediates, making CYP2E1 one of the most toxicologically significant of the P450 enzymes. The rate of formation of these toxicants is largely influenced by the level of expression of CYP2E1 in human liver. Hepatic concentrations of the enzyme may be altered genetically, physiologically, and by exposure to various xenobiotics, including ethanol.

With regard to the human fetus, adverse consequences could occur if fetal liver expressed CYP2E1. These unfavorable effects may be threefold. First, *in utero* metabolism of many substrates could occur, resulting in local formation of toxic metabolites. Second, the concentrations of metabolites formed by the fetus may be much higher than those produced by maternal metabolism and transported to the embryo. Moreover, the reactivity of many intermediates preclude their passage through the placenta. Third, maternal exposure to inducers of CYP2E1 may result in transplacental induction of the fetal enzyme, resulting in a faster rate of formation of toxic intermediates. Whether the intermediates are capable of teratogenesis is unknown at present.

One of the most well known teratogens in humans is ethanol. Consumption of alcohol during pregnancy can result in fetal alcohol syndrome (FAS) or in less severe characteristics, known as fetal alcohol effects (FAE) (Jones and Smith, 1973). Associated with either of these abnormalities is damage to the central nervous system which can occur throughout gestation (West, 1987). To date, the underlying mechanisms involved in ethanol-mediated FAS or FAE have not been resolved; however, many theories are currently under investigation (Jones and Smith, 1973). One theory exploits the genotoxic effects of acetaldehyde as a mechanism for ethanol-mediated teratogenesis. Acetaldehyde is formed during metabolism of ethanol by alcohol dehydrogenases (ADH). However, ADH levels in the human fetus are very low (Pikkarainen, 1971), negating the possibility that fetal ADH-mediated oxidation of ethanol is a mechanism for teratogenesis. Alternatively, CYP2E1 also converts ethanol to acetaldehyde. Moreover, during the oxidation of ethanol by CYP2E1, oxygen radicals are formed (Albano *et al.*, 1991). Of the ethanol oxidases, CYP2E1 is unique in its ability to form these radicals. Therefore, fetal expression of CYP2E1 could result in the local formation of not only acetaldehyde, but also oxygen radicals which are known to result in lipid peroxidation (Ekstrom and Ingelman-Sundberg, 1989). Alcohol-related liver disease is closely associated with lipid

peroxidation, suggesting that this P450 is one factor important in ethanol-mediated hepatotoxicity in adults. Furthermore, the connection between CYP2E1 and alcoholic liver disease implies that metabolism of ethanol by the fetal enzyme could be associated with certain manifestations of embryo toxicity. Enhanced expression of CYP2E1 in the fetus, due to ethanol-mediated transplacental induction, could increase the rate of formation of oxygen intermediates and acetaldehyde, which in turn would augment the risk for fetal toxicity.

Here, we demonstrate that the human fetal liver contains CYP2E1, that it is capable of metabolizing ethanol, and that it is inducible by ethanol in primary hepatocyte cultures. CYP2E1 expression in the human fetus appears to be regulated ontogenically, resulting in its appearance during the second trimester, beginning at approximately 16 weeks of gestation (Carpenter *et al.*, 1996). At 10 weeks of gestation, CYP2E1 mRNA levels were undetectable in embryo liver samples. Immunoblot analysis also showed that CYP2E1 was present between 16 and 24 weeks of gestation and that the fetal enzyme exhibits a slightly lower molecular weight than that of the adult. Despite the differences in molecular weight, CYP2E1 in fetal liver was catalytically active. Indeed, the rate of ethanol oxidation by fetal liver microsomes was between 12 and 27% of that mediated by adult liver microsomes (Carpenter *et al.*, 1996). To confirm that CYP2E1 was the primary catalyst in this reaction, antibodies to the human enzyme were utilized in immunoinhibition studies. Results indicated that anti-CYP2E1 IgG inhibited ethanol oxidation by >70% in hepatic fetal microsomes. In contrast, anti-CYP3A IgG produced negligible inhibition of ethanol metabolism.

Results were also presented which show that human fetal CYP2E1 is inducible by xenobiotics in primary cultures. Hepatocytes isolated from fetal liver samples at 24 weeks of gestation retained expression of CYP2E1 similar to that of freshly isolated cells for >40 hr in culture. CYP2E1 expression was enhanced by treating hepatocytes with inducing agents. After only 6 hr of exposure to ethanol or clofibrate, a twofold elevation of hepatocyte CYP2E1 was observed, while fetal CYP3A was unaffected by ethanol treatment (Carpenter *et al.*, 1996). Conversely, hepatocytes treated with rifampicin resulted in a fourfold elevation of fetal CYP3A with no effect on CYP2E1 levels (Carpenter *et al.*, 1996).

In summary, we have shown that CYP2E1 is present in liver samples of fetuses in their second trimester of gestation. Importantly, we have also demonstrated that the fetal enzyme is capable of converting ethanol to acetaldehyde. Whether the local production of oxygen radicals and acetaldehyde can result in certain alcohol-related teratogenic effects remains to be determined. However, results presented here suggest that selected teratogenic effects, such as central nervous system disorders, which can occur during the second and third gestational trimesters could be associated with expression of CYP2E1 in the human fetus.

FREQUENT MUTATIONS IN KI-ras IN MOUSE LUNG TUMORS FOLLOWING TRANSPLACENTAL EXPOSURE TO 3-METHYLCHOLANTHRENE (M. S. MILLER, L. L. WESSNER, M. FAN, M. F. MCENTEE, AND D. O. SCHAEFFER)

Epidemiological studies have suggested a link between maternal exposure to carcinogenic agents and an increased incidence of childhood cancers (Stjernfeldt *et al.*, 1986; Bunin *et al.*, 1987; John *et al.*, 1991). In addition, it has been demonstrated that the levels of DNA adducts in fetal and placental tissues positively correlated with the aryl hydrocarbon hydroxylase (AHH) activity of placentas from pregnant women who were exposed to environmental toxicants (Hansen *et al.*, 1992; Manchester *et al.*, 1992; Whyatt *et al.*, 1995). These results suggest that the fetus is exposed to mutagenic and carcinogenic agents, and may be at risk for the toxic effects produced by these chemicals.

Investigators have utilized C57BL/6 (B6) and DBA2/N (D2) mice, which are responsive and nonresponsive, respectively, to induction of *Cyp1a1* by polycyclic aromatic hydrocarbons (PAHs), as a model system in which to study the role of *Cyp1a1* on tumor susceptibility. Through a series of genetic crosses, a litter is produced in which fetal mice with different induction abilities reside within the same maternal environment, allowing the determination of the role of genetic differences in drug metabolic capacity on the incidence and mechanism of tumor causation in the transplacentally treated offspring. Previous studies utilizing this pharmacogenetic mouse model have shown that the incidence of lung tumors produced following *in utero* exposure to 3-methylcholanthrene correlated positively with the responsive phenotype of the fetus (Anderson *et al.*, 1985). In addition, the offspring from nonresponsive mothers had higher tumor incidences than those from responsive mothers. These differences in lung tumor incidences were shown to be related directly to the levels of expression of Cyp1a1 in the responsive and nonresponsive fetuses and mothers (Miller *et al.*, 1989,1990a,b).

In this study (Wessner *et al.*, 1996), a backcross between hybrid inducible males and noninducible D2 females was used, as fetuses residing in a nonresponsive mother were the most susceptible to 3-methylcholanthrene (MC)-mediated tumor induction (Anderson *et al.*, 1985). Pregnant D2 mice were treated with a single dose of either vehicle or 10 or 30 mg/kg of MC on Day 17 of gestation. The offspring were foster-nursed to untreated mothers and were housed for 12–13 months with no additional treatment. The mice were treated with β-naphthoflavone 48 hr prior to euthanasia and

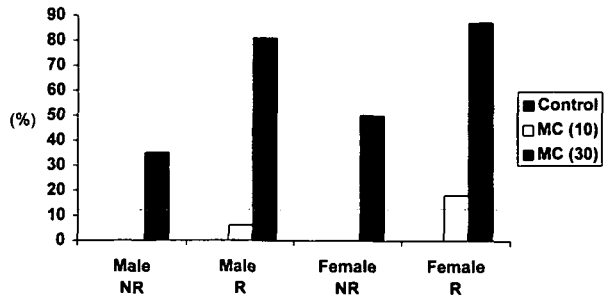

FIG. 1. Lung tumor incidence. Pregnant D2 mice containing a litter consisting of both inducible and noninducible fetuses were treated on the 17th day of gestation with a single high ip injection of either olive oil or 10 or 30 mg/kg of MC. The offspring were housed for 1 year and treated with 120 mg/kg of β-naphthoflavone 48 hr prior to euthanasia and phenotyped for Cyp1a1 expression by either the ethoxyresorufin *O*-deethylase assay or SSCP analysis of the *Ah* receptor gene. Visible lung tumors were counted and analyzed for statistical significance by analysis of variance using the Fisher test. Mice in the 30 mg/kg dose group had significantly higher tumor incidences than the control or 10 mg/kg dose groups, and responsive mice had a statistically higher incidence of lung tumors than nonresponsive mice in both male and female mice ($p < 0.05$). There was no statistically significant difference in tumor incidence between the sexes.

the metabolic phenotype determined using either the ethoxyresorufin *O*-deethylase assay or SSCP analysis of the *Ah* receptor gene.

None of the mice in the control or noninducible 10 mg/kg dose groups exhibited macroscopically visible lung tumors; however, a very low incidence of tumor formation was observed in the responsive males and females from the 10 mg/kg dose group (Fig. 1). At the higher dose (30 mg/kg) of MC, the inducible mice had a significantly higher lung tumor incidence than did their noninducible littermates ($p < 0.05$). DNA was isolated from the paraffin blocks from 16 of these tumors (from a total of 11 mice), consisting of 14 adenomas and 2 adenocarcinomas, and amplified by PCR. Slot blots of the PCR products were screened with a panel of 20-base-pair oligonucleotide probes to the 12th, 13th, and 61st codons of the Ki-*ras* gene, with each of the codons tested with at least six different mutant oligomers by allele specific oligonucleotide (ASO) hybridization.

All of the PCR products from the tumors hybridized to the wild-type probes for codons 12/13 and 61. Thirteen of the tumors (81%) exhibited binding to mutant probes to the 12th or 13th codons of exon 1; none of the tumors demonstrated mutations in the 2nd exon. These results were not due to a misincorporation error of the *Taq* polymerase, as three separate amplification reactions were performed with most of the tumors and the accompanying negative and procedure controls. The data presented in Table 2 demonstrate that most of the tumors exhibited G → T transversions in either the first or second base of codon 12, while 2 tumors from a single mouse harbored G → C transversions in the first base of codon 13. One of the tumors from a mouse containing a GGT → TGT mutation also contained a second mutation at this locus, resulting in a GGT → GAT transition. The PCR products from 5 of the amplified tumor samples were subcloned and a minimum of seven colonies were sequenced to confirm the results obtained by ASO analysis. Four of the 5 tumors contained the same G → T base pair mutation, as determined by sequence analysis, as found by ASO. One of these tumors, which had a very faint but consistent signal with the CYS^{12} mutant oligomer in the ASO analysis, did not show any mutations when sequenced. This may be due to the fact that the proportion of cells carrying the mutation was too low to detect by sequence analysis. The predominance of G → T transversions would be consistent with the known binding preference of the reactive MC metabolite produced by Cyp1a1 for guanine bases in DNA (Osborne *et al.*, 1986) and is the most prevalent lesion found in human adenocarcinomas, probably as a result of exposures to PAHs in cigarette smoke (Mitsudomi *et al.*, 1991).

Both responsive and nonresponsive mice appeared to contain the same types of mutagenic lesions—8 of the 10 tumors from responsive mice and 5 of 6 tumors from nonresponsive mice contained activating point mutations at the first or second base of codons 12 and 13 (Table 2). In addition, 7 of the 9 tumors in male mice and 6 of the 7 tumors in female mice had point mutations, indicating that there was no sex-related difference in the incidence of mutations. The results suggest that differences in the levels of *Cyp1a1* expression can modulate the individual organism's susceptibility to chemically induced cancers in a quantitative, but not qualitative, manner. The prevalence of mutations in the Ki-*ras* gene in adenomas suggests that mutation at this gene locus may be an early event in the carcinogenic process following *in utero* exposure to environmental carcinogens. Considering the marked sensitivity of the fetus to cancer-causing chemicals, fetal exposures to environmental toxicants may account for a significant portion of tumors that occur later in adult life.

TABLE 2
Mutational Spectrum of Transplacentally Produced Mouse Lung Tumors

	GTT (Val^{12})	TGT (Cys^{12})	GAT (Asp^{12})	CGC (Arg^{13})
Males				
NR	1	2	—	—
R	2	2[a]	1[a]	—
Females				
NR	2	—	—	—
R	2	—	—	2

Note. The mutational spectrum for the 13 tumors containing a mutation in the Ki-*ras* gene are shown. Three of the tumors had the wild-type sequence.
[a] One tumor exhibited two different mutations in the Ki-*ras* gene, with the Cys^{12} mutation being the most prevalent.

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