CRISPR Cas9 Mediated Generation of *Bex2* KO Mouse Model and Transcriptome Analysis of the Brain

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Abstract

Background. BEX family genes are expressed in many tissues and play significant roles in neuronal development.

Methods. In this study, a mouse model of *Bex2* gene knockout was generated, using the CRISPR-Cas9 system. A fragment of the coding exon was successfully deleted. RT-qPCR confirmed loss of gene expression at the mRNA level. A transcriptomic study of the brain was performed to identify genes and pathways under *Bex2* regulation. Essential biological functions under the control of *Bex2* related to brain development were identified.

Results. A total of 93 genes were found as differentially expressed. Among up-regulated genes, *Zfp967* and *Zfp984*, are zf protein-related genes. *Tmsb15l* is related to neuronal physiology. Among KEGG pathways, cell adhesion molecules (CAMs) and neuroactive ligand-receptor integration were most enriched. GO analysis identified cellular process, biological regulation, the metabolic process for Biological Processes. Cell, cell membrane, extracellular region and synapsis were found for Cellular Component. While binding, catalytic activity, molecular function were found for Molecular Function. A total of 53 KEGG disease terms were identified, included TNDM, Non-syndromic X-linked mental retardation, Neurodegeneration due to cerebral folate transport deficiency, Schizencephaly. Besides, HMGA, TF-Otx, RXR-like, SAND, zf-C2H2 and Homeobox transcription factors were enriched. A further study is required to confirm and explain each aspect identified.

Introduction

To date, five *BEX* genes are identified in humans (*BEX1-5*), five in chimp (*BEX1-5*), five in mice (*Bex1-4* and *Bex6*), and four in rats (*Bex1-4*) genome. According to the phylogenetic grouping, mouse and rat both are missing *Bex5* member of the Bex gene family. It is predicted that *Bex5* got lost in mice during the murid lineages after it is diverged from other mammals. All the *Bex* genes family members are positioned on the X-chromosome, excluding *Bex6*, which is present on chromosome 16 of the murine genome (1). These genes show high sequence homology and are predominantly expressed in the brain (2). A promising feature of the BEX genes is that they offer high expression in the mouse brain and are responsible for more than 12% of the rat brain's expressed sequence tags (1, 2). BEX proteins have a role in transcription regulation and signalling pathways in neurodegeneration, cell cycle, and tumour growth (3–6).

The researchers have reported the involvement of *BEX2* in various cancer types, such as glioblastoma, glioma, and breast cancer (5–7). *BEX2* is characterized in glioma development (8) and is vital to cells' tumorigenesis with activated mTOR (9). *BEX2* has a similar pro-survival function in LNT-229 glioma cells, and the downregulation of *BEX2* sensitizes LNT-229 cells to the cell death mediated by a dominant-positive variant of p53 (10). The role of *BEX2* in oncogenesis is supported by the fact that this gene's downregulation impairs neo-angiogenesis and migration of cells in oligodendroglioma (11). Due to the diverse expression pattern of *BEX2* in various tumours types, there is conflicting evidence about the role

of *BEX2* in multiple cancers. The *BEX2* expression in glioblastoma is very high and promotes the proliferation and survival of the glioblastomas mediated by NF κ B signalling (4). *BEX2* is also involved in the cell moment and invasion in glioma (5).

For the prediction of new transcripts of a particular gene expression pattern in various tissues or developmental stages, the most widely used technology is RNA sequencing (RNA-seq) compared to DNA microarray analysis (12). In this study, a *Bex2* KO mouse model has been generated using CRISPR Cas9 technology, and transcriptome analysis has been performed of the brain. Several essential pathways and KEGG diseases like TNDM, Non-syndromic X-linked mental retardation, Neurodegeneration due to cerebral folate transport deficiency, Schizencephaly are identified.

Methods

Animals

The Institutional Animal Care Committee and Animal Experimental Ethics Committee of Northeast Normal University (NENU/IACUC) have approved the study with approval number of (NENU/IACUC, AP2018011). All the recommendations for The Use of Laboratory Animals of NIH, USA, are followed strictly. Mice were kept in IVC cages (5-6 in each cage) at rotations of the 12/12 light-dark cycle in a pathogen-free environment with free access to food and water. The temperature of 21 degrees Celsius was maintained along 30-60% of humidity. Each time the mice were anaesthetized with 1% of pentobarbital sodium. The dose given was 10 mg/kg.

Designing of sgRNAs and construction of plasmid vector

The CRISPR Cas9 system is based on the 20bps nucleotide complementarity (13). These twenty nucleotides are termed as sgRNAs. These 20 nucleotides might be followed by a three bps nucleotide, NGG, where "N" can be any nucleotide, termed as Protospacer Adjacent Motif (PAM) (14). The Benchling database tool (<https://www.benchling.com>) was used to design sgRNAs. Two sgRNAs were chosen to delete the coding exon. The plasmid px330-U6-Chimeric_BB-CBhSpCas9 was purchased from Addgene (Addgene plasmid# 42230). The sgRNAs were ligated to the linearized plasmid vector according to the lab protocols. The sgRNAs designed and the respective positions on the genomic loci are shown in Table 1.

Table 1
The sgRNAs positions, and the targeted PAM sequences

sgRNA name	Locus	sgRNA seq	PAM	Remarks
<i>Bex2</i> -1	1016	ctctgtcttctaggagaaa	TGG	DSB Position 1
<i>Bex2</i> -2	1169	gactactacgtgcctagagg	AGG	DSB position 2

Microinjection and genotype

Pre-requisites were carried out before going for microinjection of the vector to the mice's oocytes strain C57BL/6J. The plasmid vector was directly injected into each oocyte's pro-nuclei. They were transferred directly to the ovary of the pseudo-pregnant mother already prepared. Olympus IX71 inverted microscope and Narishige microinjector was used. Microinjection was carried out by the labs' technician. *Bex2* specific primers targeting the upstream and downstream of sgRNAs were designed using the NCBI Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). As the pups were two weeks old, they were being labelled by cutting their fingers, which were used as templates for genotyping. The PCR was carried out according to the prescribed protocol. The PCR conditions were denaturation at 95°C for 4 minutes, 32 cycles of amplification at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 45 seconds followed by 10 mins extension at 72°C and then cooled down to 4°C. The PCR product was analyzed with 1% of agarose gel electrophoresis for up to one hour. GelDoc was used to observe the gel pictures (15). The transgenic littermates were separated accordingly. The chimaeras were obtained by crossing the littermates.

RNA extraction, synthesis of cDNA and qRT-PCR

Total RNA was extracted from brain tissues of *Bex2* KO and WT male mouse (n=3), using Trizol reagent (Takara, Dalian, China). The extracted RNA was converted to cDNA using a reverse transcription kit (Takara, Dalian). Jena Analytika (Germany) system was used for RT-qPCR. Quantitative Real-Time PCR (qPCR) was conducted with SYBR green mix in triplicates (Takara, Dalian, China). All the results were normalized according to the previously reported mechanism (16).

RNA Extraction and Library Preparation for Transcriptome analysis

Total RNA from the brain of *Bex2* KO and wild-type mice (n=3, biological replicates per sample, 6 in total) extracted using RNAiso plus reagent (Takara, Dalian) following the manufacturer's instruction and followed by an additional step of DNase I digestion to eliminate genomic DNA contamination. The sample size, and selection of the animals have been decided according to the published protocols (17). Male homozygous KO and pure WT were selected for further experiments since the *Bex2* gene is X linked. Quality and purity of RNA was checked on Nanodrop, NanoDrop™ One spectrophotometer (ThermoFisher Scientific, USA), and Agilent 2100 Bioanalyzer (Santa Clara, USA). One µg RNA for each transcript was used for the RNA-seq library. RNA-seq was performed on the BGISEQ-500 platform. The adaptor sequences and low quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. Clean reads were mapped to the mouse genome (GCF_000001635.26_GRCm38.p6) by using the Bowtie2 tool (18). The mRNA Seq raw data were deposited at the NCBI's Sequence Read Archive (ID 792087 - BioProject - NCBI (nih.gov)) under the accession number PRJNA792087.

GO and KEGG Analysis of the differentially expressed genes

All the DEGs were mapped to the Gene Ontology database. To perform GO enrichment analysis, the phyper function in the R program was used. Briefly, all DEGs were firstly mapped to each term in the Gene Ontology database (19). Genes in each term were calculated, and the hypergeometric test was applied to find GO terms that are significantly enriched in DEGs compared to the background of all genes in reference species. Bonferroni correction was used for p-value adjustment (20). Q value (corrected p-value) < 0.05 was defined as significantly enriched GO terms in DEGs. Kyoto Encyclopedia of Genes and Genome pathway classification was performed by mapping all the DEGs to the KEGG pathway database (21). The pHYPER function in the R program was used to perform the enrichment analysis accordingly (16). Pathway with Q value ≤ 0.05 was considered as significantly enriched in differentially expressed genes (22).

Statistical Analysis

Results are expressed as means $\pm$ SEM (SEM). *P-value* < 0.05 (unpaired Student's *t*-test) was considered statistically significant. All graphics were prepared with GraphPad Prism 8 for Mac (GraphPad Software).

Results

Strategy of *Bex2*^{-/-} mouse generation & Screening of mutants

The *Bex2* is a small gene and consists of only 1672 bps. The third exon contains the entire coding region. To inactivate the gene, the sgRNA_I was designed in front of the start codon, while the sgRNA_II was targeted upstream of the stop codon. To screen fragment deletion, primer pairs were designed flanking to sgRNA targets (Figure 1A). Nine pups were obtained after 19 days of the zygote transfer. Fifteen days later to the birth, these pups were weaned, cut their fingers for labelling purposes. The same biopsies were used for genotyping, following the standard protocols. Two out of nine mice were found transgenic for *Bex2*, as shown in figure 1B. The deleted (knocked-out) fragment was confirmed by ligating the PCR product fragment into a pMD18 simple vector (Takara, China). The chromatograph is shown (Figure 1C). These two mice were mated with a wild-type C57BL/6J background to segregate the alleles. F2 progenies were used as experimental organisms. The *Bex2* expression level was confirmed by qPCR in the brain and lungs (Figure 1D).

Gene expression profile of *Bex2*^{-/-} and WT brain

The cDNA libraries were constructed from brain mRNA of 7 weeks old *Bex2*^{-/-} and WT male mice in three replicates (n=3). BGISEQ platform was used for RNA-seq. Total raw reads for *Bex2*^{-/-} and WT were 23.75 M for each sample. Adapter sequences and low-quality reads were filtered out, and each sample produced an average of 1.19 Gb data or 23.74M reads each, respectively, with Q30 base percentage 93.13% and 92.97%, while Q20 percentage was 98.10% and 98.03% (Data not shown). The clean reads were then aligned to the mouse reference genome (GCF_000001635.26_GRCm38.p6), and matching efficiency between clean reads was identified using Bowtie2 (23). Transcript expression levels were calculated and presented by RSEM (24).

Identification of differentially expressed genes

The genes expressed only in KO mice are termed differentially expressed genes (DEGs). Differentially expressed genes were identified accordingly (25). The criteria set for the selection of DEGs was $\log_2$ fold change > 1 , and FDR 0.001. According to the set standards, a total of 93 genes were identified as differentially expressed between *Bex2*^{-/-} and WT, out of which 57 were up-regulated while 36 were down-regulated (Figure 2).

GO enrichment analysis

Gene Ontology (GO) is a standard gene function classification system that comprehensively explains the attributes of genes and gene products in organisms. GO has been divided into three categories; biological process, cellular components and molecular function(26). All the identified DEGs were assigned to the GO tool (Fig. 3A, 3B, 3C). The most significant GO terms enriched under *Bex2*^{-/-} regulation among Biological processes were cellular process, biological regulation, regulation of the biological process, metabolic process, multicellular organismal process, response to stimulus and signalling etc. Among the Cellular Component category, cell, cell part, organelle, organelle part, protein-containing complex, synapse, synapse part, etc. were enriched. Among Molecular Function, binding, catalytic activity, molecular function, molecular transducer activity etc. were enriched.

KEGG analysis identifies many processes are under *Bex2*^{-/-} regulation

KEGG pathway analysis helps to understand the biological function of gene networks. KEGG Pathway Enrichment Analysis was then performed to find the significantly enriched pathways in terms of DEGs mapped to the entire genome background (Figure 4). The most significant pathways enriched were cell adhesion molecules (CAMs), neuroactive ligand-receptor interaction, antigen processing and presentation, cAMP signalling pathway, cellular senescence, calcium signalling pathway etc.

KEGG Analysis of Disease-associated pathways

The identified DEGs were assigned to KEGG disease enrichment (Figure 5). The KEGG diseases enriched under *Bex2*^{-/-} regulation were Transient neonatal diabetes mellitus (TNDM), Postaxial polydactyly, Non-syndromic X-linked mental retardation, Congenital lactase deficiency, and Neurodegeneration due to cerebral folate TD, among others. The genes involved in Postaxial polydactyl, Non-syndromic X-linked mental retardation.

Genes that Encoding Transcription Factor Proteins

The essential regulatory proteins are termed transcription factors that play a role in multiple biological processes. Multiple TFs were identified in this study. TFs enriched under *Bex2*^{-/-} regulation was HMGA self-build, Otx1 transcription factor, zf-C4 self-build TFs, SAND DNA-binding protein domain, Zinc fingers, and Homeobox transcription factor genes (Figure 6). (Figure 7). Otx proteins constitute a class of

vertebrate homeodomain-containing transcription factors essential for anterior head formation, including nervous development. SAND DNA-binding protein domain is localized in the cell nucleus and has a vital function in chromatin-dependent transcriptional control. It is found solely in eukaryotes. Six3 is identified among the Homeobox transcription factors ($\text{Log}_2 \text{FC} = 2.98$), which are vital in nervous system development Fig. 6.

Discussion

The RNA-guided genome manipulation based on type II prokaryotic CRISPR/Cas system has successfully generated transgenic mouse models (27–31). The *Bex2* KO mouse model was generated using the CRISPR Cas9 gene modification system in this study. The gene was selected based on its importance in multiple physiological functions in the brain. The deletion of the fragment was confirmed by Sanger sequencing and, later on, the mRNA using RT-qPCR. A promising feature of the BEX genes is that they offer high expression in the mouse brain and are responsible for more than 12% of the rat brain's expressed sequence tags (1, 2). A brain transcriptome study was conducted in this study using RNA-seq methodology. The genes expressed only in KO mice are termed differentially expressed genes (DEGs). Differentially expressed genes were identified accordingly (25). The criteria set for the selection of DEGs was log_2 fold change > 1 , and FDR 0.001. According to the set standards, a total of 93 genes were identified as differentially expressed between *Bex2*^{-/-} and WT, out of which 57 were up-regulated while 36 were down-regulated. The most up-regulated gene is *Tmsb15l*, the $\text{log}_2 \text{FC}$ is 8.29. Other researchers also found its upregulation in fibroblast of E14 using microarray analysis (32). *H2-Q8*, DEG $\text{log}_2 \text{FC}$ is 6.62, also found up-regulated by other researchers using microarray analysis (33). *Gvin1*, 5.67 was identified IFN treatment of neuron cultures (34). *Tgtp2*, $\text{log}_2 \text{FC}$ is 5.45. *lncRNA-Tcam1* up-regulated *Tgtp2* during spermatogenesis (35). In contrast, other researchers' results were inverse to this study, where they found that *Tgtp2* was down-regulated in *Mcpt6* KO mice (36). *Serpina9*, $\text{log}_2 \text{FC}$ is 3.3. The SNP *rs11628722* in the *SERPINA9* gene was previously associated with incident ischemic stroke in the Atherosclerosis Risk in Communities (ARIC) study reviewed by (37). Other researchers identified that the SNPs in the *SERPINA9* gene showed race-specific associations with characteristics of carotid atherosclerotic plaques (38). A team of researchers suggests that along with other genes, *Serpina9* might associate with Alzheimer's disease neuropathology in the neurodegenerative process (39). *Six3*, DEG $\text{log}_2 \text{FC}$ is 2.98. Ectopic expression of *Six3* in chicks has shown that *Six3* is a direct negative regulator of Wnt1 expression (40). *Cldn2* (+2.3), It was found that *CLDN2* expression significantly inhibited the malignant phenotype of OS cells *in vitro*. *Rnaset2b*, the most down-regulated gene, $\text{log}_2 \text{FC}$ is -9.0. An inflammatory disease, Aicardi–Goutières syndrome, is related to this genes' mutation (41). Another team evaluated two patients with *RNASEH2B* mutations (42). *Rex2*, DEG $\text{log}_2 \text{FC}$ -4.90, which a group of researchers identifies as a lesser-studied gene (43). *Mss51*, DEG $\text{log}_2 \text{FC}$ -3.23 is a metabolism-related gene (44, 45). *CD300lf*, DEG $\text{log}_2 \text{FC}$ -2.7 is the primary physiologic receptor of murine norovirus (46). *Stab2*, DEG $\text{log}_2 \text{FC}$ is -2.39 is shown to be venous thromboembolic disease (47).

The most significant GO terms enriched under *Bex2*^{-/-} regulation among Biological processes were cellular process, biological regulation, regulation of the biological process, metabolic process, multicellular organismal process, response to stimulus and signalling, etc. Among the Cellular Component category, cell, cell part, organelle, organelle part, protein-containing complex, synapse, synapse part, etc., were enriched. Among Molecular Function, binding, catalytic activity, molecular function, molecular transducer activity, etc. were enriched. KEGG classification regulated under *Bex2*^{-/-}, several DEGs were annotated to multiple pathways from different categories. KEGG Pathway Enrichment Analysis was then performed to find the significantly enriched pathways in terms of DEGs mapped to the entire genome background. The most significant pathways enriched were cell adhesion molecules (CAMs), neuroactive ligand-receptor interaction, antigen processing and presentation, cAMP signalling pathway, cellular senescence, calcium signalling pathway, etc. The identified DEGs were assigned to KEGG disease enrichment. The KEGG diseases enriched under *Bex2*^{-/-} regulation were Transient neonatal diabetes mellitus (TNDM), Postaxial polydactyly, Non-syndromic X-linked mental retardation, Congenital lactase deficiency, and Neurodegeneration due to cerebral folate TD, among others. X-linked mental retardation (XLMR) is an inborn disorder that triggers malfunction to grow intellectual capabilities as of alterations in numerous genes on the X chromosome. XLMR is subdivided into syndromic and non-syndromic types (NS-XLMR), varying on whether further anomalies are observed on bodily inspection, laboratory analysis and brain tomography.

Declarations

Ethics approval and consent to participate. The Institutional Animal Care Committee and Animal Experimental Ethics Committee of Northeast Normal University (NENU/IACUC) have approved the study with approval number of (NENU/IACUC, AP2018011). All the recommendations for The Use of Laboratory Animals of NIH, USA, are followed strictly. Mice were kept in IVC cages (5-6 in each cage) at rotations of the 12/12 light-dark cycle in a pathogen-free environment with free access to food and water. The temperature of 21 degrees Celsius was maintained along 30-60% of humidity. Each time the mice were anaesthetized with 1% of pentobarbital sodium. The dose given was 10 mg/kg. We confirm that all methods are reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>) for the reporting of animal experiments.

Consent for publication. N/A

Availability of data and materials. The RNA seq raw data is available at SRA, NCBI with accession numbers SRR17327319 and SRR17327320.

Competing interests. The authors declare no competing interests.

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Author's contribution. NB designed the study, carried out the experiments, wrote the paper and analysed the data. RKS performed experiments and wrote the paper. SA performed the experiments. HU analysed the data. ZO analysed the data. MZZM analysed data. FBB analysed data. MU analysed data. FH analyzed the data. XF wrote the paper, supervised the project. YZ designed the project, supervised the study and provided funding. MZ supervised the project, revised the manuscript and funded the project.

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Figures

Figure 1

Strategic plan and screening. **(A)** Graphical representation of the *Bex2* gene and targeting sites. Exon 3 is targeted for fragment deletion by designing of two sgRNAs. Both forward and reverse primers are targeted at flanking both sgRNAs sites. **(B)** Genotyping of the pups obtained. Two transgenic mice (18,19) knocked out homozygous (*Bex2*^{-/-}) were obtained. **(C)** Sanger sequence chromatogram. **(D)** The relative mRNA levels of KO and WT mice brain and lung.

Figure 2

Statistics of differentially expressed genes. The X-axis represents the alignment scheme of DEGs for each group, and the Y-axis represents the corresponding number of differentially expressed genes (DEGs). Red bars represent the number of DEGs up-regulated, and blue bars represent the number of DEGs down-regulated.

Figure 3

3A. GO Enrichment Biological Process. The X-axis indicates the enriched terms the Y-axis indicates the rich ratio. The size of the circle indicates gene number. The colour intensity indicates Qvalue.

3B GO Enrichment Cellular Component. The X-axis indicates the enriched terms the Y-axis indicates the rich ratio. The size of the circle indicates gene number. The color intensity from indicates Qvalue.

3C GO Enrichment Molecular Function. The X-axis indicates the enriched terms the Y-axis indicates the rich ratio. The size of the circle indicates gene number. The color intensity indicates Qvalue.

Figure 4

KEGG Pathway enrichment of *Bex2*^{-/-}. The X-axis is the enrichment ratio (calculated as: Term candidate gene number/Term gene number). The Y-axis is the KEGG pathway. The size of the circle represents the number of genes annotated to the KEGG pathway. The colour represents the enriched significance.

Figure 5

KEGG Disease enrichment. The x-axis indicates the enriched terms the y-axis indicates the rich ratio. The size of the circle indicates the number of genes. The color intensity indicate the Qvalue.

Figure 6

KEGG Transcription Factors enrichment. The x-axis indicates the enriched terms the y-axis indicates the rich ratio. The size of the circle indicates the number of genes. The color intensity indicate the Qvalue.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Bex2KOBrainKEGGpathways.xlsx](#)
- [Bex2KOvsWTBrainDEGs.xlsx](#)