W3080

Phenotypic Characterization of Leucine-rich Repeat Kinase 2 (LRRK2) Knockout Rat Model: A Multi Parameter Cross Platform Biomarker Study

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Abstract

PURPOSE:
Early diagnosis and intervention are crucial for delaying disease progression. Developing and characterizing genetically modified rat Parkinson's Disease PD model(s) has proved to be a promising approach for the identification of crucial markers in clinical development. Mutations in Leucine-rich repeat kinase 2 (LRRK2) are recognized as the frequent cause associated with autosomal dominant PD. A multiple parameter biomarker feasibility study was conducted in the Lrrk2 knockout rat to further characterize the phenotypic effects of the knockout and validate the model against previous findings.

METHODS:
Male 11 weeks old Homozygous Lrrk2 Knockout Rat (LEH- Lrrk2 tm1sage; Product number: TGRL4620) and age matched controls were used. The kidney, brain, blood, serum, urine and CSF were collected for histology, bioanalytical and genomic biomarker analysis. Biomarker analysis was performed using EMD Millipore MilliplexMAP® kits for the measurement of kidney and brain neuropeptide markers. Histology and gene expression analysis (qPCR) were conducted using standard approaches.

RESULTS:
Kidney: Lrrk2 knock Rat Renal Periodic Acid-Schiff (PAS) staining indicated the abnormality morphological changed in the proximal tubule (Figure 1).
Urine samples were analyzed for the presence of nine different biomarkers. Only NGAL (Lipocalin-2) showed a statistical difference (P<0.001; Figure 2) supporting previous studies. RNA analysis of kidney tissues demonstrated a decrease (P<0.01) in Clusterin.
Brain: CSF and Serum from control and Lrrk2 rats were analyzed for neuropeptide markers by immunoassay and no discernible differences were detected. However, there were four RNA expression markers (a-synuclein, GFAP, Oxytocin, Substance P) identified in hippocampus with noticeable differences between control and Lrrk2 rats (Figure 3).
CONCLUSION: The Lrrk2 knockout phenotype provides a unique opportunity to investigate the potential for early detection biomarkers for PD as well as other conditions having mutations

PURPOSE:

Parkinson's disease (PD) is a slow, progressive neurodegenerative disease affecting 1.5% of the population over 50 years (1). PD is characterized clinically by motor symptoms such as tremors at rest, slow movements and postural instability. The motor symptoms of PD result from the death of dopamine-generating cells in the substantia nigra, however these symptoms will develop only after ~70% of the neurons in this portion of the mid-brain have already perished. The pathology of the disease is characterized by the accumulation of α-synuclein into inclusions in neurons called Lewy bodies (1). In addition there is an insufficient formation and activity of dopamine produced within parts of the midbrain. Current treatments are effective at managing the early motor symptoms of the disease, mainly through the use of synthetic dopamine and dopamine agonists.

Early diagnosis and intervention are crucial for delaying disease progression. Developing and characterizing genetically modified rat PD models have proved to be promising approaches for the identification of crucial biomarkers in target identification for drug discovery and translational clinical development (5). Although most cases of PD are idiopathic in nature, genetic models of PD are helping to provide a glimpse into this progressive neurodegenerative condition. Mutations in Leucine-rich repeat kinase 2 (LRRK2) are recognized as a frequent cause associated with autosomal dominant PD. As a joint venture, SAGE® Labs and Eurofins Bioanalytical Services conducted a multi-parameter biomarker feasibility study to evaluate the LRRK2 knockout rat model utilizing an array of biomarker testing capabilities in IHC, transcriptome analysis, multianalyte analysis and flow cytometry to further characterize the phenotypic effects of the knockout and validate the model against previous findings (2-5).

Materials & Methods:

Animal Model: SAGE® Labs uses Zinc-Finger Nuclease (ZFN) Technology, for the rapid creation of targeted gene knockout animals. Male 11 weeks old Homozygous LRRK2 Knockout Rat (LEH-LRRK2 tm1sage; Product number: TGRL4620) and age matched controls were provided by SAGE® Labs. The brain and kidney from three to six of each animal type were collected in either in 10 % Neutral buffered formalin or 4% paraformaldehyde for histology and morphological analyze respectively. Remaining tissues and blood from all animals were processed for transcriptome, flow cytometry and multiplex analysis.

Biological Fluid Biomarker Analysis: Urine, serum and cerebral spinal fluid were analyzed using EMD Millipore MilliplexMAP® kits for the measurement of kidney and brain neuropeptide markers. These assays, using xMAP® technology, give the ability to obtain multiple results from a single sample in a single well through the use of distinct fluorescence dyed beads with corresponding antibodies attached. Reporter antibodies linked to a second fluorescent dye allow for specific quantification for each bead type

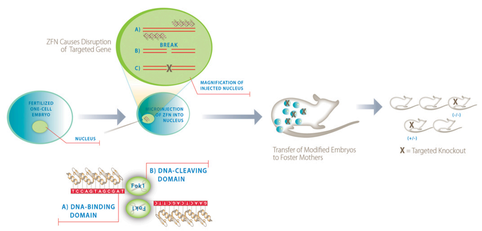
Flow Cytometry: Blood samples were collected using BD Biosciences lyse/fix according to manufacturer's instructions. Following whole blood lysis and fixation, samples are spun for approx. 5 minutes at 1000 x g and the cell pellets were frozen. Upon thawing cell pellets were resuspended in FACS buffer and then incubated with the appropriate antibody cocktails for 60 minutes. Following a wash and final centrifugation, the supernatant is removed and the cell pellet resuspended in PBS. It is then analyzed on a BD FACS CANTO II with analysis performed using FacsDiva Software.

Reverse Transcription: cDNA was synthesized from total RNA using the High Capacity RNA to cDNA kit from Life Technologies.

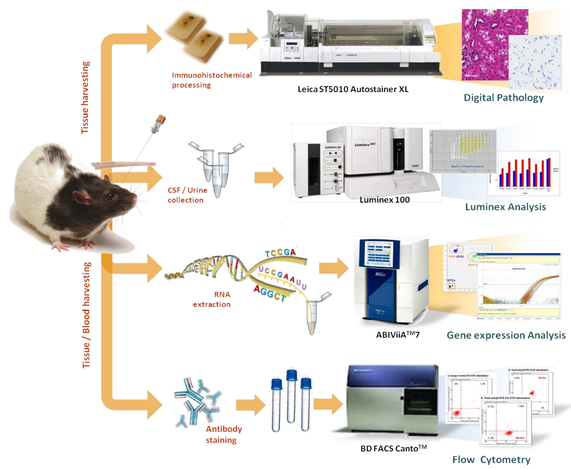
Real-Time qRT-PCR: Gene expression analysis was performed on the ABI® ViiA7 qPCR System using the custom Taqman® assay panel according to the manufacturer's instructions. cDNA was amplified using the Taqman® Fast Advanced PCR master mix. Data was analyzed using the ViiA7 software using the comparative Ct method.

Histology and Morphology The kidney and brain were collected, fixed in 10% of neutral buffered formalin (10 % NBF), paraffin embedded, sectioned, and stained by Periodic acid–Schiff stain (PAS) staining and Nissl staining for histology and morphological analyze respectively. All the slides were scanned by Aperio Digital Image scanner and analyzed.

Development of LRRK2 Knockout Rat Model by SAGE Zinc Finger Nuclease Technology



Schematic of Multi Parameter Cross Platform Biomarker Study



LRRK2 Knockout Rats Demonstrate Extensive Renal Tubular Injury

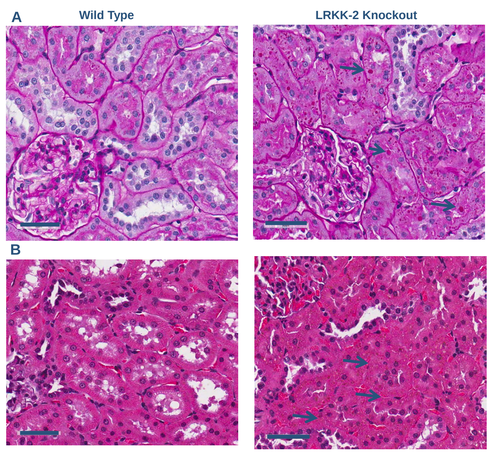
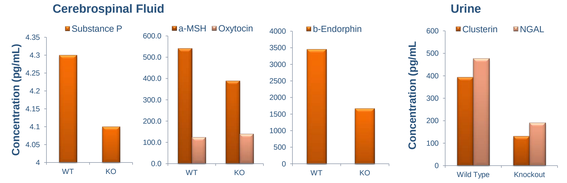


Figure 1: The Renal Periodic acid–Schiff stain (PAS) staining. Semi quantitative image analysis indicates statistical significance between two groups (P<0.01). Arrow: PAS positive staining in proximal and distal tubule (A). H.E Staining of kidney showing extensive tubular injury in K.O. mice (arrows) (B).
Scale Bar 50 µM, Magnification 40X

Luminex Analysis of Urine & CSF Show Increased Brain & Kidney Biomarkers in LRRK2 Knockouts



Urine Markers	WT						KO					
KIM-1	0.42	0.65	0.47	2.09	1.5	2.08	1.06	0.43	0.72	0.68	1.38	
OPN	1.66	0.54	1.34	0.58	1.22	1.29	0.53	0.89	0.95	1.49	0.55	
TIMP-1	2.44	27.59	24.14	140.25	104.27	14.25	22.36	49.83	90.18	14.75	124.15	
VEGF	0.85	1.79	2.71	4.91	28.83	0.35	1.36	1.77	4.59	0.81	3.21	
Albumin	574,274	> 4,000,000	124,880	> 4,000,000	96,534	> 4,000,000	197,783	> 4,000,000	313,347	3,998,087	3,331,725	
AGP	11,740.60	21,085.70	5,212.40	66,277.80	5,964.60	60,108.00	6,219.80	17,402.70	5,667.90	17,047.50	10,396.20	
β2Microglobulin	43,663.70	67,193.50	68,513.10	> 175,000	> 175,000	> 175,000	> 175,000	175,000	101,414	> 175,000	153,188.80	
Cystatin C	339.8	312.7	488.4	512.2	920.2	787.5	574.5	566.4	520.6	540.9	568.7	

Heat Map - Red (Highest), Blue (Lowest)

Figure 2: LRRK2 knockout rats demonstrate changes in neuronal and renal markers as demonstrated by multiplex analysis of cerebrospinal fluid and urine. Bar graphs and heat map table illustrate changes in biomarkers as compared to wild-type animals.

LRRK2 Knockout Rats Demonstrate an Enhanced Inflammatory & Immune Response

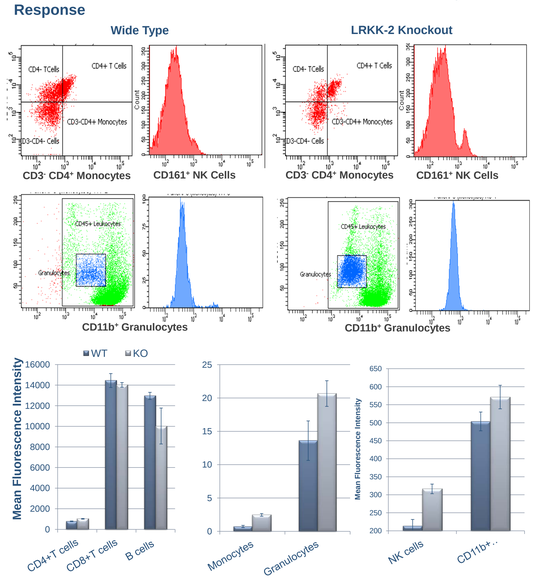
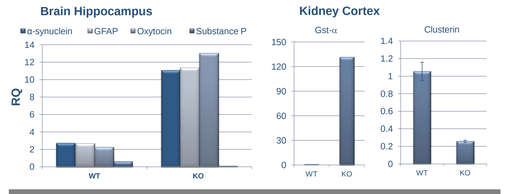


Figure 3: Blood samples from rats were processed to prepare PBMCs which were then stained with antibodies against CD3, CD4, CD161, and CD11b antigens to characterize various cell phenotypes. Mean fluorescence intensity measurements were compared across groups to access phenotypic population changes. LRRK2 knockout rats showed increased monocytes, NK cells and granulocytes suggesting an enhanced immune and inflammatory response in the knockout animals.

LRRK2 Knockout Rats Demonstrate Upregulated mRNA Expression of PD Markers in the Brain and Kidney



Hippocampus	WT			Ave	KO			Ave
α-synuclein	1.00	1.35	5.99	**2.78**	12.87	11.10	9.30	**11.09**
Glial fibrillary acidic protein	1.00	1.16	5.94	**2.70**	13.20	9.91	10.95	**11.35**
Oxytocin	1.00	1.01	4.79	**2.27**	19.48	13.69	5.95	**13.04**
Substance P	1.00	0.80	0.08	**0.63**	0.11	0.12	0.08	**0.10**
Kidney Cortex								
Clusterin	1.00	0.99	1.18	**1.06**	0.24	0.26	0.27	**0.26**
GSTα	1.00	0.68	1.08	**0.92**	268.00	11.30	115.40	**131.57**

Heat Map - Red (Highest), Blue (Lowest)

Figure 4: LRRK2 knockout rats demonstrate an increase in the transcription of Alpha Synuclein, GFAP, Oxytocin and Substance P in the brain Hippocampus. Moreover, Cortical region of kidney shows decreased levels of Clusterin and significant increased GSTα mRNA.

Summary & Conclusion

Kidney:

- LRRK2 knockout rats demonstrated extensive injury and abnormality in the proximal and distal renal tubules.
- Clusterin mRNA levels were significantly decreased in kidney cortex and there was a trend for decreased Clusterin protein and significantly decreased NGAL in urine of knockout rats.
- GSTα mRNA was significantly increased in knockout rats.

Brain:

- Histopathological changes were not observed in the hippocampus or cortex in KO rats.
- Substance P, αMSH and Oxytocin levels were decreased in the serum of KO rats.
- α-Synuclein, GFAP and Oxytocin mRNA levels were significantly upregulated whereas Substance P mRNA was decreased in the knockout rats.

Blood:

- LRRK2 knockout rats showed an enhanced inflammatory and immune response as indicated by an increased numbers of NK cells, Monocytes and CD11b+ granulocytes.

Conclusion

LRRK2 has been identified as one of the key drivers in the etiology of Parkinson's Disease (PD) therefore the LRRK2 knockout phenotype provided a unique opportunity to investigate the potential for early biomarker detection for PD as well as other LRRK2-mutation dependent conditions. Various kidney, brain, blood and histological biomarkers were detected as early as 11 weeks of age, suggesting an early differentiation in the LRRK2-knockout phenotype and defining potential early indicators for PD or other LRKK2 mutation related conditions. Increased α-synuclein mRNA expression in LRRK2 rats confirmed results from previous studies and is associated with progressive pathology of PD. Also, flow cytometry holds the potential as method for detection of PD biomarkers in blood. Future studies will focus on the further characterization of the animal model and will provide researchers with key information with potential diagnostic and therapeutic value for PD and other LRRK2-mutation linked disorders.

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