Effect of *p53* Status on Tumor Response to Antiangiogenic Therapy

Joanne L. Yu,[1,2] Janusz W. Rak,[3] Brenda L. Coomber,[4] Daniel J. Hicklin,[5] Robert S. Kerbel[1,2]*

The *p53* tumor suppressor gene is inactivated in the majority of human cancers. Tumor cells deficient in p53 display a diminished rate of apoptosis under hypoxic conditions, a circumstance that might reduce their reliance on vascular supply, and hence their responsiveness to antiangiogenic therapy. Here, we report that mice bearing tumors derived from $p53^{-/-}$ HCT116 human colorectal cancer cells were less responsive to antiangiogenic combination therapy than mice bearing isogenic $p53^{+/+}$ tumors. Thus, although antiangiogenic therapy targets genetically stable endothelial cells in the tumor vasculature, genetic alterations that decrease the vascular dependence of tumor cells can influence the therapeutic response of tumors to this therapy.

Genetic instability, a defining hallmark of the cancer cell, constitutes the major driving force behind acquired drug resistance. Angiogenesis inhibitors are new anticancer drugs considered potentially capable of circumventing or significantly delaying acquired drug resistance, because they target the normal—and hence genetically stable—host endothelial cell of a tumor's growing vasculature (*1*, *2*). The lack of acquired resistance has been reported in preclinical studies with certain direct-acting antiangiogenic agents (*3*, *4*) and when various angiogenesis inhibitory drugs are used in certain combinations (*5*), and likewise in the treatment of certain nonmalignant tumors in the clinic (*6*, *7*). However, there are examples of gradual loss of response, and perhaps acquired resistance to antiangiogenic drugs or treatment strategies, especially when the drugs are administered as monotherapies (*8*).

Genetic mutations commonly detected in cancer cells, such as those leading to inactivation of the wild-type *p53* tumor suppressor gene (*9*) can render cells less susceptible to apoptosis induced by hypoxic stress (*10*). Transformed $p53^{-/-}$ cells have a survival advantage over their $p53^{+/+}$ counterparts when cultured in vitro under hypoxic conditions (*10*). It is therefore conceivable that the selection and overgrowth of subpopulations with a reduced dependence on blood vessels could occur over time in the face of antiangiogenic therapy (*11*, *12*).

To investigate whether *p53* loss confers on tumor cells a resistance to hypoxia that might reduce the efficacy of antiangiogenic therapy, we compared the response of tumors derived from paired isogenic $p53^{+/+}$ and $p53^{-/-}$ HCT116 colorectal carcinoma cells (*13*). The cells were injected subcutaneously into SCID (severe combined immunodeficiency) mice, and once the tumors reached 100 mm^3 in size, treatment was initiated with DC101, an antibody directed against vascular endothelial growth factor receptor-2 (VEGFR-2), in combination with continuous low-dose vinblastine chemotherapy (*5*, *14*). Although the growth of all tumors was inhibited by treatment, there was a dramatic difference in response based on *p53* status (Fig. 1A). At 42 days after the initiation of treatment, the volume of the $p53^{-/-}$ tumors had increased sevenfold, compared with only a twofold increase in the $p53^{+/+}$ tumors. Similar results were obtained after treatment with DC101 alone, i.e., the results are not dependent or due exclusively to the low-dose vinblastine treatment (*15*).

The $p53^{-/-}$ tumors were much slower to respond to the DC101 and vinblastine therapy from the onset of treatment (Fig. 1B). The volume of treated $p53^{+/+}$ tumors decreased to 39.0 ± 7.0% that of untreated controls after only 7 days of therapy, whereas the volume of treated $p53^{-/-}$ tumors remained at 91.5 ± 13.7% that of untreated controls. It was not until after 21 days of therapy that the $p53^{-/-}$ tumors reached a similar 60% reduction in tumor volume as a percentage of controls.

Cancer cells that have lost *p53* function are thought to be selected for during tumor progression. To recapitulate this process, we established tumors from 1:1 mixtures of $p53^{+/+}$ and $p53^{-/-}$ cells, and treated them with DC101 and vinblastine (Fig. 2A). The composition of mixed tumors at the time of removal was ascertained by Southern blotting of tumor genomic DNA with a *p53*-specific probe (*16*). The percentage of $p53^{+/+}$ and $p53^{-/-}$ cells was determined from a standard curve after densi-

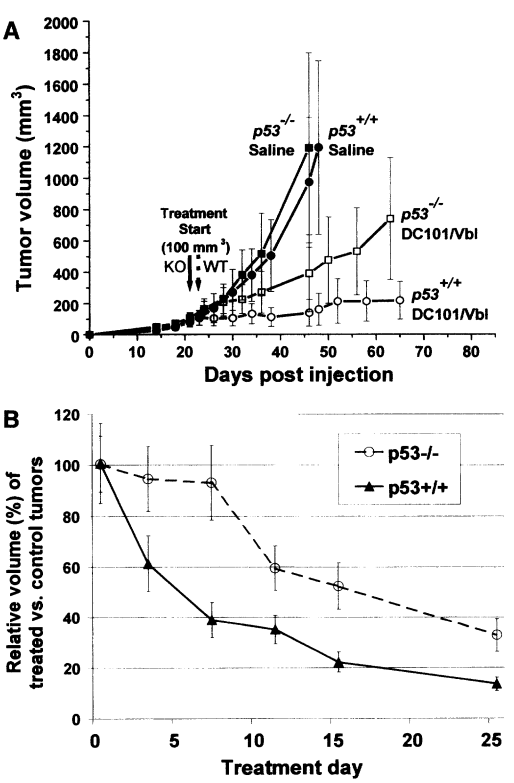

Fig. 1. Disruption of *p53* decreases sensitivity of HCT116 tumors to antiangiogenic therapy. (**A**) Mice bearing tumors established by subcutaneous injection of 10^6 cells were treated with twice weekly intraperitoneal injections of the DC101 VEGF receptor-2–specific (flk-1) neutralizing antibody (800 μg per mouse) and low-dose vinblastine (0.5 mg/kg), or an equal volume of saline (controls). Tumor volume (mm^3) was estimated from caliper measurements using the standard formula: (length × width^2)/2. For $p53^{+/+}$ (WT) tumors, n = 15 (control), n = 17 (treatment); for $p53^{-/-}$ (KO) tumors, n = 15 (control), n = 16 (treatment). (**B**) $p53^{-/-}$ tumors are slower to respond to DC101 and vinblastine treatment from the start, as demonstrated by plotting treated tumor volumes as a percentage of controls. All error bars represent SD.

[1]Sunnybrook and Women's College Health Sciences Centre, Molecular and Cellular Biology Research, Room S-218, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5. [2]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M5S 1A1. [3]Hamilton Civic Hospitals Research Centre, McMaster University, Hamilton, Ontario, Canada L8V 1C3. [4]Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1. [5]ImClone Systems, Inc., 180 Varick Street, 7th Floor, New York, NY 10014, USA.

*To whom correspondence should be addressed. E-mail: robert.kerbel@swchsc.on.ca

tometric analysis of bands corresponding to the wild-type *p53* allele and each disrupted $p53^{-/-}$ allele (*16*) (Fig. 2B). We found that the proportion of $p53^{+/+}$ cells decreased dramatically after antiangiogenic therapy (Fig. 2C). Although $p53^{+/+}$ cells made up 43.4 ± 3.0% of saline-treated tumors (slightly decreased from the initial 50%), they made up only 19.0 ± 11.0% ($P < 0.001$, *t* test) of the tumors that had received antiangiogenic treatment for 35 days (Fig. 2D). In 2 of the 12 treated mixed tumors, the band corresponding to the wild-type *p53* allele could not even be detected. Thus $p53^{-/-}$ cells showed a clear selective advantage during therapy.

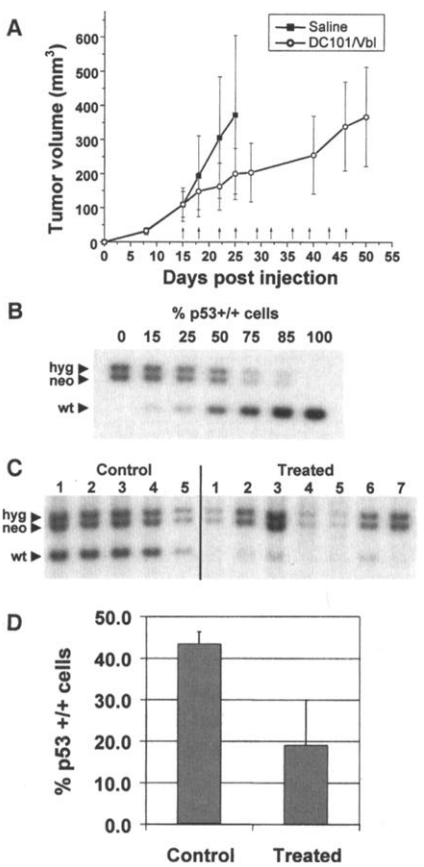

Fig. 2. Differential response of $p53^{+/+}$ and $p53^{-/-}$ tumors can be explained by decreased survival of $p53^{+/+}$ cells during treatment with antiangiogenic therapy. (**A**) Growth of mixed tumors initially comprised of a 1:1 mixture of $p53^{+/+}$ and $p53^{-/-}$ HCT116 colon carcinoma cells, and inhibition by DC101 and vinblastine treatment. Arrows indicate injections of DC101 and vinblastine or saline. (**B**) Southern blot of genomic DNA extracted from mixtures of known proportions of $p53^{+/+}$ and $p53^{-/-}$ cells, used to establish a standard curve by which the percentage of $p53^{+/+}$ cells in a tumor could be calculated from the relative intensities of the bands (*16*). (**C**) Southern blot of DNA extracted from tumors after saline (control) or DC101-vinblastine treatment, showing a marked decrease in intensity of $p53^{+/+}$ bands in treated mixed tumors. (**D**) Percentage of $p53^{+/+}$ cells is diminished in mixed tumors treated with antiangiogenic therapy ($P <$ 0.001, *t* test). $n = 12$ for control and treated groups. All error bars represent SD.

We next examined the distribution of $p53^{+/+}$ and $p53^{-/-}$ cells relative to the vasculature within a heterogeneous tumor. If $p53^{+/+}$ cells are more sensitive to hypoxia-induced death, one would predict that these cells would be localized predominantly in oxygen-rich perivascular tumor regions. To test this hypothesis, we established tumors from 1:1 mixtures of $p53^{+/+}$ and $p53^{-/-}$ cells, and labeled the cells most proximal to perfused vessels by intravenous injection of Hoechst 33342 dye (*12*). This procedure yields highly fluorescent cells immediately surrounding the vasculature and low fluorescence intensity in more distal, hypoxic areas. After enzymatic disaggregation of each tumor into a single-cell suspension, samples were analyzed by fluorescence-activated cell sorting (FACS), and the cells displaying the 5% highest and lowest Hoechst fluorescence intensities were collected and assayed for *p53* status (*17*). This analysis revealed that the proportion of $p53^{+/+}$ cells was diminished in the distal (Hoechst-dim) subpopulations (Fig. 3). The average relative percentage of $p53^{+/+}$ cells was 43.4 ± 1.7% in the proximal (Hoechst-bright) tumor cell samples, but this value was reduced to 20.6 ± 1.6% in cells collected from the distal, more hypoxic tumor regions ($P < 0.05$, *t* test). Thus, within a heterogeneous tumor, a greater proportion of $p53^{+/+}$ cells are found immediately surrounding perfused vasculature and are selectively lost from tumor regions distal to these vessels, an observation consistent with the possibility that $p53^{+/+}$ cells are more sensitive to hypoxia-induced apoptosis.

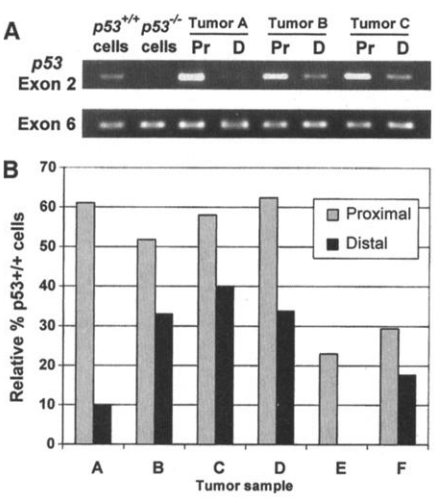

Fig. 3. Differential perivascular distribution of $p53^{+/+}$ and $p53^{-/-}$ cells in mixed tumors. (**A**) PCR analysis of p53 status in tumor cell populations (*17*). Genomic DNA amplified from $p53^{+/+}$ but not $p53^{-/-}$ cells contains exon 2, whereas exon 6 is present in both. Both bands are present after amplification of vessel-proximal and distal cells sorted from mixed tumors, but the exon 2 band is diminished in distal cells isolated from hypoxic tumor regions. Pr, proximal; D, distal. (**B**) The relative intensity of the exon 2 PCR product versus the exon 6 product for each DNA sample is proportional to the relative percentage of $p53^{+/+}$ cells and is decreased in cells "distal" to perfused vasculature ($n = 6$).

To examine the extent of apoptosis in hypoxic regions of $p53^{+/+}$ or $p53^{-/-}$ tumors, we intravenously injected EF5, a 2-nitroimidazole compound, into mice 3 hours before tumor excision. Under low-oxygen conditions, EF5 forms adducts with cellular macromolecules that are detectable by monoclonal antibodies (*18*). To assess apoptosis, we stained cryosections of $p53^{+/+}$ and $p53^{-/-}$ tumors first by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay using fluorescein-labeled nucleotides, and then with Cy5-labeled antibodies against EF5 to visualize hypoxic cells (*14*, *18*). As the TUNEL assay is not exclusive for apoptotic cells, much of the positive TUNEL signal occurred in necrotic, nonviable tumor regions. However, EF5 binding only occurs in viable cells (*18*). Thus, maximal EF5 binding occurred at the boundary between necrotic and apoptotic regions. We found that the frequency of apoptosis in hypoxic areas of $p53^{+/+}$ tumors was greater than in hypoxic regions of *p53*-deficient tumors, in agreement with previous studies (*10*, *14*).

We also found the above consequences of *p53* deletion to be unrelated to p53-dependent effects mediated by p21, a cell-cycle inhibitor that is essential for *p53*-mediated growth arrest after DNA damage (*19*). By performing similar Hoechst labeling experiments with $p21^{-/-}$ HCT116 cells (parental cells are $p21^{+/+}$) (*19*), we observed that disruption of *p21* did not have a significant effect on the capacity of these cells to survive in hypoxic or nutrient-deprived tumor regions (*14*). Indeed, the growth of both parental HCT116 ($p53^{+/+}$, $p21^{+/+}$) and $p21^{-/-}$ tumors was inhibited with similar kinetics after treatment with the DC101-vinblastine combination therapy (*14*).

These experiments demonstrate that the genetic background of a tumor cell, in this case the presence or absence of wild-type *p53*, may be an important determinant of response to antiangiogenic therapy. This study examined only one type of antiangiogenic therapy; however, one might expect that this mechanism would similarly influence the efficacy of other angiogenesis antagonists that cause increases in hypoxia. Although *p53* inactivation, either by direct mutation or by indirect alterations in *p53*-interacting genes, is a common feature in human malignancies, it is clear that alterations in *p53* represent only one example of the types of genetic lesion that could affect the vascular dependence or hypoxia-sensitivity of tumor cells. For example, changes in the hypoxia-inducible factor-1α (HIF-1α) pathway (*12*, *20*, *21*), potentially as a result of upstream oncogenic changes (e.g., ac-

tivated *ras*, *src*, or *HER-2*) could also result in altered cellular responses to hypoxia and could enhance the survival capacity of tumor cells under conditions of stress (*22–24*). Hence, one method of improving the efficacy of angiogenesis inhibitors might be to combine them with inhibitors of oncogene-mediated signal transduction, with the goal of counteracting such decreases in vascular dependence. A second method would be to combine antiangiogenic drugs with hypoxic cell cytotoxins, a new class of drugs that can overcome resistance of hypoxic cells to radiation and chemotherapy and can induce cytotoxic effects on their own (*25–27*). Combination bacteriolytic therapy (COBALT) could also be exploited in this manner (*28*). A third approach would be to use drugs such as vascular targeting agents, which can destroy existing tumor blood vessels and/or acutely disrupt blood flow (*29*, *30*) in combination with antiangiogenic drugs that block new blood vessel formation. These various combinations also highlight the possibility that, although tumor cell variants may be able to survive under certain conditions of hypoxia, they could not do so in anoxic conditions (*31*).

In summary, although tumor growth and progression are angiogenesis-dependent, our results suggest that it is essential to consider the possibility that the vascular dependence of tumor cell populations may be heterogeneous, variable, and quantitative, rather than absolute and qualitative in nature. These considerations not only have important implications for the design, scheduling, and monitoring of antiangiogenic therapies in future clinical trials, but also for the interpretation of results obtained from such trials.

References and Notes

1. R. S. Kerbel, *BioEssays* **13**, 31 (1991).
2. J. Folkman, P. Hahnfeldt, L. Hlatky, *Nature Rev. Mol. Cell. Biol.* **1**, 76 (2000).
3. T. Boehm, J. Folkman, T. Browder, M. S. O'Reilly, *Nature* **390**, 404 (1997).
4. T. Browder *et al.*, *Cancer Res.* **60**, 1878 (2000).
5. G. Klement *et al.*, *J. Clin. Invest.* **105**, R15 (2000).
6. R. A. Ezekowitz, J. B. Mulliken, J. Folkman, *N. Engl. J. Med.* **326**, 1456 (1992).
7. L. B. Kaban *et al.*, *Pediatrics* **103**, 1145 (1999).
8. R. S. Kerbel *et al.*, *Cancer Metastasis Rev.* **20**, 79 (2001).
9. E. R. Fearon, B. Vogelstein, *Cell* **61**, 759 (1990).
10. T. G. Graeber *et al.*, *Nature* **379**, 88 (1996).
11. J. Rak, R. S. Kerbel, *Cancer Metastasis Rev.* **15**, 231 (1996).
12. J. L. Yu *et al.*, *Am. J. Pathol.* **158**, 1325 (2001).
13. F. Bunz *et al.*, *Science* **282**, 1497 (1998).
14. Supplemental material is available on *Science* Online at www.sciencemag.org/cgi/content/full/295/5559/1526/DC1
15. J. L. Yu, unpublished observations.
16. Tumors were established by subcutaneous injection of a mixture of 10^6 $p53^{+/+}$ and 10^6 $p53^{-/-}$ cells. Genomic DNA was extracted from snap-frozen tumors after pulverizing the frozen tissue by using a prechilled mortar and pestle, and 15 μg of DNA digested with Hind III, followed by Southern blotting and hybridization with a genomic *p53* probe (*32*). Densitometry was performed using the Image Quant system (Molecular Dynamics), and the measured percentage of $p53^{+/+}$ cells was determined by dividing the intensity of the 2.5-kb wild-type band by the total intensity of the wild-type plus hyg/neo bands (3.5/3.7 kb) (*13*). The actual percentage of $p53^{+/+}$ cells in a tumor was determined from a standard curve established from Fig. 2B.
17. Tumor-bearing mice were injected via the tail vein with Hoechst 33342 dye, which was allowed to circulate for 20 min before tumor removal. A single-cell suspension suitable for FACS analysis was produced by disaggregation of tumor tissue with collagenase type III, hyaluronidase, and collagenase type IV, and then resuspension in PBS. Tumor cell samples were sorted on the basis of Hoechst fluorescence intensity on an EPICS Elite V flow cytometer. Genomic DNA extracted from sorted cells was subject to polymerase chain reaction (PCR) by using the following primers: *p53* (exon 2) 5′-TGG AAG TGT CTC ATG CTG GA-3′ and 5′-CAG AAC GTT GTT TTC AGG AA-3′; *p53* (exon 6) 5′-TTG CTC TTA GGT CTG GCC CC-3′ and 5′-CAG ACC TCA GGC GGC TCA TA-3′. After genomic DNA extraction from sorted cells, the relative proportion of $p53^{+/+}$ cells in the proximal and distal tumor subpopulations was determined using PCR to amplify genomic sequences in exon 2 and exon 6 of the *p53* gene. The relative intensity of the exon 2 PCR product versus the exon 6 product for each DNA sample would be proportional to the relative percentage of $p53^{+/+}$ cells in each population of sorted cells. Gel band intensities were quantified using the Quantity One Gel Doc system (Bio-Rad).
18. E. M. Lord, L. Harwell, C. J. Koch, *Cancer Res.* **53**, 5721 (1993).
19. T. Waldman, K. W. Kinzler, B. Vogelstein, *Cancer Res.* **55**, 5187 (1995).
20. P. Carmeliet *et al.*, *Nature* **394**, 485 (1998).
21. N. Akakura *et al.*, *Cancer Res.* **61**, 6548 (2001).
22. G. L. Semenza, *Crit. Rev. Biochem. Mol. Biol.* **35**, 71 (2000).
23. J. Rak, J. Filmus, R. S. Kerbel, *Eur. J. Cancer* **32A**, 2438 (1996).
24. M. V. Blagosklonny, *Int. J. Oncol.* **19**, 257 (2001).
25. J. M. Brown, *Drug Resist. Updat.* **3**, 7 (2000).
26. W. A. Denny, W. R. Wilson, *Expert. Opin. Investig. Drugs* **9**, 2889 (2000).
27. S. Masunaga *et al.*, *Int. J. Radiat. Oncol. Biol. Phys.* **47**, 799 (2000).
28. L. H. Dang, C. Bettegowda, D. L. Huso, K. W. Kinzler, B. Vogelstein, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 15155 (2001).
29. J. Griggs, J. C. Metcalfe, R. Hesketh, *Lancet Oncol.* **2**, 82 (2001).
30. D. J. Chaplin, G. R. Pettit, S. A. Hill, *Anticancer Res.* **19**, 189 (1999).
31. R. S. Kerbel, J. Folkman, *Nature Rev. Cancer*, in press.

32. We thank B. Vogelstein for the HCT116 cell lines and the genomic *p53* probe; S. Green, A. Viloria-Petit, and B. Mayer for animal care assistance; and L. Woodcock and C. Cheng for excellent secretarial assistance. Supported primarily by NIH grant CA-41233 (R.S.K.), a Canadian Institutes of Health Research doctoral research award (J.L.Y.), and separate National Cancer Institute of Canada grants to B.L.C. and J.R.

26 November 2001; accepted 16 January 2002

Transcriptional Regulation of Cortical Neuron Migration by POU Domain Factors

Robert J. McEvilly,* Marcela Ortiz de Diaz, Marcus D. Schonemann, Farideh Hooshmand, Michael G. Rosenfeld*

The identification of pathways mediated by the kinase Cdk5 and the ligand reelin has provided a conceptual framework for exploring the molecular mechanisms underlying proper lamination of the developing mammalian cerebral cortex. In this report, we identify a component of the regulation of Cdk5-mediated cortical lamination by genetic analysis of the roles of the class III POU domain transcription factors, Brn-1 and Brn-2, expressed during the development of the forebrain and coexpressed in most layer II-V cortical neurons. Brn-1 and Brn-2 appear to critically control the initiation of radial migration, redundantly regulating the cell-autonomous expression of the p35 and p39 regulatory subunits of Cdk5 in migrating cortical neurons, with *Brn-1*($^{-}$/$-$)/*Brn-2*($^{-}$/$-$) mice exhibiting cortical inversion.

Development of the mammalian cerebral cortex involves both radial migration of postmitotic neurons arising from the dorsal telencephalon and tangential migration of inhibitory interneurons originating in the ganglionic eminence (*1*, *2*). Analysis of murine mutations has partially defined two signaling pathways regulating radial migration of cortical neurons, one involving reelin, expressed in marginal zone Cajal-Retzius cells, which binds the receptors for reelin and activates the downstream effector molecule mDab1 in radially migrating neurons (*3–5*), and the second mediated by Cdk5 and its regulatory subunits, p35 and p39 (*6*, *7*).

To assess the potential roles of the class III POU domain transcription factors Brn-1 and Brn-2 (*8–10*) in cortex development, we examined the consequences of the deletion of the *Brn-1* (Web fig. 1, A to D) (*11*) and *Brn-2* (*10*) genomic loci, both separately and together. *Brn-1*($^{+}$/$-$) heterozygous mice were phenotypically normal; *Brn-1* gene–deleted mice were present at expected Mendelian ratios at birth but survived no longer than 36 hours postpartum, probably reflecting a defect in renal develop-

Howard Hughes Medical Institute, Department and School of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92037–0648, USA.

*To whom correspondence should be addressed. E-mail: rmcevilly@ucsd.edu, mrosenfeld@ucsd.edu