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Tight regulation of p53 activity by Mdm2 is required for ureteric bud growth and branching

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ABSTRACT

Mdm2 (Murine Double Minute-2) is required to control cellular p53 activity and protein levels. *Mdm2* null embryos die of p53-mediated growth arrest and apoptosis at the peri-implantation stage. Thus, the absolute requirement for Mdm2 in organogenesis is unknown. This study examined the role of Mdm2 in kidney development, an organ which develops via epithelial–mesenchymal interactions and branching morphogenesis. *Mdm2* mRNA and protein are expressed in the ureteric bud (UB) epithelium and metanephric mesenchyme (MM) lineages. We report here the results of conditional deletion of *Mdm2* from the UB epithelium. $UB^{mdm2-/-}$ mice die soon after birth and uniformly display severe renal hypodysplasia due to defective UB branching and underdeveloped nephrogenic zone. Ex vivo cultured $UB^{mdm2-/-}$ explants exhibit arrested development of the UB and its branches and consequently develop few nephron progenitors. $UB^{mdm2-/-}$ cells have reduced proliferation rate and enhanced apoptosis. Although markedly reduced in number, the UB tips of $UB^{mdm2-/-}$metanephroi continue to express *c-ret* and *Wnt11*; however, there was a notable reduction in *Wnt9b*, *Lhx-1* and *Pax-2* expression levels. We further show that the $UB^{mdm2-/-}$ mutant phenotype is mediated by aberrant p53 activity because it is rescued by UB-specific deletion of the *p53* gene. These results demonstrate a critical and cell autonomous role for Mdm2 in the UB lineage. Mdm2-mediated inhibition of p53 activity is a prerequisite for renal organogenesis.

Introduction

Congenital abnormalities of the kidney and urinary tract account for 30–40% of end-stage renal disease in infants and children (Bates, 2000; Cain et al., 2010). The etiology of a majority of them arises from defects in the fine balance between proliferation, survival, differentiation and the acquisition of physiological function. The definitive kidney or metanephros develops through reciprocal signaling between the ureteric bud (UB), an epithelial outgrowth of the Wolffian or mesonephric duct, and the metanephric mesenchyme (MM) (Costantini and Kopan, 2010; Lechner and Dressler, 1997). These proximate interactions result in reiterative branching of the UB leading to the formation of an elaborate collecting duct system. Meanwhile the MM assumes one of three fates, namely, epithelial renal vesicles (which will ultimately form the mature nephrons), stroma, or vascular elements (Dressler, 2009). Disruptions, either genetic or chemical, of the reciprocal signaling pathways result in impaired growth and/or differentiation of the component tissues leading to agenesis or dysgenesis of the kidney (Cain et al., 2010).

Tightly coordinated cell death and survival decisions have a direct impact on embryonic development and in adult tissue homeostasis. Deregulation of these processes results in pathological conditions. Owing to the growth inhibitory and apoptosis-promoting functions of the tumor suppressor p53, there are strict mechanisms in place to regulate p53 levels and activity under normal physiological conditions (Harris and Levine, 2005; Oren et al., 2002; Poyurovsky and Prives, 2006). Mdm2 is recognized as a potent negative regulator of p53 stability and function (Lozano and Montes de Oca Luna, 1998; Wade et al., 2010).Mdm2 was originally identified as an amplified gene on double-minute chromosomes in transformed mouse fibroblasts (Fakharzadeh et al., 1991). Subsequently, it was found to interact with p53 and to inhibit its function through two main mechanisms: first, the direct binding of Mdm2 to the N-terminal domain of p53 inhibits the transcriptional activation function of p53 (Momand et al., 1992; Oliner et al., 1993); second, Mdm2 possesses E3 ubiquitin activity that targets p53 for degradation through the 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1997). The vital interaction between Mdm2 and p53 was clearly revealed by elegant genetic studies which showed that the peri-implantation lethality of *Mdm2* null mice can be circumvented by the concomitant deletion of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). Remarkably, *Mdm2* itself is in turn a transcriptional target of p53 and together they form an autoregulatory feedback loop (Barak et al., 1993; Perry et al., 1993). In contrast, the closely related partner, Mdm4, is not transcriptionally regulated by p53 (Marine et al., 2007).

The Mdm2–p53 signaling network has been implicated in the development and/or homeostasis of several cell lineages. Genetic

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mouse models that are homozygous null, hypomorphic or haploinsufficient for *Mdm2* demonstrate that the Mdm2–p53 signaling network supports the timely proliferation, differentiation, and survival of several cell lineages namely hematopoietic, lymphopoietic, cardiomyocyte, osteoblast, hepatocyte, neural, and intestinal cells (Francoz et al., 2006; Grier et al., 2006; Lengner et al., 2006; Mendrysa et al., 2003; Terzian et al., 2007; Xiong et al., 2007). Notably, most (but not all) phenotypes associated with either a partial or total loss of Mdm2 from selected tissues are efficiently rescued by the concomitant deletion of *p53* or a reduction in *p53*dosage. In certain tissues (e.g., heart), other members of the Mdm2 family, such as *Mdm4*, exert redundant developmental functions with *Mdm2*. With regard to the kidney, there is suggestive evidence that *Mdm2* or *Mdm4* gene function is necessary for normal kidney development. $Mdm2^{+/-}$; $Mdm4^{+/-}$ double heterozygous mice that survived to postnatal day (P) 20 have small kidneys with very few glomeruli in addition to marked hypoplasia and/or atrophy of the thymus and spleen (Terzian et al., 2007). Using transgenic mice that express wild type p53 within the ureteric bud, Godley et al. (1996) demonstrated the need to titrate the expression levels of p53 for kidney development to proceed normally. These transgenic mice exhibit acute kidney degeneration at E17.5 reaching half the size of normal kidneys with fewer glomeruli that are hypertrophic and fibrotic. Excessive p53 interferes with the differentiation of the ureteric buds and secondarily causes apoptosis in the adjacent metanephric mesenchyme and its limited conversion to epithelium (Godley et al., 1996). Given that Mdm2 is a potent negative regulator of p53, by extrapolation it may be inferred that Mdm2 could have a significant role in embryonic kidney morphogenesis. Here, we have characterized the spatial and temporal expression of Mdm2 in the developing kidney in mice. Utilizing a conditional approach, we examined the consequences of *Mdm2* loss exclusively from the UB lineage on metanephric development. We demonstrate that Mdm2 has an essential role in maintaining normal branching morphogenesis and/or survival of the UB epithelium. Strikingly, the loss of *Mdm2* appears to disrupt *Wnt9b*, *Lhx-1* and Pax2 signaling, essential for the reciprocal induction of metanephric mesenchyme and the organization of epithelial progenitors of renal vesicles. Genetic rescue experiments suggest that the renal phenotypes encountered were largely p53-dependent.

Materials and methods

Animals

All animal protocols utilized were in strict adherence to guidelines established by the Institutional Animal Care and Use Committee. For timed pregnancies, noon of the day on which the vaginal plug was detected was regarded as embryonic day (E) 0.5. The *Mdm-2flox* mice (01XH9, Dr. Mary Ellen Perry) were obtained from the NCI mouse repository (Frederick, MD) while the *R26R-EYFP* reporter mice (006148, Dr. Frank Constantini) were purchased from the Jackson Laboratory (Maine, USA). The specifications for genotyping were furnished by the respective companies. The *Hoxb7–Egfp–Cre* mice (Zhao et al., 2004) were a kind gift from Dr. Carlton Bates. The breeding strategy used involved crossing *Hoxb7–Egfp–Cre*; $mdm2^{flox/+}$ transgenic mice to either $mdm2^{flox/flox}$ or $R26R^{EYFP/+}$; $mdm2^{flox/+}$ mice. For our rescue experiments we used both conventional and conditional approaches: accordingly we bred (1) *Hoxb7–Cre*$^{+}$; $p53^{+/-}$; $mdm2^{flox/+}$ mice to *Hoxb7–Cre*$^{-}$; $p53^{+/-}$; $mdm2^{flox/+}$ mice or (2) *Hoxb7–Cre*$^{+}$; $p53^{flox/+}$; $mdm2^{flox/+}$mice to *Hoxb7–Cre*$^{-}$; $p53^{flox/+}$; $mdm2^{flox/+}$ mice.

Reverse transcriptase-PCR

RNA was isolated from the kidneys using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was reverse transcribed using Random Hexamers and a first-strand synthesis kit (SuperScript II; Invitrogen) according the manufacturer's recommendations. The forward and reverse primers for *Mdm2* had the following sequence: 5′ ATG TGC AAT ACC AAC ATC TCT GTG TC 3′ and 5′ GCT GAC TTA CAG CCA CTA AAT TTC 3′ respectively and yielded a 337 bp PCR product. The PCR product corresponded to nucleotides 202–538 of the mouse *Mdm2* sequence deposited under GenBank Accession number X58876. The cycling parameters used were 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min.

Quantitative reverse transcriptase–PCR

Quantitative reverse transcriptase–PCR was performed on total RNA isolated from embryonic kidneys using the RNeasy Mini Kit (Qiagen). Real-time primer-probe mixes for *Mdm2* (ID Mm00487652-g1 Mdm2), *p53* (ID Mm01731287_m1), and *Gapdh* (Mm99999915-g1) were ordered from Applied Biosystems. The Taqman expression assay was constituted using reagents from the Brilliant II QRT–PCR 1-step Master mix kit (Cat #600809, Agilent Technologies). The thermal profile used was as follows: 50 °C for 30 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s, 56 °C for 1 min and 72 °C for 30 s. The reactions were done in triplicate. The scale bars represent the standard error of mean.

Gross morphology

All bright field images were captured on a Nikon SMZ1000 stereomicroscope mounted with a DS-Fi 1 camera with the aid of NIS-Elements F2.20 software.

Histological staining

Periodic acid-Schiff staining (PAS)

PAS staining on paraffin tissue sections was done using the Periodic Acid-Schiff Stain Kit (Richard-Allan Scientific). Briefly, deparaffinized and rehydrated sections were incubated in Periodic acid solution for 5 min and Schiff Reagent for 15 min. Hematoxylin 1 was used to stain the nuclei and Bluing Reagent to intensify the color. The slides were finally dehydrated in graded alcohol series, cleared in Xylene and mounted using Permount mounting media.

Hematoxylin and Eosin staining

Routine histological staining using H and E involved incubation in Hematoxylin 2 for 2 min and Eosin Y (Richard-Allan Scientific) for 1 min.

Organ culture

Ex vivo kidney explants were grown in 6-well transwell plates with 0.4 μm pore size filters (Corning Inc). E12.5 kidneys were cultured in Advanced DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin streptomycin at the air and medium interface in 5% $C0_2$ atmosphere at 37 °C. To study branching morphogenesis the kidney explants were cultured for 48 h and images captured using an Olympus BX51 fluorescence microscope.

Immunohistochemistry

The kidneys were fixed in 10% buffered formalin and processed for paraffin embedding. Four micrometer paraffin sections were subjected to antigen retrieval (10 mM sodium citrate, pH 6.0) after deparaffinization and rehydration steps. Primary antibodies used were polyclonal rabbit anti-Mdm2 at 1:100 dilution (ab15471-1 Abcam), *Dolichos biflorus* lectin (1:40 dilution, Sigma) and rabbit anti-Pax2 at 1:100 dilution (Zymed), Fluorescein Lotus Tetragonolobus lectin at 1:100 dilution (FL-1321, Vector Laboratories, Inc.), polyclonal goat anti-AQP2 at 1:500 dilution(sc-9882, Santa cruz), rabbit anti-