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The Significance of Expression Pattern of Dab1 and Reelin in the Postnatal Bladder of Dab1 -/- (Yotari) Mice

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ABSTRACT

Background. The aim of our study is to provide an insight into the genetic expression landscape of Dab1 and Reelin, which are important in human genitourinary tract development and could help elucidate the critical stages of the onset of bladder anomalies.
Methods. Yotari, heterozygote and wild type mice were sacrificed on the 4th, 11th and 14th postnatal day. Morphological parameters were analyzed using immunohistochemistry on mice bladder samples.
Results. Significant expression of Dab1 was observed in the bladder epithelium during the 4th postnatal day and in the wt - wild type, but also in the yot -/- mouse. Reelin expression was significant in bladder epithelium during the 4th postnatal day in the wild-type and mutant mouse type. A significant expression of Dab1 positive cells in lamina propria was observed on the 4th postnatal day in yot -/- and in wt. The percentage of Reelin positive cells in lamina propria was significant on the 4th postnatal day in mutated and wild-type mice.
Conclusions. An increase in the expression of Dab1 and Reelin proteins in the bladder of a yotari mouse may indicate bladder damage, due to congenital renal abnormalities and caused by a mutation in the Dab1 gene.

Key words: Dab1; Reelin, CAKUT, bladder development, yotari mice

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INTRODUCTION

Congenital anomalies of the kidneys and urinary system (CAKUT) account for around 20% of all congenital malformations that occur in one in 500 live births. CAKUT causes chronic renal failure in 40-50% of pediatric and 7% of adult patients diagnosed with CAKUT (1). Lower urinary tract abnormalities can be identified by in utero ultrasound in approximately 50% of cases (2). These anomalies include, but are not limited to, renal agenesis, renal hypodysplasia, multicystic dysplastic kidney, hydronephrosis, ureteropelvic joint obstruction, megaureter, ureteral duplex, vesicoureteral reflux and posterior urethral valve (3). Numerous studies support the influence of epigenetic and environmental factors on renal development and the natural history of CAKUT, suggesting a multifactorial pathogenesis of this syndrome (4). An intrauterine diet low in protein can cause congenital anomalies of the kidney and the urinary system phenotype (5). Moreover, the offspring of hyperglycemic or diabetic females showed a significant deficit in the number of nephrons in rats (6). It has long been shown that in utero exposure to alcohol and drugs also increases the incidence of the development of certain CAKUT phenotypes (7,8). Some findings, however, suggest that pre-pregnancy obesity may be associated with an increased risk of CAKUT at the population level (9). Nevertheless, based on studies conducted on experimental animals, it is considered that gene mutations could be the leading cause of CAKUT anomalies. In approximately 30% of cases, CAKUT or some form of CAKUT, occurs as part of a multiorgan gene syndrome, such as Fraser syndrome, Alagille syndrome, Apert syndrome, etc. (10). To date, it has been confirmed that mutations in around 30 genes cause syndromes that include CAKUT, such as: JAGGED1, FGFR2, GATA3, DSTYK, etc. (10, 11). Reelin is a large, extracellular glycoprotein that is necessary for proper neurological development and plays an important role in the plasticity of the adult brain. In addition, Reelin is expressed in many extraneural tissues. However, its role on the periphery has been insufficiently explored. In the brain, many Reelin functions are performed by signaling downstream pathways that include two lipoprotein receptors, apolipoprotein E receptor-2 (Apoer2) and very low-density lipoprotein (VLDL), then neuronal phosphoprotein Disabled-1 (Dab1), and members of the Src family of tyrosine kinases as crucial elements (12). Disabled-1 (Dab1) is a phosphoprotein that acts as an intracellular adapter in protein kinase pathways (13). DAB1 is found in the cytoplasm and plays a vital role as an adapter protein, specifically associated with neuronal migration and polarization. This discovery is further supported by the yotari or DAB1 - / - mouse model. The yotari mouse model produces the DAB1 protein, although an aberrant form because it cannot be phosphorylated. Expression analysis shows that DAB1 protein is expressed in a population of neurons exposed to Reelin. Similar phenotypes of reeler, scrambler, yotari and mdab1 null mice suggest that Reelin and DAB1 act as signaling molecules that regulate the position of cells in the developing brain (14). However, outside of nervous tissue, DAB1 has been confirmed as occurring in rodent small intestines, mouse retina, human breast cancer, and in mouse kidneys with special expression in podocytes and in the distal convoluted tubule. A minimal positive expression of Dab1 was found in the yotari mice in the proximal convoluted tubule (15). The DAB1 gene plays a major role in renal development and its mutation potentially causes damage (13). With respect to the kidney, DAB1 expression appears to be more important in the fetal period (and thus kidney development), than in the post-natal period. DAB1 also plays a potentially significant role during fetal kidney development. It shows strong expression in the proximal and distal tubules and in the glomeruli, suggesting its regulatory role in tubule formation or in maintaining function during development. In the postnatal kidney, the percentage of Reelin positive cells decreased

dramatically in all observed structures. The decreased expression of DAB1 and Reelin after birth implies a gradual loss of their role in the postnatal period. Poor localization of these two markers suggests their diverse role during renal development (16). In the autosomal recessive mutated yotari mouse, there is a replacement of part of the Dab1 gene with a long-interspersed fragment of the nuclear element (17). Yotari mice express a mutated form of mdab1 mRNA and little or no mDAB1 protein (2). Yotari mutants are recognizable by unstable gait ("Yotaru" in Japanese means "unstable gait") and shivering, they are also characterized by an early death during weaning from the mother and have a lower body weight compared to the healthy group. The cerebellum of homozygous yotari mice is hypoplastic (18).

Numerous studies show that Dab1 and Reelin play a key role during brain development, not only in mice but also in humans, especially in the organization of brain architecture patterns. In addition, DAB1 and Reelin are also expressed in non-brain tissues, therefore, more systematic data on their extraneural localization during development are required. Given the fact that the expression of these proteins in the kidneys has been proven, there is a reasonable suspicion that they are localized in other parts of the urinary system. We hypothesize that DAB1 and Reelin may play an important role during bladder development. Furthermore, numerous studies suggest that mutations in the Dab1 gene could cause CAKUT.

The aim of this study was to determine the pattern of expression of Dab1 and Reelin proteins in the 4-, 11- and 14-day-old postnatal bladders of yotari, heterozygous and wild-type mice.

PARTICIPANTS AND METHODS

Experimental animals

Three groups of pups were observed according to their Dab1 gene status: yotari (Dab1 -/-), heterozygotes (Dab1 +/-) and wild type (Dab1 +/+) controls. The yotari mice were produced by PGK-neo cassette, which resulted in the target disruption of the first 47 codons of the gene coding for the protein-interlacing domain (PI-PTB). Heterozygotes were produced by standard manipulation of blastocysts and mouse breeding. In standard polycarbonate cages, at least one of each genotype of mouse were group-housed and raised. Their access to water and food was ad libitum, and their environment was a temperature-controlled (23±2°C) room with a 12-h light/dark cycle.

Tissue collection and immunohistochemistry

On postnatal days 4, 11 and 14, mice were deeply anesthetized with pentobarbital and transcardially perfused with a saline solution of phosphate buffer (PBS, pH 7.2) and 4% paraformaldehyde (PFA) in 0.1 M PBS. The bladders were isolated and fixed in 4% PFA in 0.1 M PBS overnight.

After fixation in 4% PFA in 0.1 M PBS overnight and rinsing with working PBS, the bladder tissue is dehydrated in a growing series of solutions of ethanol (25%, 50%, 75%, 90%, 100%) and xylene, fitted into paraffin blocks. 5 µm thick sections were cut with a micro-tome and placed on slides. Within each yotari, heterozygous and wild group, four glasses consisting of two bladder sections were assigned per group for further processing and analysis. Prior to histological analysis, samples were deparaffinized in xylene, rehydrated in a series of growing ethanol solutions (100%, 90%, 75%, 25%) and washed in water.

After dewaxing and rehydration, the samples were boiled in citrate buffer (pH=6.2) in a kettle for 30 minutes, cooled to room temperature, washed with working PBS, separated with PAP foam and incubated with Protein block solution for 20 minutes. The samples were incubated overnight in a humid chamber with primary antibodies: Rabbit Polyclonal An-ti-Dab1 and Mouse Monoclonal Anti Reelin. After washing with PBS, secondary antibodies were applied and incubated in a humid chamber, in the dark, for 1 hour. After rinsing with PBS, DAP1, core,

dye was applied, and the samples were fitted into Immu-Mount medium and covered with a coverslip.

Samples were examined on a fluorescence microscope (Olympus BX51, Tokyo, Japan) and imaged on an objective magnification x40 digital camera (DP71, Olympus). Microphotographs were processed and overlapped using Adobe Photoshop software (Adobe, San Jose, CA, USA) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA), by counting the number of positive or immunoreactive and negative cells. Immunoreactive cells were determined by the intensity of color staining (green for Dab1) in bladder tissue. Colocalization studies of Dab1 and Reelin proteins in the bladder tissue were also performed.

Statistical analysis

A qualitative analysis of staining intensity was determined semiquantitatively and organized into four groups: no staining (-), mild staining (+), moderate staining (++), strong staining (+++). Each level of nuclear, membrane or cytoplasmic signal was characterized as positive. The Kruskal-Wallis test with Dunn's post hoc analysis was used for quantitative analysis. The percentage of Dab1 and Reelin positive cells was calculated in two layers (epithelium, submucosa) of the bladder, and was compared between groups of yotari, heterozygous and wild-type mice. Three different sequences of animal sacrifice were performed (4th, 11th and 14th postnatal day). The percentage of positive cells is expressed as mean ± standard deviation (SD). The level of statistical significance was set at $p<0.05$.

RESULTS

Since yotari mice were produced by the PGK-neo cassette, resulting in a targeted disruption of the domain responsible for the phosphorylation of the Dab1 protein, the expression of the DAB1 protein is also present in yotari mice, but it is dysfunctional.

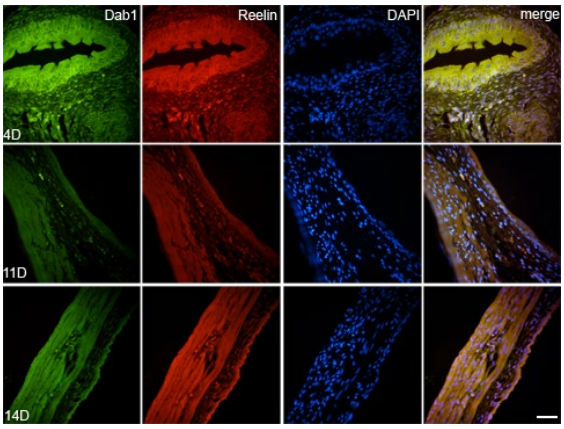

Figure 1.a. Immunofluorescence staining for Dab1 (green), Reelin (red) and DAPI (blue stained nuclei) of the postnatal bladder of a wild-type mouse (wt); merge - folded coloring images on DAB1 and Reelin and DAPI. Representative images of the 3 postnatal days 4D, 11D and 14D were taken; D - postnatal day. The 20 μm scale applies to all panels.

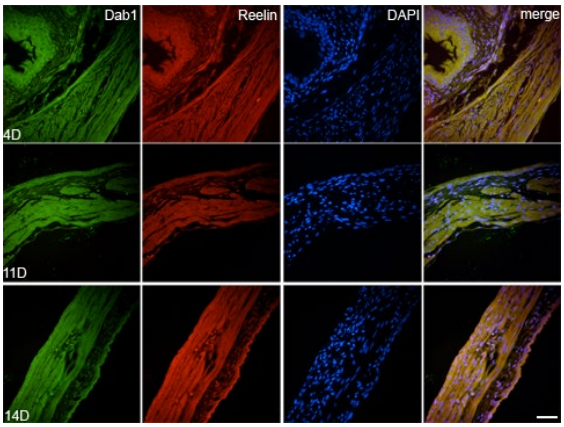

Figure 1.b. Immunofluorescence staining for Dab1 (green) and Reelin (red) and DAPI (blue stained nuclei) of the postnatal bladder of a heterozygous mouse (yot +/-); merge - folded coloring images on Dab1 and Reelin and DAPI. Representative images of the 3 post-natal days 4D, 11D, and 14D were taken; D - postnatal day. The 20 μm scale applies to all panels.

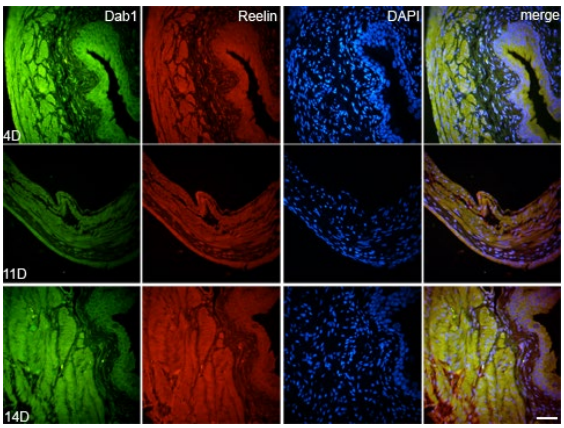

Figure 1.c. Immunofluorescence staining for Dab1 (green), Reelin (red) and DAPI (blue stained nuclei) of the postnatal bladder of mutated mice (yot. - / -); merge - folded coloring images on Dab1 and Reelin and DAPI. Representative images of the 3 postnatal days 4D, 11D and 14D were taken; D - postnatal day. The 20 µm scale applies to all panels.

A significant expression of DAB1 was observed in bladder epithelium during the 4th post-natal day and in wt - wild type mice (98%), but also in yot. - / - mice (81%). However, DAB1 expression decreased significantly in wild-type mice after 11 (8%) and 14 (7%) postnatal days, whereas this percentage decreased more slowly in yotari mice at 61% on the 11th postnatal day and 52% on the 14th postnatal day (Figure 2.a, 2.c).

Reelin protein expression was significant in bladder epithelium during the 4th postnatal day in the wild (60%) and mutated (28%) mouse types. On the 11th postnatal day, the percentage of cells decreased, therefore, in the wild-type mice it was 4% and in the mutated mouse 21%. The percentage decrease continued on the 14th postnatal day; in yot. - / - mice, it was 18%, while in wt mice, there was a slight increase (5%) (Figure 2.a, 2.c).

A significant expression of DAB1 positive cells in lamina propria was observed on the 4th postnatal day in yot - / - (15%), and in wt (11%) mice. There was no significant difference in the following days (Figure 2.a, 2.c).

The percentage of Reelin-positive cells in lamina propria was significant on the 4th post-natal day in mutated (8%) and wild (7%) mouse types (Figure 2.a, 2.c).

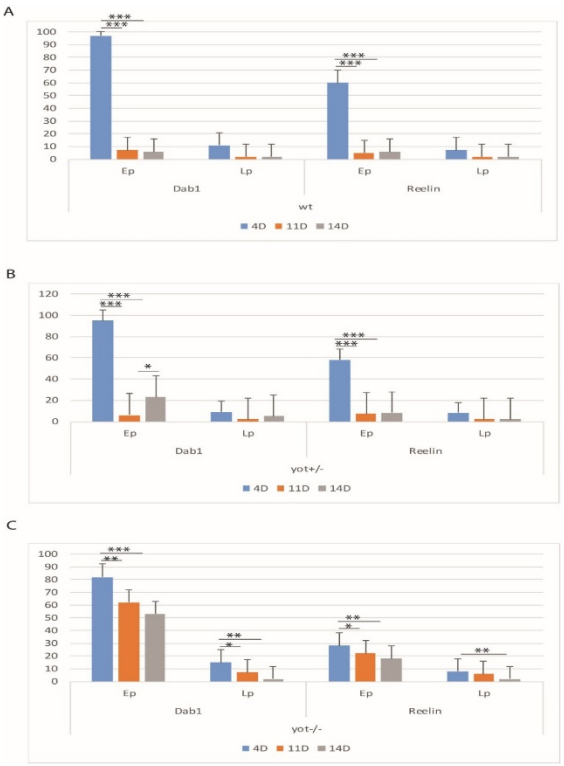

Figure 2. Distribution of the percentage of DAB1-positive cells and Reelin-positive cells during the 4th, 11th and 14th postnatal day (D) in the bladder tissue of mice of the wt - wild-type (a), heterozygous mice (yot +/-) (b) and mice off Dab1 (yot - / -) (c). Ep - epithelium, Lp - lamina propria. Data were tested by the Kruskal – Wallis test and presented as median ± interquartile range. A significant difference is shown as *** p<0.0001.

DISCUSSION

After searching the current literature, it was observed that no research has been conducted to date on the expression of DAB1 and Reelin protein in the postnatal bladder, therefore, our results form a good basis for further research in this area. Although research into the expression and role of DAB1 and Reelin proteins in the bladder is only in its infancy, their importance in establishing central nervous system function has long been known. The importance of the Reelin signaling pathway in the migration of several hindbrain nuclei (19) as well as in the aging process, and its potential role in the pathogenesis of neurological diseases, such as schizophrenia, autism and Alzheimer's disease has been demonstrated (20,21). More An increasing number of scientists are regarding DAB1 and Reelin proteins as potential

therapeutic targets for mental illness. Dab1 and Reelin proteins also play a role in the digestive system, where their expression in the colonic mucosa has been demonstrated. The amount of Reelin protein expressed decreases in colon cancer before an epithelial-mesenchymal transition occurs, making it a promising biomarker for colon cancer (22). In addition to the colon, Reelin is also expressed in small bowel myofibroblasts (23). Recent studies have confirmed the presence of Dab1 and Reelin proteins during fetal and post-natal kidney development in humans and mice. It has been shown that the kidneys of mouse yotari are affected by hypoplasia, a disorder from the CAKUT spectrum, as well as congenital nephrotic syndrome, caused by the alignment of the podocyte legs (24,25). Thanks to these results, it can be assumed that Dab1 and Reelin proteins also play a role in the development of the bladder, so we decided to prove their expression by immunofluorescence. By analyzing immunofluorescence staining images, we concluded that the percentage of Dab1 and Reelin-positive cells was significantly higher in the epithelium compared to lamina propria, suggesting the importance of these proteins in maintaining bladder function. Similar results have so far been found in the kidney, where these proteins are almost exclusively expressed in the epithelial components of the kidney (24,25). It was also observed that the percentage of Dab1 and Reelin positive cells decreased significantly in the epithelium and lamina propria of the bladder, among all genotypes, in the later stages of the postnatal period. It is important to note that these results are consistent with the results of previous studies stating that Dab1 and Reelin protein expression is more significant during fetal and early postnatal development of the human kidney compared to the later stages of postnatal development. These results may suggest that Dab1 and Reelin proteins are factors influencing the establishment of the proper function of both the kidney and bladder during development, while postnatally, their role is gradually lost (25). Moreover, our study showed that in yotari mice of 11D and 14D, the percentage of Reelin positive cells in the epithelium and lamina propria was significantly higher than in wild-type mice. These results are also consistent with the results of a study by Racetin et al., who found a significant increase in Reelin protein expression in the glomeruli of yotari mice compared to wild-type mice (24). It has been shown that the kidneys of mouse yotari are affected by hypoplasia and congenital nephrotic syndrome (24) and it can be assumed that the increase in Reelin-positive cells in the glomeruli, and consequently, in the bladder is due to adaptation to changes in glomeruli. The limitation of our study is that only one immunohistochemistry was used to investigate protein expression and, therefore, the quantitative level cannot be established. It is necessary to investigate the prognostic and diagnostic potential of Reelin and DAB1 markers, as well as the potential for targeted therapeutic action in the treatment of bladder tumors.

CONCLUSIONS

Our study showed that Dab1 and Reelin proteins are expressed in the bladder, with different expression patterns between the observed structures, epithelium and lamina propria, but also between the observed time points (4P, 11P, 14P). An increase in Dab1 and Reelin protein expression in the bladder of a yotari mouse may indicate bladder damage, due to congenital renal abnormalities caused by a mutation in the Dab1 gene. To confirm this conclusion, further examinations of the morphological structure, but also the function of both the kidney and the bladder, are needed.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Katarina Vukojević - conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, resources, validation, visualization, software, writing of original draft.

Jelena Čurčić wrote the manuscript with support from Katarina Vukojević, Anita Racetin, and Nela Kelam. All authors have read and agreed upon the published version of the manuscript.

ETHICAL BACKGROUND

Institutional review board statement: The experimental protocol was approved by the Ethics Committee of the University of Split School of Medicine and conducted according to the Croatian Animal Welfare Act (Classification No. 910-08-17-02-0009; Registry No.:2181-198-03-04-0024). The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board No. 910-08-17-02-0009 ur.br. 2181-198-01-01-17-0003 from 10.02.2017.

Informed Consent Statement: Non applicable.

Data Availability Statement: The work did not form databases.

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