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Inheritance of susceptibility to induction of nephroblastomas in the Noble rat

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Abstract

Noble (Nb) strain rats are susceptible to nephroblastoma induction with transplacental exposure to direct-acting alkylating agent N-nitrosoethylurea (ENU), while F344 strain rats are highly resistant. To study the inheritance of susceptibility to induction of these embryonal renal tumors, fetal Nb and F344 rats and F1, F2 and reciprocal backcross hybrids were exposed transplacentally to ENU once on day 18 of gestation. Nephroblastomas developed in 53 percent of Nb offspring with no apparent gender difference, while no nephroblastomas developed in inbred F344 offspring. F1 and F2 hybrid offspring had intermediate responses, 28 and 30 percent, respectively. Nephroblastoma incidence in the offspring of F1 hybrids backcrossed to the susceptible strain Nb was 46 percent, while that in F1 hybrids backcrossed to resistant strain F344 was much lower (16 percent). Carcinogenic susceptibility is therefore consistent with the involvement of one major autosomal locus; the operation of a gene dosage effect; and a lack of simple Mendelian dominance for either susceptibility or resistance. Since established Wilms tumor-associated suppressor genes, Wt1 and Wtx, were not mutated in normal or neoplastic tissues, genomic profiling was performed on isolated Nb and F344 metanephric progenitors to identify possible predisposing factors to nephroblastoma induction. Genes preferentially elevated in expression in Nb rat progenitors included Wnt target genes *Epidermal growth factor receptor*, *Inhibitor of DNA binding 2*, and *Jagged1*, which were further increased in nephroblastomas. These studies demonstrate the value of this model for genetic analysis of nephroblastoma development and implicate both the Wnt and Notch pathways in its pathogenesis.

INTRODUCTION

While human cancer susceptibility is dependent in a limited number of cases on high-penetrance germline mutations, e.g., *RB* or *BRACA1/2*, the majority of cancers probably involve complex interactions between the host's genetic background and the environment. Furthermore, it is now widely appreciated from studies of twins that genetic background

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contributes significantly to many cancers previously described as nonhereditary (Lichtenstein et al., 2000). Because of the difficulty in sorting relevant genetic modifiers in heterogeneous backgrounds, investigators recently began studying inbred rodent strains with established divergent predispositions for organ-specific neoplastic disease in order to focus on the intrinsic susceptibility factors and to minimize complicating environmental factors (Demant, 2005). Such studies revealed differential gene expression profiles in susceptible versus resistant tissues in inbred rodent strains and further implicated specific transcripts previously linked to the neoplastic process (Bianchi-Frias et al., 2007).

The human pediatric kidney tumor called nephroblastoma or Wilms tumor contains both embryonic epithelial and mesenchymal cellular elements and bears some morphological resemblance to the structure of the embryonic renal cortex. The vast majority of Wilms tumors arise sporadically, but about 2% have a familial origin. Even in those cases, however, the genetic factors remain undefined. Recent elucidation of the X-linked Wilms tumor-associated suppressor gene called *WTX* has shed some light on the disease mechanism in sporadic neoplasms, although WTX has been implicated in only a third of those tumors. It is therefore of value to further develop and evaluate established rodent models in order to better understand the mechanism(s) of tumorigenesis in this relatively common pediatric neoplasm.

Nephroblastomas can arise spontaneously in rats and certain other nonhuman species, and these most closely resemble classic Wilms tumors with a triphasic histology. Moreover, they can be induced in some rodents (but not mice) and lagomorph species by prenatal exposure to alkylating agents (Hard, 1984; 1986). The direct-acting alkylating agents, *N*-nitrosoethylurea (ENU) or *N*-nitrosomethylurea (MNU), when administered transplacentally cause nephroblastomas and other tumors in offspring in a number of experimental animal species, including some strains of rats (Fox et al., 1975; Rice et al., 1989; Hara et al., 1982; Sharma et al., 1994). Inbred Noble strain (Nb) rats are especially susceptible to development of nephroblastoma after a single transplacental exposure to ENU late in gestation, maximally on day 18 (Hard, 1985). In the outbred Sprague-Dawley rat, MNU induced both nephroblastomas and renal mesenchymal tumors that contain mutations in the Wilms tumor suppressor gene *Wt1*, suggesting the involvement of this gene in rat nephroblastoma pathogenesis (Sharma et al., 1994).

Our previous studies (Diwan and Rice, 1995) demonstrated that susceptibility of the developing Nb rat kidney to carcinogenesis by ENU varies with age and the state of differentiation of the target organ. No nephroblastomas were observed in Nb rat offspring exposed to ENU on day 10 or 12 of gestation, at the time metanephric development is initiated. In contrast, nephroblastomas commonly occurred in rats exposed to ENU on gestation days 14 (when nephronic differentiation has been induced), 16 or 18 of gestation with the highest frequency after treatment on day 18 (when the entire enlarged cortical region is morphogenetically active).

The F344 rat has been widely used for bioassay of suspected chemical carcinogens. Relatively few reports have been published on transplacental carcinogenesis by alkylnitrosoureas in this strain of rat (Perantoni et al., 1987; Diwan et al., 1989). The most prevalent types of tumors observed in F344 rat offspring exposed transplacentally to ENU included neurogenic tumors of the cranial, spinal and peripheral nerves, the spinal cord, and the brain(Perantoni et al., 1987), in common with most other strains of rats. In contrast to Nb rats, tumors of the kidney, particularly nephroblastomas, are extremely infrequent in F344 rats given ENU during the final week of gestation. To study the pattern of inheritance of susceptibility to the chemical induction of nephroblastomas, we compared the incidence of such tumors in segregating crosses between Nb and F344 rats after transplacental exposure to ENU on day 18 of gestation. We also evaluated known Wilms tumor-associated suppressor genes, *Wt1* and *Wtx*, to rule out their involvement in Noble rat tumorigenesis. Furthermore, we applied global genomic profiling to

metanephric mesenchymes and nephroblastomas in order to detect genes that are differentially regulated in Nb versus F344 rat tissues with the expectation that such efforts may provide clues to the predisposing factors in Wilms tumorigenesis.

MATERIALS AND METHODS

Chemicals

ENU was synthesized by Dr Gary Muschik (Chemical Synthesis and Analysis Laboratory, SAIC, Frederick, MD). It was stored at -20° C and was dissolved in physiological saline buffered to pH 3.5 just before use.

Animals

Animal studies were performed in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals", NIH Publication No. 86-23 and under a NCI-Frederick Animal Care and Use Committee approved protocol. Male and female Nb rats were obtained from Charles River (Kingston, NY) at 6-8 weeks of age. F344/NCr (F344) rats of both sexes, 6-8 weeks old, were obtained from Animal Production Services at the NCI-Frederick, Frederick, MD. These animals were given a laboratory diet of commercial pellet diet (NIH-3, autoclavable) and tap water *ad libitum*. Reciprocal matings between susceptible Nb and resistant F344 rats produced F_1 hybrids. Female F_1 hybrids were mated to males of both parent strains to produce susceptible-parent and resistant-parent backcross hybrids. F_2 rats were derived from crosses between F_1 animals.

Treatment

Five pregnant rats of strains F344 and Nb, their F_1 hybrids, and F_1 hybrids backcrossed to each parent strain were given 0.5 mmole ENU/kg body weight, intraperitoneally, on day 18 of gestation. Rats were allowed to deliver naturally and to nurse their own offspring which were weaned and separated by gender at 4 weeks of age. Untreated controls were not included since prior studies had demonstrated an absence of nephroblastoma development even in Noble rats (Diwan and Rice, 1995).

Necropsy and Histopathology

Offspring were euthanized by CO_2 gas when tumors were palpated or when they showed clinical signs of illness. Remaining rats were euthanized by CO_2 at 70 weeks of age when the experiment was terminated. Complete necropsies were performed. All lesions and kidneys, lungs, brain, spinal cord, thyroid gland and spleen were fixed in 10% buffered formalin. Lesions and representative samples from each major organ were embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin (H&E) for histological evaluation. Kidneys were sliced in a sagittal direction so that four separate sections of each kidney were generally available for microscopic examination.

RNA preparation and microarray analysis

For gene profiling, metanephric rudiments were dissected from Nb and F344 rat embryos at 14-days gestation, and metanephric mesenchymes (MM) were separated from the ureteric bud as previously described (Karavanova et al., 1996). Buds were comparably branched in Nb and F344 embryos at the time of dissection, indicating a similar stage of development. However, to minimize possible variations between litters and embryos, MMs (>60) from four timed-pregnant Nb or F344 rats were pooled. Rat nephroblastomas were removed from euthanized moribund animals, quick frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Total RNA was isolated from normal and tumor tissues using an RNeasy kit (Qiagen). Synthesis of cDNA and labeled cRNA, hybridizations to the Rat Expression Arrays 230 2.0

chips, and measurements of hybridization intensities were carried out according to manufacturer's protocols (Affymetrix Inc., San Diego, CA, USA). Triplicate sets of GeneChips were included to allow statistical evaluation (*t*-test and Change Call) using Data Mining Tool software (Affymetrix Inc., San Diego, CA, USA).

RT-PCR and Sequencing

For semi-quantitative RT-PCR confirmation of microarray results, total RNA (1 μg) was used for the synthesis of single-stranded cDNA. Briefly, RNA samples were reverse transcribed at 42° C for 1 hr by incubation with 20 μl of a reverse transcription mixture containing the following components: 5 μM of hexamer primer; 1X reverse transcriptase buffer; 10 mM 1,4-dithiothreitol; 5 mM $MgCl_2$; 20 units of RNasin; 0.5 mM deoxynucleotide triphosphates; 200 units of SuperScript II (Invitrogen, CA). The resulting single-stranded cDNA was used for PCR. The oligonucleotide primers for selected genes were designed using GeneFisher software (sequences are available upon request). Repeated PCR reactions for each primer pair were performed to optimize the number of cycles required to remain within the exponential phase of the amplification curve (data not shown). At least three independent PCRs were performed in a total volume of 20 μl using a FastPCR kit (Roche, NJ), 20 pmol of each 5′ and 3′ primer; and 1μl cDNA (corresponding to 50 ng/μl total RNA). PCR reactions were conducted for 1 min at 94° C, 0.5 min at 55° C, and 1 min at 72° C for 27 cycles using an iCycler (BioRad, CA).

For *Wt1* sequencing, two regions, exon 1 and the zinc-finger domain, were evaluated based upon Sharma et al. (Sharma et al., 1994). PCR amplification of rat tumor genomic DNA was performed using exon 1 and adjacent intronic gene primers for rat (FW: 5' TCCGACGTGCGGGACCTGAAC 3'; RV:5'CAAGTCGCGGAGCTACTACC). This yielded a fragment of 486 bp. For the zinc-finger region, fragments were amplified from cDNA with primers FW: 5'GCATCTGAAACCAGTGAGAA and RV: 5'CCCTCAAAGCGCCACGTGGAG, yielding a 393-bp (nt 950-1343) sequence that covers the entire zinc-finger domain. PCR reactions were conducted for 45 sec at 95° C, 30 sec at 58° C, and 2 min at 72° C for 30 cycles.

The exon 1 PCR products were analyzed on 0.8% agarose gel. Specific fragments were gel purified using an Ultra-free DA column (Millipore) and sequenced directly using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit. Products from the zinc-finger region were subcloned into a Topo TA cloning vector (Invitrogen), and multiple clones were analyzed by sequencing with T3 forward/T7 reverse primers from the vector.

For *Wtx* sequencing, PCR was carried out using the PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) under the following conditions: 94° C 0.5 min, 55° C 0.5 min, 72° C 1 min for 34 cycles. The following two sets of primers were used to produce and amplify rat *Wtx* cDNA from mRNA sequences involving base pairs 1 – 762 and 743 –1586, respectively: set 1: (I) ATG GAG ATT CAA CAG GAT GA; (II) ACA AGC TGT CTT CTC AGT AG and set 2: (III) CTA CTG AGA AGA CAG CTT GT; (IV) AGG TCA TAA AGG CAG TCA TC. The PCR fragments were purified from agarose gels using a NucleoTrap gel extraction kit (BD Biosciences, Palo Alto, CA). They were cloned into pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). For each strain of rat and each tumor, 8 colonies were picked for DNA purification using a miniprep kit (Qiagen, Valencia, CA). For each preparation, sequencing was carried out with both T3 primer and T7 primer using the Big Dye terminator (Laboratory of Molecular Technology, SAIC, Frederick, MD).

Western blot analysis

Immunoblotting was performed as previously described (Levashova et al., 2003). Briefly, tumor tissues were homogenized in ice-cold RIPA cell lysis buffer [50 mmol/L Tris-HCl,pH7.4, 150 mmol/L NaCl,1%Nonidet P-40, 0.25% Na deoxycholate, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 0.1 mmol/L phenyl methylsulfonyl fluoride (PMSF)] containing a complete protease inhibitor cocktail (Roche Diagnostic GmbH, Mannheim, Germany). Homogenates (30 μg total protein) were then run in reducing buffer in 10% Tricine-SDS PAGE gels and blotted to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBST) (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 0.1% Tween 20), incubated with Jagged1 antibody (1:1000, rabbit mAB #2620, Cell Signaling Technology, Inc, Danvers, MA) for 2 hours, washed, and visualized with an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

RESULTS

Renal neoplasms

The incidence of renal tumors induced by transplacental ENU in parental strain and hybrid rats is summarized in Table 1. Histologically, three types of renal tumors were observed: epithelial (renal cell), mesenchymal, and nephroblastic. No differences between male and female offspring were observed in frequency of any of these distinctive kinds of tumors in either of the inbred parental strains or in any of the interstrain crosses. The incidence of epithelial and mesenchymal tumors was low in both parental strains and in hybrids. No significant differences were observed in the frequency of such tumors among various crosses, and their frequency was not related to the genotype of the individual rat. No urothelial tumors of the renal pelvis occurred in this study. Histologically, the nephroblastomas (example in Figure 1) observed in this study were similar to those described earlier by us (Diwan and Rice, 1995) and others (Hard, 1985;1986).

Inheritance of susceptibility to induction of nephroblastoma

No nephroblastomas were observed in (F344 × F344) rat offspring (0/30) (Table 1). In contrast, the frequency of nephroblastomas in offspring of Nb rats (both sexes combined) was 16/30 (53%). The frequency of nephroblastomas in F_1 and F_2 hybrids was intermediate to that of the parental strains, with neither susceptibility nor resistance displaying a simple Mendelian dominance pattern of inheritance but instead a pattern more consistent with incomplete dominance (Table 2). The frequency in F_1 hybrids (the two reciprocal F_1 crosses combined) was 18/63 (28%); there was no significant difference in incidence between (F344 × Nb) F_1 offspring (8/31, 26%) and (Nb × F344) F_1 offspring (10/32, 31%). The frequency of nephroblastomas in the offspring of F_1 hybrids backcrossed to the susceptible parent strain Nb was high (15/33, 46%) and nearly the same as in the Nb strain itself, while the nephroblastoma frequency in offspring of F_1 hybrids backcrossed to the resistant parent strain F344 was low (5/32, 16%). Again, these results are more consistent with predicted patterns based on incomplete rather than simple dominance.

Other tumors

The frequency of tumors in organs other than the kidney is summarized in Table 3. The most common types of non-renal tumors observed in offspring of both parental strain and hybrid crosses included neurogenic tumors of various kinds and follicular cell tumors of the thyroid gland. The incidence of tumors of the peripheral nervous system was high in both parental strains and in hybrids. These tumors were mostly anaplastic schwannomas. There was no significant difference in the incidences of schwannomas between the parental strains or their

hybrids. The most frequent type of tumor in the central nervous system was oligodendroglioma. Small numbers of mixed and anaplastic gliomas were also observed. Thyroid gland tumors related to ENU treatment included follicular cell adenomas and carcinomas. Their frequency was not significantly different either between parental strains or among the various crosses, although the incidence appeared slightly greater in Nb offspring and in F_1 and backcross progeny than in inbred F344 offspring. Numbers of these tumors were too low to analyze inheritance of susceptibility.

Other tumors either occurred with similar incidence across all strains and hybrids studied, or were characteristic of aged F344 rats (e.g., large granular lymphocytic leukemia; Leydig cell tumors of the testis). These are not listed in Table 3; among them were pituitary tumors, a few mammary tumors, lymphocytic leukemia and testicular tumors. A very small number of miscellaneous tumors also occurred, including single tumors of the prostate, pancreas, jaw, ovary and uterus in one or more treatment groups. These tumors also were too few to analyze according to pattern of inheritance.

The Wt1 and Wtx loci

Sharma et al. previously identified point mutations in *Wt1* in MNU-induced renal tumors from Sprague-Dawley rats (Sharma et al., 1994). In nephroblastomas, these were restricted to exon 1 (codon 111) and were found in all four nephroblastomas examined. On the other hand, renal mesenchymal tumors contained mutations (three of seven listed) in exon 1 and in the second zinc-finger. To rule out the involvement of these regions in Noble rat nephroblastoma pathogenesis, we analyzed 10 tumors for point mutations. Sequencing of PCR amplified fragments revealed no mutations either in exon 1 or in any portion of the zinc-finger domain, suggesting that the locus involved in the Noble rat nephroblastoma is distinct from *Wt1* (Table 4).

The X-linked locus *Wtx* has recently been implicated in about one third of human Wilms tumors (Rivera et al., 2007), and it apparently functions in the regulation of canonical Wnt signaling (Major et al., 2007). It encodes a protein of 1135 amino acid residues that can interact with β-catenin, a key component of the Wnt signaling pathway, and promotes its ubiquitination and degradation. Since a polymorphism in the gene might alter the encoded protein's ability to interact with components that regulate Wnt signaling and therefore account for differential susceptibilities, we sequenced the region of *Wtx* associated with Wilms tumorigenesis (Rivera et al., 2007) in both rat strains. As the mutations in human Wilms tumors were all detected in the N-terminal portion of encoded protein, we designed two sets of primers to amplify cDNA from the corresponding coding region in rat (nt 1 – 1586). Following RT-PCR with the two sets of primers, DNA fragments of predicted sizes were generated in RNA from both strains. Sequencing of the fragments revealed that normal Nb and F344 rat sequences are identical to one another. Additionally, 0/8 Nb rat nephroblastomas contained missense mutations in these regions (Table 4). Thus, the *Wtx* gene is unlikely to be responsible for the differential susceptibility of Noble and F344 rats to nephroblastomas induced by ENU. Furthermore, these findings are consistent with the observed autosomal dominant mode of inherited susceptibility.

Genomic profiling

In efforts to identify possible predisposing factors in nephroblastoma development, a comparison of gene expression profiles was made between metanephric mesenchymes isolated from 14-gestation day (gd) embryos from the susceptible Nb rat and the resistant F344 rat. To minimize embryonic variation, tissues were collected from the litter mates of four separate timed-pregnant females and pooled for Affymetrix microarray analysis. These studies revealed a series of genes that were significantly upregulated (127) or downregulated (141) in Nb versus F344 rats. An annotated list of particularly interesting candidates is provided (Table 5), and

these have been confirmed by semi-quantitative RT-PCR (Table 5 and Figure 2). The table identifies genes implicated in cell proliferation, signal transduction, and transcriptional activation and includes genes that have previously been implicated in Wilms tumorigenesis and associated with Wnt activation, e.g., the *Epidermal growth factor receptor* (*Egfr*). Moreover, an evaluation of nephroblastomas derived from ENU-treated Nb rats revealed elevated expressions of some of the same genes that were increased in normal embryonic tissues. Levels often significantly exceeded those observed even in normal Nb rat MMs, for example, for *Egfr*, *Id2*, *CK2α*, *Cux1*, *Jag1*, *Hes1*, *Zfp347*, and *RGD1309362* (Table 5 and Figure 3), suggesting that these were further selected in tumorigenesis. Jagged1 expression was also confirmed using Western blot analysis (Figure 4). Strong protein expression is apparent for all nephroblastomas but absent from adult kidney tissue. Others, such as *Drg1*, *Sgk*, or *Six1*, which showed rat strain differentials in gene expression, were either not increased further in tumors or actually decreased in expression, suggesting that they are not involved in tumor development or are involved only in early stages. Alternatively, they might play a suppressor role (Bandyopadhyay et al., 2006;Maiyar et al., 1996), and hence their decreased expression could be contributing to tumor development at later stages by allowing the tumor to evade growth control restrictions.

DISCUSSION

Nephroblastomas in Nb rats are histologically distinctive embryonal neoplasms of the renal cortex which morphologically resemble human Wilms tumor (Hard, 1986; Turusov et al., 1980), but which develop after the attainment of sexual maturity rather than in infancy and characteristically present as a single tumor mass. The genetic pathway(s) to nephroblastoma in Nb rats has not been established and may differ from the pathway to Wilms tumor in humans. Nephroblastoma is uncommon in most strains of rat as either a chemically induced or naturally occurring tumor.

The results of this study clearly show that genetic factors control the susceptibility of inbred rats to induction of nephroblastomas by transplacental administration of ENU. F344 rats are totally resistant to the induction of these tumors while their incidence was approximately 50% in Nb rat offspring of ENU-treated mothers. A similar frequency of nephroblastomas in ENU-treated offspring was reported in previous studies with Nb rats (Hard, 1985; Diwan and Rice, 1995). F_1 rats were less sensitive by a factor of two than the susceptible parental strain Nb. Thus, neither susceptibility nor resistance to chemical induction of nephroblastoma exhibits simple Mendelian dominance. Nephroblastoma incidence in F_2 offspring was approximately 30%, again intermediate between the tumor frequencies seen in resistant and susceptible parent strains. The frequency of nephroblastomas in offspring of F_1 hybrid females backcrossed to Nb males was approximately equal to that of the susceptible parental strain Nb. A much lower incidence (16%) of such tumors was observed in the offspring of F_1 hybrid females backcrossed to resistant F344 males. A pattern of inheritance that closely fits these data is presented in Table 2, which gives the observed and expected numbers of nephroblastomas in offspring of the various crosses, assuming that two alleles from one genetic locus in the Nb parent contribute equally to nephroblastoma susceptibility in that strain and that a single autosomal Nb allele, inherited from the Nb parent or grandparent, confers one-half the degree of susceptibility of the Nb parent strain in hybrid rats which are heterozygous for that locus, i.e., incomplete dominance.

The exceptional sensitivity of Nb rats to transplacental induction of nephroblastoma and the inherited pattern of susceptibility in F_1, F_2, and backcross hybrids with strain F344 are unlikely to result from any differences in the stage of development at the time of exposure to the carcinogen, since the gestation period in Nb rats, 21-22 days, is the same as that of strain F344 (Diwan and Rice, 1995) and since metanephroi from the two strains showed comparable

branching of the ureteric bud at the time of tissue harvesting (14 gd). It is most unlikely that there are significant toxicokinetic differences in delivery of carcinogen to the gravid uterus in different strains, as ENU is a direct-acting alkylating agent with no dependence on metabolism for its activation and is rapidly absorbed and distributed throughout the body (Koestner et al., 1971; Rice, 1979).

Wikland et al. (Wiklund et al., 1981) studied genetic factors that confer susceptibility to the natural occurrence of pituitary tumors in rats. A series of crosses was performed between F344 (susceptible) and Holtzman (nonsusceptible) rats to produce the F_1 hybrid, the F_2 generation, and backcrosses of the F_1 hybrid to each parent. Their data were compatible with the involvement of three independently segregating genetic loci in determining pituitary tumor susceptibility. Naito et al. (Naito et al., 1985) undertook genetic analysis of susceptibility to chemical induction of trigeminal nerve schwannomas in rats by neonatal administration of ENU. Their studies of susceptible strain LE rats, resistant strain WF rats, and their F_1, F_2 and backcross hybrids suggested that three independently segregating loci are involved in determining susceptibility to induction of nerve tumors by this alkylating agent.

Naito et al. (Naito et al., 1985) suggested that the susceptibility of LE rats to induction of trigeminal schwannomas was probably related to defective capacity to repair DNA damage. Since LE rats are also more susceptible than WF rats to colon carcinogenesis induced by 1,2-dimethylhydrazine(Takizawa et al., 1978), the genetic factors that regulate the capacity of the peripheral nervous system to repair alkylation damage to DNA in this strain appear also to control this activity in other organs. The susceptibility of Nb rats to induction of nephroblastomas might also be related to defective DNA repair, resulting in accumulation of O^6-ethylguanine or other promutagenic adducts in the target cells of fetal kidney DNA (Loveless, 1969; Goth and Rajewsky, 1974; Mehta et al., 1981). In contrast to the findings of Naito et al. (Naito et al., 1985), however, we observed no consistent differences between Nb and F344 rats in the frequencies of tumors in other target organs including the central and peripheral nervous systems. Moreover, only nephroblastoma susceptibility varied with parental genotype in our study, and not the frequencies of renal tumors of adult epithelial or mesenchymal origin. A heritable DNA repair defect that could account for the increased susceptibility of rats with Nb strain parents or grandparents would have to be expressed only in target cells that give rise to tumors with an embryonal phenotype, which seems unlikely.

In humans, deletions and mutations in the tumor suppressor genes *WT1* (Huff and Saunders, 1993; Rauscher et al., 1990) or *WTX* (Rivera et al., 2007) appear to play an important role in development of about one third of Wilms tumors. The WT1 gene product is expressed at high levels in the developing kidney in humans (Werner et al., 1993) as well as rats (Sharma et al., 1992). It has been reported (Rauscher et al., 1990; Drummond et al., 1992; Wang et al., 1993) that the WT1 protein functions as a potent repressor and/or activator of transcription in vivo by binding to multiple sites in the promoter sequence of several growth related genes (*EGR1*, *IGF2*, *PDGF* A chain and *IGFIR*). If the *Wt1* allele in Nb rats is more susceptible to adduction and mutagenesis by ENU than the corresponding allele in F344 rats, the resulting mutations in the WT1 protein might play a role in neoplastic transformation of embryonal renal tissue and the subsequent development of nephroblastomas. However, our own assessment for possible mutations in tumors from Nb rats has revealed no evidence for such lesions. Alternatively, susceptibility may depend upon dysregulation of canonical Wnt signaling through stabilization of secondary messenger β-catenin. Constitutive activation of β-catenin has been reported in some Wilms tumors (Koesters et al., 2003). Moreover, loss of tumor suppressor function WTX has recently been implicated in Wilms tumors and its mechanism appears to involve β-catenin ubiquitination and degradation through direct interaction with the APC/Axin degradation complex (Major et al., 2007). Our studies reveal the absence of any polymorphisms between F344 and Nb rats nor missense mutations in nephroblastomas in the

mutation-prone portion of the *Wtx* gene, suggesting that it is not involved in susceptibility or tumor pathogenesis; however, an examination of Nb rat nephroblastomas has revealed nuclear localization of β-catenin (Ehrlich et al., 2008) as occurs in Wilms tumorigenesis (in >60% of rat and human tumors), so the Wnt pathway may be dysregulated in the rat tumors as well.

To further address the issue of possible susceptibility genes, we performed microarray studies to compare isolated metanephric mesenchymes from F344 and Nb metanephroi at a similar stage of development. Furthermore, we compared these results with microarray analysis of Nb rat nephroblastomas. These studies identified a set of genes that were differentially expressed normally and were further increased in tumors. Included in this list is the membrane-bound tyrosine kinase *Egfr*. This gene, when inactivated, has already been associated with strain-dependent differential embryonic lethality in the mouse (Threadgill et al., 1995). It is also upregulated in about one third of Wilms tumors (Ghanem et al., 2001), and its overexpression is often associated with the anaplastic form of the disease (Little et al., 2007). Moreover, in the liver, it is a direct target of Wnt/β-catenin signaling (Tan et al., 2005). Its potential role in the neoplastic process has in fact led to clinical trials using the EGFR inhibitor Gefitinib (Freeman et al., 2006).

Inhibitor of DNA-binding/differentiation *Id2* was similarly elevated in expression both in normal Nb rat MMs and nephroblastomas. This transcriptional regulator can function as a dominant-negative inhibitor of basic Helix-Loop-Helix (HLH) transcription factors, since it consists of a HLH domain without a DNA-binding domain. These oncogenic factors can block differentiation and stimulate cell proliferation in a variety of tissues (Sikder et al., 2003). *Id1-3* are expressed in the developing metanephros (Jen et al., 1996), and nuclear localization is associated with polycystic kidney disease, a disorder of cell proliferation (Li et al., 2005). In such cases, Id2 inhibits cyclin-dependent kinase inhibitor p21 through interaction with polycystins 1 or 2. Id2 is also a target of canonical Wnt signaling in colon carcinoma,(Rockman et al., 2001) so its dysregulation may result from a lesion in the Wnt pathway as well.

Another potential susceptibility gene identified in our genomic profiling is the transcriptional repressor *Cux1* (also known as CCAAT displacement protein/CDP). Ectopic expression of this gene in transgenic mice causes renal hyperplasia possibly through repression of cyclin-dependent kinase inhibitor p27 (Ledford et al., 2002). In Drosophila, the gene is upregulated by Notch activation (Micchelli et al., 1997), and Wnt (Wingless) also regulates this process by inducing expression of Notch ligands. In the mouse metanephros, expression of Notch pathway components is demonstrable in nephronic progenitors, and overexpression of Notch in rat kidney cells upregulates *Cux1* expression,(Sharma et al., 2004) suggesting it is also a target of Notch signaling in mammalian cells. In the current studies, elevated *Cux1* expression may be indicative of activation of Notch signaling in the nephroblastomas, which is consistent with the observed increased expression of the Notch ligand *Jag1* and Notch target gene *Hes1* in our microarray studies.

More intriguing is the link between tumor susceptibility/development and *Jag1* and *Hes1* expression. In the mouse, *Jag1* is also a target of Wnt signaling (Estrach et al., 2006) and along with *Hes1*, a direct downstream target of Notch signaling, are both highly upregulated in induced MM found in the cap condensates (Kuure et al., 2005). Previously, we reported the expression of transcriptional co-activator CITED1 specifically in cap condensate cells during metanephric development (Plisov et al., 2005). This population, which overlays the inductive ureteric bud tip, undergoes mesenchymal-epithelial transition beneath the tip to form the epithelia of the nephron (Boyle et al., 2008). It is also highly expressed in blastemal populations in Wilms tumors and rat nephroblastomas (Lovvorn et al., 2007), suggesting that blastemal elements are largely locked in this stage of development in these tumors. While *Jag1* and *Hes1* may simply function as markers for this developmental stage in both normal and tumor

tissues, the misexpression of *Jag1* has a profound effect upon normal metanephric development (Kuure et al., 2005), so it is not unreasonable to think that it may also function in tumorigenesis. In the case of intestinal development and tumorigenesis, studies have clearly implicated Wnt signaling through TCF4 activation in crypt progenitor maintenance and adenoma formation. Notch signaling, while similarly active in intestinal crypts, is involved in progenitor cell determination by regulating the decision between adsorptive and secretory fates (van Es and Clevers, 2005). Exactly how these two signaling mechanisms might interact in the MM is unclear; however, the successful application of Notch pathway inhibitors to intestinal adenomas, inducing the proliferative cells to a post-mitotic differentiated state, indicates that Notch signaling is required in those tissues for maintenance of proliferation and that inhibition of this pathway may have important therapeutic implications (van Es et al., 2005). If blastemal populations in Wilms tumor are similarly dependent upon Notch signaling for proliferation, then anti-Notch therapies may prove efficacious for their treatment. This is currently under investigation.

These studies indicate first that the Nb rat may provide a powerful genetic tool for the identification of a major susceptibility gene in nephroblastoma development. With the availability of sufficient markers for the rat genome, it is now possible to pursue the specific genetic locus involved. Secondly, the rat model may be of value in determining human drug efficacy, as there are several similarities with the human disease and tumors are rapidly induced at high incidence in the Nb rat. Finally, while these studies do not identify the mechanism(s) of dysregulation in nephroblastoma development, they provide additional support for a role for Wnt activation in tumor development and/or maintenance. Multiple targets of Wnt activation were identified, including Egfr, Id2, and Jag1, which further implicates the Notch pathway in this process. It is important now to determine if the same factors are dysregulated in human tumors and to characterize their involvement in Wilms tumor pathogenesis.

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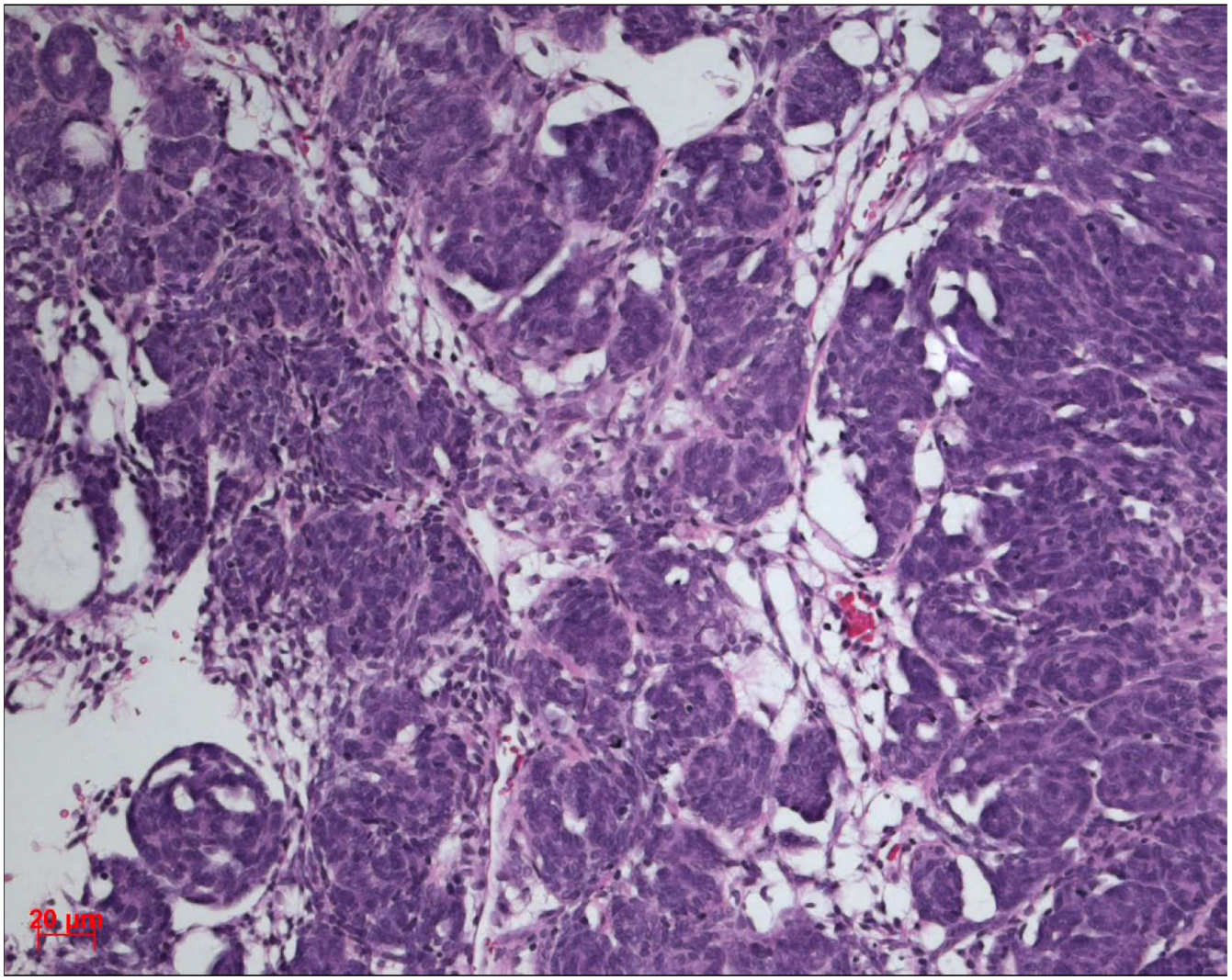

Figure 1.
Rat nephroblastomas exhibit a triphasic histology with elements of blastema and embryonic epithelial tubules and stroma.

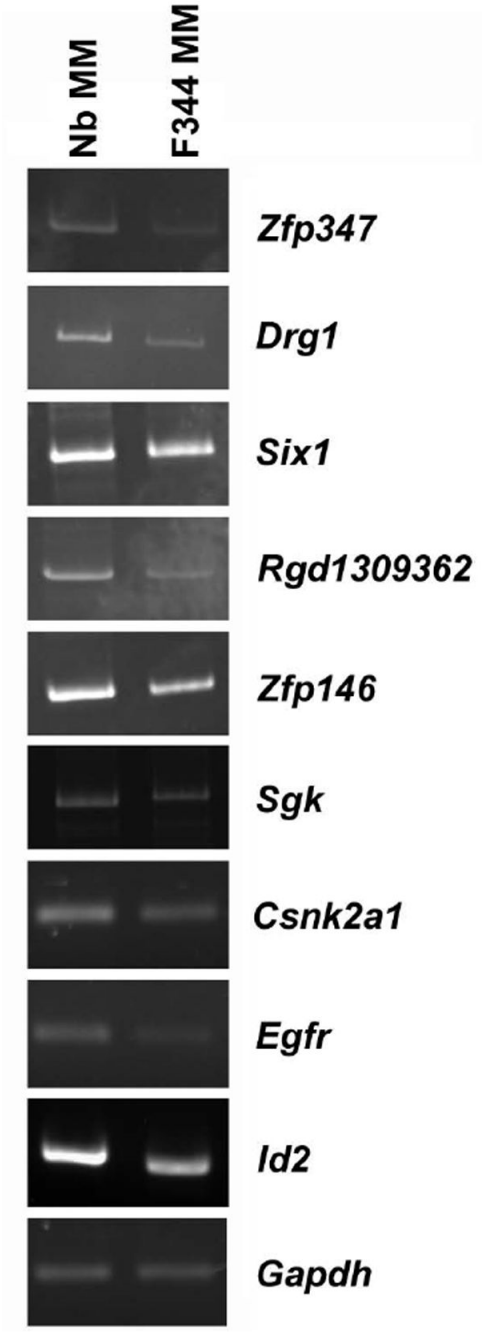

Figure 2.
Differential expression of genes in F344 and Nb rat metanephric mesenchymes. RNA was amplified by semi-quantitative RT-PCR to confirm differential gene expression patterns observed by microarray analysis. Sequences were separated in 1XTBE 10% PAGE gels.

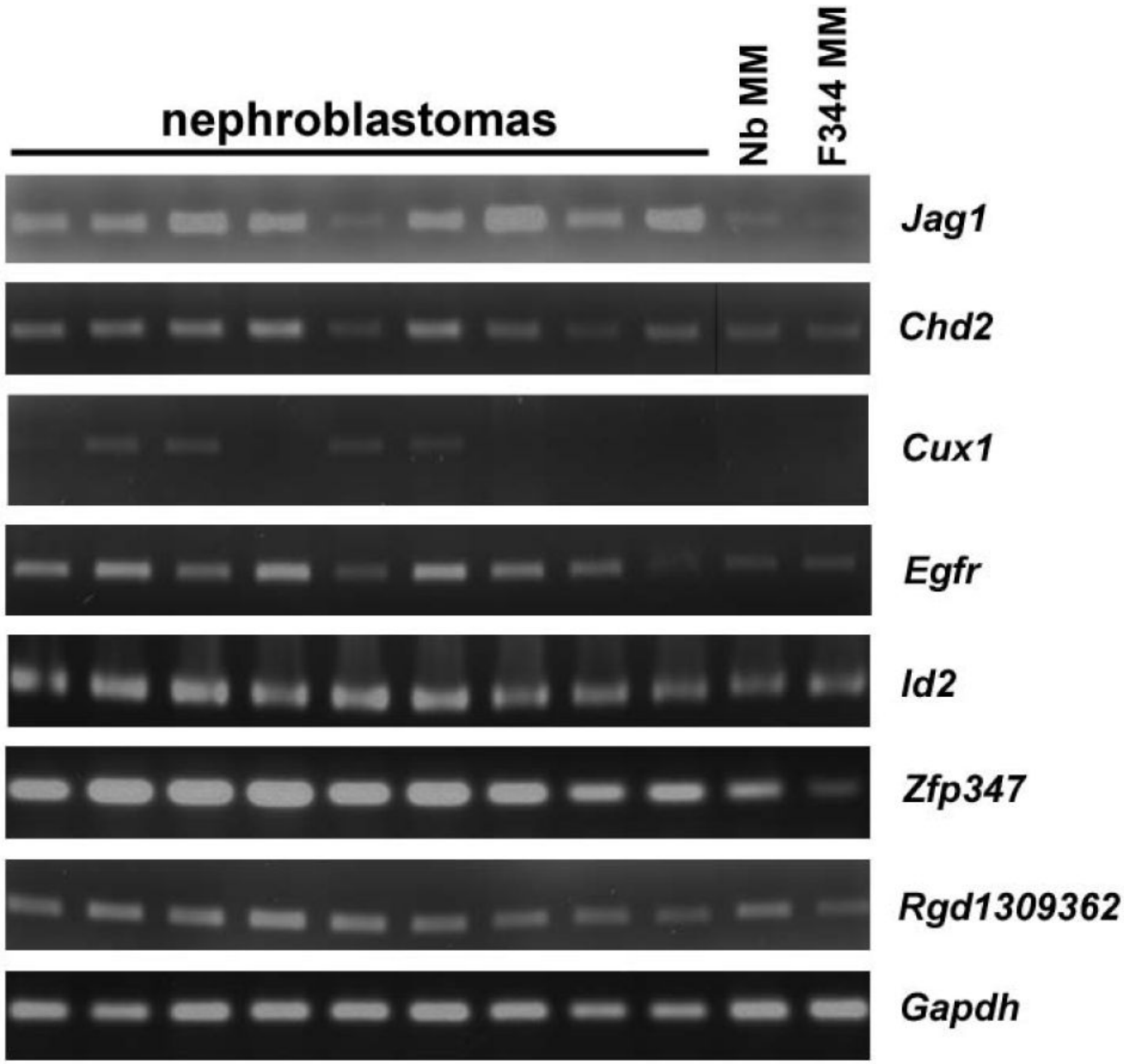

Figure 3.
Differential expression of genes in F344 or Nb rat metanephric mesenchymes and ENU-induced Nb rat nephroblastomas. Sequences were separated in 1% agarose gels.

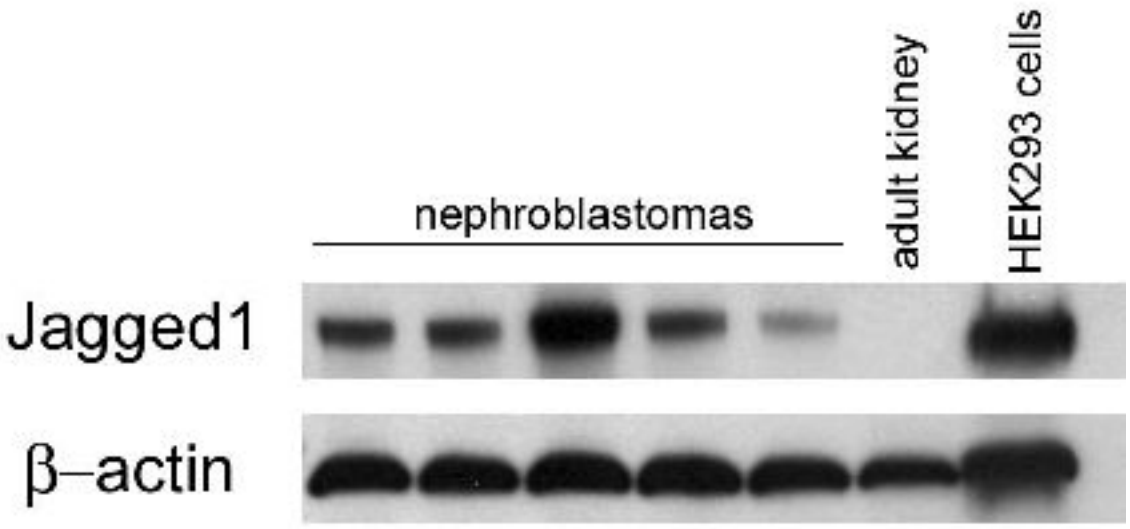

Figure 4.
Jagged1 is expressed in rat nephroblastomas. Western blot analysis demonstrates high levels of protein expression relative to adult rat kidney. HEK293 cells were used as a positive control.

Table 1

Incidence of kidney tumors induced in Nb and F344 rats and their hybrids by transplacental exposure to ENU.

Rat strain or hybrid	Effective number of animals	Number of rats with kidney tumors by histological type			
		Epithelial	Mesenchymal	Nephroblastic	Total
F344	30	0	2 (6%)	0	2 (6%)
Nb	30	1 (3%)	0	16 (53%)	17 (57%)
F_1: F344 × Nb	31	2 (6%)	0	8 (26%)	10 (32%)
F_1: Nb × F344	32	0	3 (9%)	10 (31%)	13 (41%)
F_1 × F344	32	2 (6%)	2 (6%)	5 (16%)	9 (28%)
F_1 × Nb	33	2 (6%)	1 (3%)	15 (46%)	18 (55%)
F_2	30	0	2 (6%)	9 (30%)	11 (37%)

Table 2

Observed frequency of transplacentally induced nephroblastomas in Nb and F344 rats and their hybrids exposed transplacentally to ENU, 0.5 mmole/kg body weight on day 18 of gestation, and expected numbers of nephroblastomas in the offspring assuming that each allele from the susceptible Nb parent contributes equally to nephroblastoma frequency in offspring with an Nb parent or grandparent.

Rat strain or hybrid	Number of offspring with nephroblastoma / effective number of animals	Percent offspring with nephroblastoma	
		Observed	Expected
F344	0/30	0	0
Nb	16/30	53	53
F_1	18/63	28	27
F_1 × F344	5/32	16	13
F_1 × Nb	15/33	46	41
F_2	9/30	30	28

Table 3

Non-renal tumors considered related to carcinogen exposure in F344 and Nb strain rats and their F_1, F_2 and backcross hybrids exposed prenatally to ENU.

Rat strain/hybrid	Effective number of animals	Neurogenic tumors		Thyroid follicular cell tumors
		CNS	PNS	
F344	30	14	15	3
Nb	30	16	18	8
F344 × Nb	31	13	20	8
Nb × F344	32	19	22	7
F_1 × F344	32	20	14	6
F_1 × Nb	33	21	17	8
F_2	30	19	15	5

Table 4

No missense mutations were found in *Wt1* or *Wtx* in Nb rat nephroblastomas. Previously, *Wt1* exon 1 mutations were reported in chemically induced Sprague-Dawley rat tumors (Sharma et al., 1994).

	Wt1		Wtx	
	Exon 1	**Zinc finger**	**bp 1-762**	**bp 743 –1586**
Nb rat nephroblastoma, ENU, transplacental, 18 gd	0/10	0/10	0/8	0/8
Sprague-Dawley rat nephroblastoma, MNU, subcutaneous, neonates (ref. 9)	4/4*	0/4	ND	ND

* all codon 111, T-to-A transversions

ND= not determined

Table 5

Annotated list* of genes differentially expressed between F344 and Nb rat 14-gd metanephric mesenchymes (MMs) or nephroblastomas. Highlighted genes are shown in Figure 2.

Affy ID	Gene Symbol	Gene Name	Microarray change fold (Nb MMs vs. F344 MMs)	Microarray change fold (Nephroblastomas vs. F344 MMs)
	Positive regulation of cell proliferation			
1370830_at	*Egfr*	Epidermal growth factor receptor	2.2	3.7
1392341_at	*Capns1*	Calpain, small subunit 1	2.1	-7.0
1375532_at	*Id2*	Inhibitor of DNA binding 2	2.0	8.0
	Cell surface receptor linked signal transduction			
1377950_at	*RGD1309362*	Similar to Interferon-inducible GTPase	8.0	18.4
1391602_at	*Drg1*	Developmentally regulated GTP binding protein1	2.6	1.4
1390723_at	*Catna1*	Catenin (Cadherin-associated protein), alpha 1	2.4	3.5
1384339_s_at	*Csnk2a1*	Casein kinase II, alpha 1 polypeptide	2.2	6.5
1390398_at	*Bmpr1a*	Bone morphogenetic protein receptor, type 1A	2.0	1.2
1388994_at	*Fzd6*	Frizzled homolog 6 (Drosophila)	1.9	1.1
1370418_s_at	*Bk*	Brain and kidney protein	1.9	-2.3
1392588_at	*Ripk5*	Receptor interacting protein kinase 5	1.7	-1.2
1368197_at	*Oprk1*	Opioid receptor, kappa 1	1.7	-21
1368725_at	*Jag1*	Jagged1	1.6	16.0
1387480_at	*Notch2*	Notch gene homolog 2	NC	2.6
1367802_at	*Sgk*	Serum/Glucocorticoid regulated kinase	1.7	1.1
	Regulation of transcription, DNA-dependent			
1368726_a_at	*Zfp347*	zinc finger protein 347	9.0	16.0
1380747_at	*Pbx1_predicted*	Pre-B-Cell Leukemia transcription factor 1	3.2	2.6
1379830_at	*Chd2_predicted*	Chromodomain helicase DNA binding protein 2	3.2	17.0
1385888_at	*Cux1/Cutl1*	Cut-like 1 (Drosophila)	2.3	5.7
1375661_at	*Sox11*	Sry-box containing gene 11	2.2	-1.1
1389419_at	*Zfp146*	Similar to zinc finger protein 146 (OZF)	1.9	1.6
1375426_a_at	*Khsrp*	Kh-type splicing regulatory protein	1.7	1.0
1374904_at	*Six1*	Sine oculis homeobox homolog 1 (Drosophila)	1.7	-1.3
1387036_at	*Hes1*	Hairy and enhancer of Split 1	NC	2.1
370656_a_at	*Homer1*	Homer homolog 1 (Drosophila)	1.6	-1.1

Affy ID	Gene Symbol	Gene Name	Microarray change fold (Nb MMs vs. F344 MMs)	Microarray change fold (Nephroblastomas vs. F344 MMs)

* The full list of genes differentially regulated is available upon request.