Transcriptome analysis of a rat PKD model: Importance of genes involved in extracellular matrix metabolism

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Transcriptome analysis of a rat polycystic kidney disease (PKD) model: importance of genes involved in extracellular matrix metabolism. PKD is a common genetic cause of chronic renal failure, and is characterized by the accumulation of fluid-filled cysts in the kidneys and other organs. Abnormalities in the expression of selected genes thought to be involved in cystogenesis have been described, but no systematic analysis of the global transcriptomal pattern has been reported. With this aim, a rat oligomicroarray was used to identify variations in gene expression in Han:Sprague–Dawley Cy/Cy rats, an animal model presenting a severe PKD phenotype. Some upregulated genes were validated using real-time polymerase chain reaction in Cy/Cy and Cy/+ rats. Among the 350 genes identified as being upregulated, we found about 30 genes involved in extracellular matrix metabolism. These genes encoded proteins or peptides that could be implicated into two different biological processes: molecules involved in fibrosis and proteins involved in adhesion to the extracellular matrix. In heterozygotes, some genes (*glypican 3, fibronectin 1*) were already upregulated in early stages of the disease. We conclude that differential regulation of genes linked to extracellular matrix metabolism may be one of the first events leading to tubule enlargement and subsequent cyst formation in PKD.

Autosomal-dominant polycystic kidney disease (PKD) and the other genetic forms of cystic kidney disease are responsible for more than 5% of the worldwide total of end-stage renal failure cases.[1] Despite the identification of the genes responsible for these diseases and the elucidation of numerous key events in the development of PKD, particularly the role of the primary cilium, there is still no treatment available for PKD patients. Detailed knowledge of the different pathways associated with the progression of renal failure in autosomal-dominant PKD will undoubtedly provide new therapeutic avenues for the management of this condition.

PKD may be caused by different mechanisms, genetic or not, and is characterized by a progressive evolution involving the following phases. The first phase involves dilatation of the tubules. Subsequently, invaginations appear within the dilated tubules, followed by the formation of cysts, which are no longer related to the original tubule. The growth of the cysts characterizes the final phase.[1] The formation and growth of large numbers of cysts leads to chronic renal failure and ultimately end-stage renal failure.

Although the histopathology of the progression of the disorder has been described in detail, few data are available regarding the potential role of variations in gene expression in the progression of PKD. Moreover, most of these data concern only part of the cellular and tissue-specific transcriptome.[2] Grace of the international efforts in genome and EST sequencing, most of the transcriptional units have been identified in the three most commonly used mammalian systems: humans, mice and rats.[3,4] This has allowed the performance of large-scale transcriptional analysis in these models.

We have performed a transcriptional analysis of normal and polycystic kidneys, in order to track changes in gene expression that may be associated with cystic disease. Our strategy was to use total kidneys, in order to represent the natural situation as closely as possible. This choice was dictated by the difficulty of culturing kidney cystic cells, and to avoid the possibility of artefactual transcriptional changes associated with the *in vitro* culture conditions necessary for the promotion of kidney cell growth. Obviously, our biological samples are not homogeneous in term of their

constituent tissues, but *in situ* hybridization may be used to refine the analysis, as well as immunocytochemistry on tissues sections.

In order for our analysis to be significant, it was necessary to use a model in which the number of cysts per kidney was high. For this reason, we have chosen Han:Sprague–Dawley (SPRD) rats, a model of inherited polycystic kidney disorder.[5,6] The gene involved in this disorder is located on chromosome 5, but has not yet been isolated.[7] Han:SPRD rats provide a model of severe PKD, and homozygotes (Cy/Cy) usually die within the first month after birth. Heterozygotes (Cy/+) develop a progressive autosomal-dominant PKD in which cysts appear after 2 to 3 months of life, and increase in number and size throughout the lifespan.[6] Variations in the expression of genes involved in several signalling pathways have been previously described in this model.[8–11] These different pathways have been confirmed in other models or in the human disease.[12–14] Hence, despite the lack of identification of the mutant gene, Han:SPRD rats provide insights into human autosomal-dominant PKD.

RNAs from normal and Cy/Cy homozygote rats were used to perform a transcriptomal analysis using rat oligonucleotide microarrays. We observed that a large number genes involved in extracellular matrix metabolism were overexpressed. We also used some of this information to describe variations in gene expression related to progression of the disease in Cy/+ rats. Here, we report these data and discuss the potential impact of these variations in terms of two important events in PKD: fibrosis and loss of adherence to cell matrix.

RESULTS AND DISCUSSION
In order to identify variations in gene expression associated with PKD, RNAs were extracted from the kidneys of normal and 3-week-old Cy/Cy rats and a transcriptomal analysis was performed using rat oligomicrochips. For the synthesis of the probes used in the initial screen, RNA samples from the normal rats were labelled with Cy3 and those from Cy/Cy rats with Cy5. In a reciprocal experiment, the same samples were labelled in the inverse sense, that is, a dye-swap approach. Genes that were found to be over- or underexpressed in the initial screen and that behaved in a reciprocal manner in the second screen (e.g. underexpressed in the first screen and overexpressed in the second screen) were selected and included in a minilibrary. The expression profiles of these genes were then compared to hybridization data from four different microarrays hybridized with RNAs originating from four different Cy/Cy animals. The reference sample was the RNA isolated from the wild-type littermates used in the first two screening experiments. Genes that maintained their expression profiles in the initial dye-swap experiment as well as the subsequent microarray screens were analyzed in more detail; this final subset of differentially regulated genes represented more than 90% of those selected for inclusion in the dye-swap minilibrary. A total of 440 genes presented variations in their expression profiles with a *P*-value <0.05 as

predicted by the bioinformatic software; of these, 350 were upregulated and 90 were downregulated.

We then grouped these genes according to their molecular function, and evaluated the percentage on these different groups present in the up- and downregulated genes sets (Figure 1). Three different functional categories of genes present in the upregulated group were of particular interest to us: the cytoskeletal/structural genes, the extracellular matrix genes, and the cell communication genes. As the frequency of these groups of genes in the transcriptome of Cy/Cy rats is higher than the expected frequency in transcriptome of normal animals, we chose to focus on this aspect. To note, the relevance of fibrotic processes in PKD disease has been discussed extensively.[15–17] We observed that around 30 genes with either a structural function or a role in extracellular matrix protein synthesis or degradation were overexpressed in the kidneys of the Cy/Cy animals (Table 1). Interestingly, these genes could be further separated into two different biological processes. The first comprises genes involved in fibrosis. These encode either signalling proteins, such as connective tissue growth factor (CTGF) or transforming growth factor-β (TGF-β) or small peptides such as dermatan sulfate proteoglycan-II, glypican, and
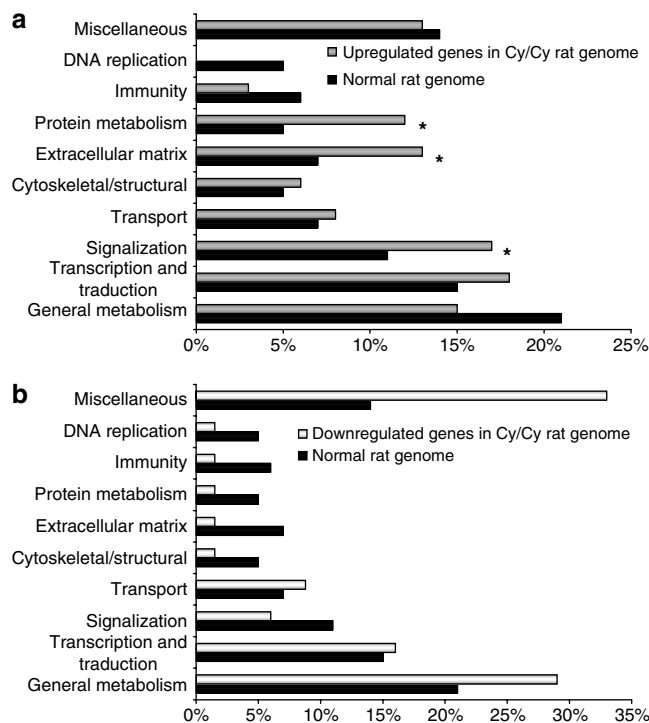
Figure 1 | Gene classification by their molecular function. Genes identified as either up- or downregulated were classified according to the information obtained from Gene Ontology and compared with the classification in normal rat kidney transcriptome. Percentages were calculated using the total numbers of up- or downregulated genes. (**a**) Groups of upregulated genes. (**b**) Sets of downregulated genes. An asterisk indicates the molecular function groups that highly differ from overall normal rat transcriptome and up/down-regulated transcripts. Only three classes, all upregulated in pathological kidneys, diverge from the normal distribution.

Table 1 | Relevant upregulated genes in Cy/Cy rats, identified using rat oligo-microarrays, divided into molecular function categories

| Sequence description	P-value	Fold change
Extracellular matrix proteins		
Cytoplasmic linker 2 (*Cyln2*)	8.11E−04	1.48
Glycoprotein (transmb) nmb (*Gpnmb*)	1.82E−11	2.40
Vimentin (*Vim*)	2.70E−08	1.99
Lectin galactose-inding, soluble 3 (*Lgals3*)	1.50E−05	1.68
Transmembrane 4 (*Tm4sf3*)	4.57E−09	2.08
Integrin alpha v subunit	4.86E−05	1.73
Syndecan 1 (*Sdc1*)	2.82E−06	1.53
Glypican 3 (*Gpc3*)	6.80E−6	1.71
Collagen XVIII	1.65E−05	1.67
Collagen XII alpha 1 (*Col12a1*)	1.71E−04	1.56
Leucyl-spc. aminopeptidase PILS (*APPILS*)	1.16E−04	1.52
Fibromodulin (*Fmod*)	2.09E−10	2.26
Biglycan (*Bgn*)	8.65E−06	1.70
Dermatan sulfate proteoglycan-II (*decorin*)	1.51E−07	1.90
Fibronectin 1 (*Fn1*)	2.88E−04	1.53
Matrix Gla protein (*Mgp*)	2.46E−06	1.76
Extracellular matrix protein 2 (*Ecm2*)	0.00E+00	1.31
Fibrinogen, gamma polypeptide (*Fgg*)	3.04E−12	2.51
Matrix protein metabolism		
Matrix metalloproteinase 14, m-inserted (*Mmp14*)	6.82E−05	1.43
Matrix metalloproteinase 7 (matrilysin) (*Mmp7*)	1.72E−08	2.12
Tissue inhibitor of Mmp 2 (*Timp2*)	4.25E−06	1.74
A disintegrin and metalloproteinase with thrombospondin motifs 1 (*ADAMTS-1*)	2.84E−04	1.53
Ser (or cys) proteinase inhibitor, clade E, member 1 (*Serpine1*)	3.04E−09	2.14
Cathepsin D (*Ctsd*)	7.68E−09	2.06
Cathepsin K (*Ctsk*)	1.67E−08	2.04
Ser (or Cys) proteinase inhibitor (*PAI*)	3.04E−09	2.14
Lysozyme (*Lyz*)	1.05E−08	2.04
Cell–cell communication		
Interferon gamma receptor (*Ifngr*)	3.66E−07	1.86
Tumor necrosis factor receptor (*Tnfr1*)	2.16E−07	1.89
Connective tissue growth factor (*Ctgf*)	7.37E−13	2.60
Latent TGF beta-inding protein 2 (*Ltbp2*)	1.71E−12	2.55
Latent TGF beta-inding protein 1 (*Ltbp1*)	6.52E−04	1.36
TGF-β-timulated clone 22 (*Tgfb1i4*)	8.36E−11	1.81
TGF-β2 (*Tgfb2*)	1.03E−05	1.71
Neuregulin 1 (*Nrg1*)	3.34E−04	1.57
Ciliary neurotropic factor (*Cntf*)	2.27E−05	1.67

Fold changes indicated the logarithm of the ratio in intensities of hybridization of the Cy/Cy versus the normal kidney RNAs. The *P*-values were calculated by the Rosetta software, taking into account the relative intensites as well as the ratio of these intensities.

syndecan.[18–21] The second category represents genes corresponding to proteins associated with cell migration and adhesion during embryonic/morphogenetic processes, such as fibronectin 1 (Fn1), integrin alpha v subunit, and collagen XII.[22] Finally, a series of genes encoding metalloproteases that are involved in extracellular matrix protein proteolysis were also found to be overexpressed.[23–25] Surprisingly, almost none of the genes identified as being downregulated were found to be associated with extracellular matrix protein metabolism.

In order to verify these data, we performed real-time polymerase chain reaction (RT-PCR) analysis on RNAs from Cy/Cy and +/+ rats. Analyzed genes were chosen among which are shown the highest and the lowest fold change values. An example is shown in Figure 2. We found that all the genes tested presented similar variations in their expression patterns upon either microarray screening or

RT-PCR analysis (Table 2), thereby validating the expression data obtained from the initial microarray screens.

As our data related to only the most severely affected animals (Cy/Cy), we also analyzed RNAs for the *CTGF*, *glypican 3*, *syndecan 1 (Sdc1)*, *Fn1* and *dermatan sulfate proteoglycan-II* genes in 1-, 3-, and 8-month-old Cy/+ rats using the real-time PCR assay. We correlated the expression profiles of these genes with the pathophysiology of the disease by measuring the serum creatinine levels of the animals over the period of the experiment. Figure 3 shows the levels of serum creatinine in the Cy/+ animals throughout their lifespan. At 4–6 months, the animals presented the first signs of renal failure, and their kidney function was dramatically impaired by 8 months of age. As cyst progression has already been described in this model by means of histological analysis,[6] few studies have used serum creatinine levels as a marker of renal function.
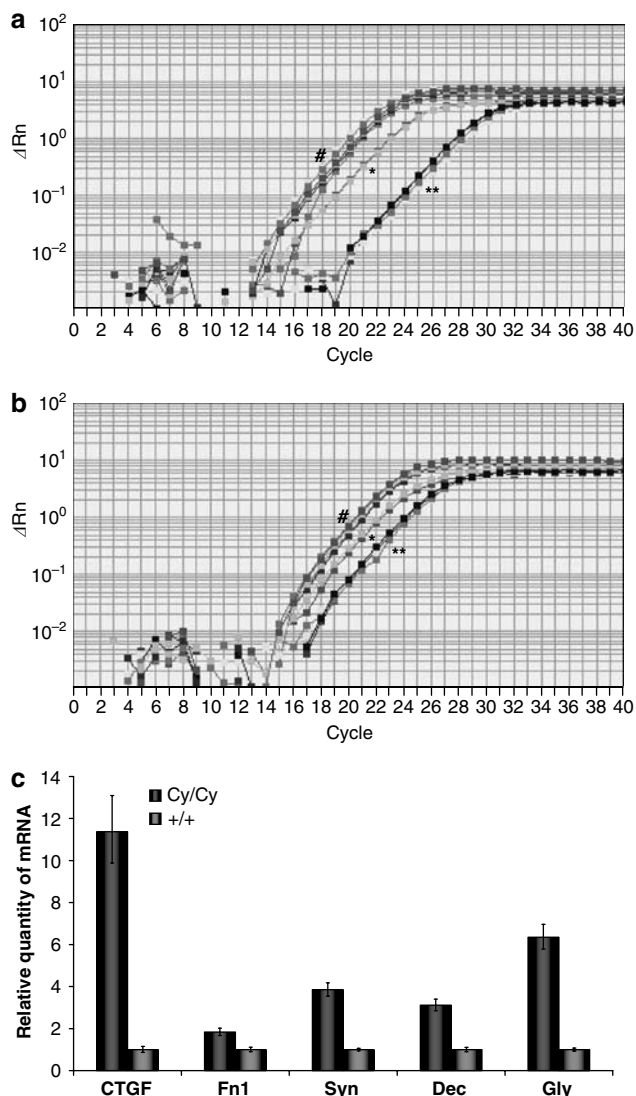
Figure 2 | Quantitative PCR. (**a** and **b**) Amplification plots for the *CTGF* and *glypican 3* cDNAs. #Amplification curves for *Rps29*, the housekeeping gene: *Amplification curves for the test gene in Cy/Cy rats. **Amplification curves for the test gene in normal rats (+/+). (**c**) Relative amounts of the mRNAs of several genes in Cy/Cy rats.

Table 2 | Real-time PCR validation of the microarray results

	$\Delta\Delta C_T$ in Cy/Cy rats relative to normal (+/+)	Relative quantity Cy/Cy versus (+/+)	*P*-value
CTGF	3.20 ± 0.20	11.37 ± 1.61	0.0143
Fn1	0.88 ± 0.14	1.84 ± 0.17	0.0143
Sdc1	1.95 ± 0.12	3.85 ± 0.32	0.0143
Decorin	1.64 ± 0.13	3.11 ± 0.18	0.0143
Gpc3	2.67 ± 0.13	6.35 ± 0.59	0.0143

CTGF=connective tissue growth factor; decorin=dermatan sulfate proteoglycan-II; Fn1=fibronectin 1; Gpc3=glypican 3; Sdc1=syndecan 1.
The same RNAs samples, originating from Cy/Cy or normal rat kidneys, were subjected to real-time PCR quantification, using specific primers corresponding to the genes identified as being overexpressed in the microarray experiments (see Materials and Methods). The expression of selected genes was compared to that of *Rps29*. Results were expressed related to those of the normal rats (+/+). $\Delta\Delta C_T$ indicates the logarithm of the relative ratio of the hybridization signal between Cy/Cy versus normal rats. Relative quantity represents the ratio, in terms of numbers of molecules, between the two samples. The *P-value* indicates the statistical significance of these results.

Figure 3 | Serum creatinine profile evolution in Cy/+ rats during the lifespan follow-up. Numbers on the *X*-axis represent the ages of animals in months. The basal levels calculated from +/+ animals are represented by the control column.
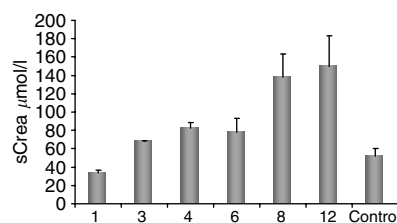
From expression data presented in Table 3, we were able to divide the genes tested into two categories. The expression of the genes involved in inflammation/fibrosis (*CTGF* and *dermatan sulfate proteoglycan-II*) was only slightly increased in the Cy/+ males at 1 month of age. However, the expression of these genes increased significantly with the onset of renal failure and followed the progression of the disorder. *Fn1*, *Sdc1*, and *glypican 3* expressions were increased in the male heterozygotes at 1 and 3 months of age, before the appearance of cysts and/or the onset of renal failure. These genes are clearly involved in developmental processes. *Fn1* is a major player in early embryonic stages and cell migration,[26] whereas *glypican-3* is implicated in Simpson–Golabi–Behmel syndrome and has recently been linked to renal development.[27,28]

What do these results tell us in terms of the pathophysiology of the renal cystic disorders? They indicate the occurrence of two types of events. The first requires the genes encoding the proteins or peptides involved in inflammatory processes and fibrosis. It has long been known that fibrosis and necrosis are associated with the cystic disorders.[29] Moreover, abundant fibrosis has been described in the Han:SPRD rat.[30] Although it is difficult to deduce from these observations which event may trigger the onset of fibrosis, it is generally assumed that the expression of *CTGF*, *dermatan sulfate proteoglycan-II*, and *Sdc1* is stimulated by *TGF-β*, which may be the initial event in this whole process.[31] Stimulation of the expression of *TGF-β* itself could be owing to an increase in the cAMP pool, as has already been described.[32] Interestingly, the expression of these genes increased with progression of the disorder, indicating a link between this increase and progression of the fibrosis. Furthermore, the increase in expression of these genes is low in young Cy/+ animals, where few cysts are detected, indicating that fibrosis/inflammation occurs at a later stage, and may be linked to the increase in the number and size of the cysts seen during disease progression.

The second type of event involves the extracellular matrix proteins linked to morphogenic processes. It is possible that variations in the expression of these proteins may be involved

Table 3 | Real-time PCR results from Cy/+ rats compared to +/+ animals

	1 month			3 months			8 months		
	$\Delta\Delta C_T$	RQ	*P*-value	$\Delta\Delta C_T$	RQ	*P*-value	$\Delta\Delta C_T$	RQ	*P*-value
CTGF	0.05 ± 0.27	1.04	NS	1.16 ± 0.18	2.23	0.02	1.40 ± 0.11	2.64	0.02
Fn1	1.87 ± 0.12	3.66	0.0014	1.61 ± 0.08	3.04	0.0014	1.42 ± 0.11	2.67	0.0014
Sdc1	0.60 ± 0.10	1.51	0.025	1.73 ± 0.17	3.31	0.002	2.11 ± 0.14	4.32	0.002
Decorin	0.29 ± 0.37	1.22	NS	—	—	—	2.05 ± 0.06	4.15	0.0028
Gpc3	0.84 ± 0.16	1.79	0.0043	1.87 ± 0.06	3.65	0.0043	2.67 ± 0.54	6.36	0.0043

CTGF=connective tissue growth factor; decorin=dermatan sulfate proteoglycan-II; Fn1=fibronectin 1; Gpc3=glypican 3; Sdc1=syndecan 1; NS=not significant.
RNA samples were isolated from the Cy/+ group animals at different ages then subjected to RT-PCR quantification, using specific primers corresponding to the genes previously tested in Cy/Cy animals. The housekeeping gene used as a control was *Rps29*. All results are expressed as relative to those in the normal rats. *P* indicates the *P*-value of the results when compared to those of the control animals. RQ indicates relative quantity of RNA molecules in Cy/+ rats, where RQ=1 corresponds to +/+ rats.

in the loss of adhesion of tubular cells to the matrix, leading to dilatation of the tubules and subsequent cyst formation. These genes may thus be good candidates for one of the first events that occur during the asymptomatic phase of PKD, a stage that is currently poorly understood. Interestingly, variation in gene expression is high in young Cy/+ rats, and for *Fn1*, no variation was seen during the progression of the disorder. This could be an indication that upregulation of these genes is an early event that could be linked leading to cyst formation.

In conclusion, if similar variations in gene expression can be validated in other PKD models, we propose that the genes that we have identified in this study will prove to be appropriate targets for new rational therapies for this disorder.

MATERIALS AND METHODS
Animals and tissue samples
Han:SPRD rats were obtained from Dr N Gretz and were kept in our local core animal facility. At 3 weeks of age, before the Cy/Cy rats usually die, males were anesthetized with isoflurane and their kidneys were removed and snap-frozen in liquid nitrogen. Clear morphological evidence led us to choose Cy/Cy animals, but DNA analysis was used for genotyping Cy/+ and +/+ rats. DNA samples were isolated from hair specimens, and were then genotyped by amplification using the d5rat9 primers (UniSTS: 122170; 127–136 bp)[33] Kidneys from male Cy/+ and +/+ rats were also obtained at 1, 3, and 8 months of age (five in each group). In order to assess renal function in Cy/+ rats over their lifespan, blood samples were collected from their tail veins and the plasma creatinine levels were determined using an autoanalyzer (Olympus AU400).

RNA extraction and analysis
After pulverization of the renal tissue in liquid nitrogen, total RNA was extracted using Trizol in accordance with the manufacturer's instructions. The quality and concentration of each sample was tested using an Agilent 2100 bioanalyzer. Five samples were prepared for each group (+/+, Cy/+, and Cy/Cy).

Hybridization and microarray scanning
The following materials were purchased from Agilent Technologies, France: the Low RNA Input fluorescent linear amplification kit (5185-818), the *in situ* hybridization kit Plus (5184-568), and the 60-mer rat oligomicroarray kit (G4130A) (for additional information on the oligomicroarrays visit www.agilent.com). The reverse transcription labelling reactions and hybridizations essentially followed the protocol recommended by Agilent Technologies. Briefly, a 10 µg aliquot of each total RNA sample was reverse transcribed into a cDNA probe using an oligo(dT) primer. The reaction was carried out in a solution containing 500 µM dNTP and 400 U MMLV reverse transcriptase at 40°C for 2 h, and then terminated by incubation at 65°C for 15 min. The reverse transcription and incorporation reactions were performed at 40°C for 2 h in a mixture containing either 400 µM cyanine 3-carbamylated protein (Cy3; for the +/+ samples) or 400 µM cyanine 5-carb-amylated protein (Cy5; for the Cy/Cy samples), the transcription mix, and T7 RNA polymerase. For purification of the amplified labelled cRNA samples, RNeasy spin columns (Qiagen, SA, France) were used according to the manufacturer's protocol. Hybridization was carried out in 440 µl of a mixture containing 750 ng of Cy3-and Cy5-labelled cRNA probes, control targets, and the fragmented DNA supplied by the manufacturer at 60°C for 17 h. The microarrays were then washed according to the manufacturer's protocol, dried, and scanned using an Agilent microarray scanner with a 532 nm laser for Cy3 measurement and a 635 nm laser for Cy5 measurement. The results were analyzed using a Luminator bioinformatics platform (Rosetta Inc., Rosetta Biosoftware, WA, USA).

Reverse transcriptase-PCR analysis
To confirm the differential gene expression patterns, we observed between the Cy/Cy and +/+ animals, we selected five upregulated genes, namely, *CTGF, Fn1, Sdc1, dermatan sulfate proteoglycan-II,* and *glypican 3,* and verified their levels of expression by quantitative reverse transcriptase-PCR using an ABI PRISM 7700 sequence detection system. The housekeeping gene *Rps29* was used for data normalization.[34] The results were treated using the comparative C_T method, where the amount of the target, normalized to the endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_T}$ We also followed the changes in the expression of these genes in Cy/+ rats of 1, 3, and 8 months of age.

Statistical analysis
The reverse transcriptase-PCR data were analyzed with the non-parametric Mann–Whitney test owing to the small number of samples. We used the Prism v4 software to perform the statistical analysis.

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