MicroRNA-362-3p attenuates motor deficit following spinal cord injury via targeting paired box gene 2

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Original Research

Spinal cord injury is a disabling disorder, leading to neurological impairments. Although some microRNAs have been reported to be associated with spinal cord injury, the function of microRNA-362-3p, as one of downregulated miRNAs after spinal cord injury, is still unclear. In current study, spinal cord injury models were established. Then, we performed microRNA-362-3p overexpression in spinal cord injury rats, which expressed the low microRNA-362-3p. Results from behavioral testing, hematoxylin and eosin staining and Nissl staining revealed that microRNA-362-3p overexpression improved the functional resoration in spinal cord injury rats. Furthermore, it caused the decrease of neuronal apoptosis and inhibition of the neuronal inflammation in these rats. Besides, Paired box gene 2 was verified as a target gene of microRNA-362-3p using luciferase assay, which predicted via bioinformatics technology. Moreover, microRNA-362-3p alleviated the neuralgia and reduced the activation of ERK and p38 through inhibition of Paired box gene 2. In conclusion, the findings demonstrated that microRNA-362-3p attenuated neuropathic pain following spinal cord injury through targeting Paired box gene 2. It provides us the new biomarker to diagnose and monitor spinal cord injury.

Keywords
Spinal cord injury; miR-362-3p; PAX2; neuralgia

1 Introduction
Spinal cord injury (SCI) is considered as a disabling disorder, and the patients with it often lead to neurological impairments (Eckert and Martin, 2017). There are about 17,000 new cases to happen from 280,000 patients with SCI in the United States alone every year (Spinal Cord Injury 2016 Facts and Figures at a Glance, 2016). Among patients with spinal cord injury, 80% of individuals experienced a severe sensory deficit neuropathic pain (Cragg et al., 2015; Finnerup et al., 2014; Siddall and Loeser, 2001; Siddall et al., 2003). Currently, stabilizing the cord and restoring homeostasis immediately following injury for patients with SCI was the most acute treatments (Chang et al., 2013; Edgerton and Harke, 2011; Khurana and Garg, 2014). Furthermore, for patients with neuropathic pain resulted from SCI, pharmacotherapy was mainly used for treatment approaches such as pregabalin (Cardenas et al., 2013; Siddall et al., 2006). Although there are some treatments for neuropathic pain following SCI, it is necessary to get more the knowledge of it.

It is reported that microRNAs (miRNAs) are related with sensory neurons and regulates stress signaling pathways (Mendell and Olson, 2012; Weston et al., 2006; Xu et al., 2007). MiRNAs (20-23 nucleotide), single-stranded and non-coding RNA molecules, take part in regulating post-transcriptional gene expression (Bartel, 2004; Smalheiser and Lugli, 2009). In the previous study, miRNAs were found to be abundant in the nervous system (Kosik, 2006). In addition, the previous evidence confirmed that miRNAs were also involved in regulating neuronal inflammation (Taras-
sishin et al., 2011). Interestingly, Zhu et al. reported that miR-362-3p, as one of 46 downregulated miRNAs, was indicated in rat with spinal cord after contusion SCI (Zhu et al., 2017). However, the function and molecular mechanism of miR-362-3p involved in neuropathic pain following SCI are still unclear.

Paired box gene 2 (PAX2), as a second member of PAX gene family, is a novel paired-box-containing gene. It is found to be broadly expressed in the intermediate region from the development spinal cord (Nornes et al., 1990; Puschel et al., 1992). PAX2 was required for the development of interneurons, and involved in neurotransmitter expression, axon morphology and dendritic arborization control (Burrill et al., 1997). Several lines of evidence revealed that PAX2 controlled γ-aminobutyric acid (GABA)ergic differentiation and was the marker of inhibitory neuron, and functioned in pain processing from central nervous system (Cheng et al., 2004; Gutierrez-Mecinas et al., 2017). Huang et al. demonstrated that PAX2 overexpression caused the promotion of ectopic glycnergic and GABAergic neuron differentiation in the dorsal spinal cord (Huang et al., 2008). Larsson et al. revealed that a GABAergic phenotype could be maintained by PAX2 in mature inhibitory dorsal horn neurons (Larsson, 2017). Furthermore, previous studies showed that rats could display allodynia and hyperalgesia after partial sciatic nerve ligation. In addition, there were the enhancement of PAX2, endothelin-1 (ET-1), endothelin receptor type A (ETAR) (not endothelin receptor type B (ETBR)) and nuclear factor of activated T-cells 5 (NFAT5) levels in these rats. Subsequently, the mitogen-activated proteins kinases (MAPK) and nuclear factor-kappa B (NF-κB) signaling pathways were activated. The PAX2-ET-1-ETAR-NFAT5 axis was demonstrated to be the novel regulatory mechanism in neuropathic pain (Tai et al., 2018). It suggests that PAX2 plays an important role in neuropathic pain following SCI. Interestingly, PAX2 was reported to be regulated by miRNAs to participate in the progression of some disease. For instance, PAX2, as a target of miR-497, could take part in cell proliferation to affect the development of disease (Lin et al., 2016). Therefore, we hypothesized that miR-362-3p may show an important role in neuropathic pain following SCI by targeting PAX2.

In current study, we revealed that the miR-362-3p was associated with neuropathic pain following SCI through PAX2. The findings suggest that miR-362-3p may serve as the new therapeutic avenue in treating neuropathic pain following SCI.

2. Materials and methods
2.1 SCI model
A total of 70 male Sprague-Dawley rats, which aged 8-9 weeks and weighed between 250 and 300 g, were obtained from the Center for Animal Experimental of Gansu Provincial Hospital. The rats were housed six to eight rats per cage. They were fed at a room temperature (25 ± 3°C), 12 h light and 12 h dark cycles, 55%-75% relative humidity, and free access to food and water. The rats were firstly assigned to four groups: sham; SCI only (SCI); SCI + negative control (NC) agomiR; SCI + miR-362-3p agomiR. The SCI operation was conducted under intraperitoneal anesthesia with a mixture of 40 mg/kg ketamine and 10 mg/kg xylazine. Then rats were placed in a stereotaxic system. A 20 g rod was dropped onto the exposed spinal cord after laminectomy of the T8 thoracic vertebrae. After that, SCI rats were intravenously injected with NC agomiR or miR-362-3p agomiR (20 nmol/L, 1 μL/h) via the tail vein for 3 days. In sham group, rats were also anesthetized, and they were not subjected to a spinal cord contusion injury after laminectomy of the T8 thoracic vertebrae. An equal volume of saline was injected into sham and SCI rats at the same time and speed for 3 days. All experimental procedures were carried out according to the protocol approved by the Guideline for the care and use of laboratory animals.

2.2 Behavioral testing
Behavioral tests for hindlimb locomotor function were conducted at 1, 3, 7, 14, 21 and 28 days after SCI with the Basso, Beattie, Bresnahan locomotor rating scale (BBB scale) open-field locomotion scale (Basso, 1995; Basso et al., 1996). At each time point, the outcome value for each rat was obtained by averaging motor score from the observation and scoring of behaviors involving the hindlimbs. All behavioral tests were performed by three independent observers who masked to SCI severity.

2.3 Quantitative real-time polymerase chain reaction (PCR)
Total RNA from dorsal spinal cord was extracted via TRizoll® reagent (Invitrogen, Thermo Fisher Scientific, Inc.). Reverse transcription was then performed and cDNAs were generated with a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Subsequently, miR-362-3p, cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α) and IL-6 levels were determined by PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan). Relative expression of miR-362-3p, COX-2, IL-1β, TNF-α and IL-6 was calculated via 2-∆∆CT method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was an internal control. Primers for quantitative real-time PCR were shown in Table 1.

| Gene      | Sequence (5’-3’)          |
|-----------|---------------------------|
| miR-362-3p| F: GGGAACACACACTTATTAAG  |
|           | R: TTTGGCCATACTAGCACATT  |
| COX-2     | F: CAGGCCCATATAAGCTCGGA  |
|           | R: GGATGCTTGTCGCTGCTTC   |
| IL-1β     | F: GCTGCTTCCAAAACCTTTTGC|
|           | R: AGCTTCTACACAGCACAAT   |
| TNF-α     | F: AGACACAAAGTGCCACAACG  |
|           | R: CATGGCGTCGATGCCTCATA  |
| IL-6      | F: CGTTTCTACCTGGGATTTTG  |
|           | R: GTTTGCGGAATGACCTCAT   |
| GAPDH     | F: TGCGCTTCGGTGTTCCTAC   |
|           | R: GAGTGCGTGGTGAAGTGGCA  |

2.4 Hematoxylin and eosin (HE) staining
The rat brain tissues were fixed using 4% paraformaldehyde. They were embedded by paraffin, cut into 5 micrometers sections with a slicer (Leica RM2265, Beijing, China) and stained with HE. The histopathologic changes from specimens were observed under a microscope with a digital zoom (micro, 400 x).
2.5 Nissl staining

The rat spinal cord tissues were collected and cut into 10 μm-thick sections. The samples were then stained using 0.1% cresyl violet solution (Sigma-Aldrich, St. Louis, MO). The cells positive for Nissl staining were measured and the numbers of survival neurons were counted.

2.6 Western blot analysis

Total protein from rat neurons was extracted via a protein extract kit (Cytoplasmic Protein Extraction Kit; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Protein concentrations were determined with the Bradford method. Subsequently, the 30 μg of protein samples were used for SDS-PAGE electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Afterwards, the 5% non-fat dried milk was used to block the membranes in a 37°C oven for 1 h. The membranes were incubated using primary antibodies, including cleaved-caspase-3 (1:200; Cell Signaling Technology, Danvers, MA), cleaved-poly (ADP-ribose) polymerases (PARP) (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA), Bax (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (1:1000, Abcam, Cambridge, UK), pAX2 (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA), caspase-3 (1:1000; Cell Signaling Technology, Danvers, MA), PARP (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA), extracellular signal-regulated kinase 1/2 (ERK1/2) (1:1000, Cell Signaling Technology Inc., Danvers, MA, USA), phospho-ERK 1/2 (p-ERK1/2) (1:1000, Cell Signaling Technology Inc., Danvers, MA, USA), mitogen-activated protein kinase 1/2 (MEK1/2) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-MEK1/2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-p38 (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), p38 (1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (1:1000, Sigma, St. Louis, MO), overnight at 4°C. Subsequently, the secondary antibody was used to incubate the membranes at 37°C for 1 h. Finally, the bands was visualized and quantified with Chemiluminescent Detection Kit (Advansta, Menlo Park, CA) and Image J software.

2.7 Enzyme linked immunosorbent assays (ELISA)

The rat spinal cord tissues were collected and the COX-2, IL-1β, TNF-α and IL-6 levels were measured using ELISA kit (Quantikine; R&D, Minneapolis, MN) on microplate reader. Briefly, the supernatant of rat spinal cord tissues were added into microwell plate. Subsequently, the plates were incubated with biotin and streptavidin-HRP buffer for 1 h at 37°C. After the substrate reaction, the stop solutions were added and absorbance in each well was assayed via microplate reader (BioTek Elx800, America) at a wavelength of 450 nm. The levels of COX-2, IL-1β, TNF-α and IL-6 were calculated, respectively.

2.8 Luciferase assays

The wild or mutant sequence of 3'-UTR of PAX2 were amplified and cloned into luciferase reporter plasmids (Promega, Madison, WI, USA). Subsequently, HEK293 cells co-transfected with miRNA (miR-NC or miR-362-3p) and the generated wild-type plasmid (PAX2-WT) or mutant-type plasmid (PAX2-MUT) using Lipofectamine 2000, respectively. The luciferase activities were measured via the Dual-Luciferase Reporter Assay System (GeneCopoeia, Rockville, MD, USA) after 48 h.

2.9 Statistical analysis

All statistical tests were conducted by GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Each experiment was performed in repeated three times. Data are presented as the mean ± standard error of the mean (SEM). The one-way or two-way analysis of variance (ANOVA) by Tukey was used for differences. P values less than 0.05 was set as statistically significant (*, p < 0.05, **, p < 0.01, ***, p < 0.001).

3. Results

3.1 miR-362-3p level is down-regulated in SCI rats

In order to detect miR-362-3p level in SCI rats, we established the SCI models. The animal locomotor activity was scored using the BBB scale 1-28 d after SCI. The results showed that BBB scores were significantly decreased in SCI rats, compared with sham rats (Fig. 1A). In addition, we further found that the miR-362-3p level was lower in SCI group than sham group (Fig. 1B). These results indicated that the miR-362-3p level was down-regulated in SCI rats.

3.2 miR-362-3p overexpression improves the functional reoration in SCI rats

To explore the effect of miR-362-3p on functional resoration in SCI rats, we performed the miR-362-3p overexpression. We first detected the miR-362-3p level in SCI rats with NC agomiR or miR-362-3p agomiR using quantitative real-time PCR. The results showed that miR-362-3p was successfully overexpressed in SCI rats (Fig. 2A). We then measured the animal locomotor activity according to BBB scale 1-28 d in SCI rats with NC agomiR or miR-362-3p agomiR. The BBB scores revealed that the SCI rats with NC agomiR or miR-362-3p agomiR were decreased, compared with sham-operated rats, whereas the SCI rats with NC agomiR were lower than those rats with miR-362-3p agomiR (Fig. 2B). In addition, HE staining and Nissl staining found that the SCI rats with NC agomiR could lead to the increase of lesion cavity area and the decrease of the numbers of Nissl-positive cells, whereas the SCI rats with miR-362-3p agomiR could reverse the changes of lesion cavity area and the numbers of Nissl-positive cells (Fig. 2C). The findings revealed that miR-362-3p overexpression improved the functional resoration in SCI rats.

![Figure 1](https://example.com/figure1.png)  
Figure 1. miR-362-3p level is down-regulated in SCI rats. (A) BBB scores were used to detect the animal locomotor activity. (B) Quantitative real-time PCR measured the miR-362-3p level. * indicated p < 0.05, ** indicated p < 0.01, *** indicated p < 0.001.
miR-362-3p overexpression inhibited the neuronal inflammation in SCI rats. Nevertheless, miR-362-3p agomiR suppressed the increase of PAX2 level using quantitative real-time PCR and western blot analysis (Fig. 5B, C). Moreover, the negative correlation between miR-362-3p and PAX2 was analyzed (Fig. 5D). The data suggested that PAX2 was verified as the target of miR-362-3p.

3.6 miR-362-3p alleviates the neuralgia by inhibiting PAX2 in SCI rats

We then examined the effect of miR-362-3p on neuralgia in SCI rats. In addition to the miR-362-3p agomiR in SCI rats, quantitative real-time PCR and western blot analysis showed that PAX2 overexpression was also successfully performed in SCI rats with miR-362-3p agomiR (Fig. 6A, B). Although the SCI rats with miR-362-3p agomiR increased the BBB scores comparing to those rats with NC agomiR, PAX2 overexpression in SCI rats with miR-362-3p agomiR reversed the change (Fig. 6C). Furthermore, quantitative real-time PCR and ELISA assay demonstrated that PAX2 overexpression in SCI rats with miR-362-3p agomiR rescued the suppression of COX-2, IL-1β, TNF-α and IL-6 induced by miR-362-3p agomiR (Fig. 6D, E). The findings demonstrated that miR-362-3p alleviated the neuralgia by inhibiting PAX2 in SCI rats.

3.7 miR-362-3p reduces the activation of ERK and p38 by inhibition of PAX2 in SCI rats

To further explore whether miR-362-3p had effect on the activation of ERK and p38 by PAX2, western blot analysis was conducted to detect the series of protein levels. We found that the suppression of p-ERK1/2, p-MEK1/2 and p-p38 protein levels were caused by miR-362-3p agomiR in SCI rats. However, the PAX2 overexpression in SCI rats with miR-362-3p agomiR could reverse the decrease of those protein levels (Fig. 7). The results revealed that miR-362-3p reduced the activation of ERK and p38 by inhibition of PAX2 in SCI rats.

4. Discussion

In this study, the SCI models were established and the down-regulation of miR-362-3p level was detected in SCI rats. Subsequently, in order to explore the function of miR-362-3p in SCI rats, the miR-362-3p overexpression was performed. The improvement of functional restoration, decrease of the neuronal apoptosis and inhibition of the neuronal inflammation were induced by miR-362-3p overexpression in SCI rats. In addition, the PAX2 level was suppressed by miR-362-3p overexpression. Interestingly, PAX2 was verified as the target of miR-362-3p. The interaction between miR-362-3p and PAX2 was demonstrated. Furthermore, we further indicated that miR-362-3p can alleviate the neuralgia and reduce the activation of ERK and p38 by inhibition of PAX2 in SCI rats. Hence, the results suggest that miR-362-3p plays an important role in neuropathic pain following SCI via targeting PAX2.

Previously, miRNAs were found to be involved in the regulation of SCI. For example, miR-544a level was significantly reduced after SCI and its high expression level alleviated the inflammation and took part in the recovery of SCI (Yang et al., 2018). Moreover, previous studies revealed that miR-137 could show the suppressive effect on the inflammatory response and apoptosis after SCI (Gao et al., 2018). Importantly, it is interesting that miR-362-3p could be regulated after SCI, which demonstrated by previous research (Zhu et al., 2017). Notably, in the present study, miR-362-3p was used to explore the effect of it on neuropathic pain following SCI. The consistent results indicated that miR-362-3p level was down-regulated in SCI rats. MiR-362-3p overexpression

Figure 2. miR-362-3p overexpression improves the functional restoration in SCI rats. (A) MiR-362-3p level was analyzed by quantitative real-time PCR. (B) The animal locomotor activity was examined using BBB scores. (C) HE staining measured the lesion cavity area. (D) Nissl staining assayed the numbers of Nissl-positive cells. * indicated p < 0.05, *** indicated p < 0.001.

3.3 miR-362-3p overexpression decreases the neuronal apoptosis in SCI rats

In order to evaluate whether miR-362-3p overexpression can affect the neuronal apoptosis in SCI rats, western blot analysis was carried out. The results from it demonstrated that the cleaved-caspase-3, cleaved-PARP and Bax protein levels were enhanced, whereas Bcl-2 protein level was inhibited in the SCI rats with NC agomiR. However, the SCI rats with miR-362-3p agomiR could alleviate those protein levels (Fig. 3). These data suggested that miR-362-3p overexpression decreased the neuronal apoptosis in SCI rats.

3.4 miR-362-3p overexpression inhibits the neuronal inflammation in SCI rats

We further examined the effect of miR-362-3p on neuronal inflammation in SCI rats, quantitative real-time PCR and ELISA assay were both performed. The quantitative real-time PCR demonstrated that there were higher level of COX-2, IL-1β, TNF-α and IL-6 in the rats with NC agomiR than in sham group at 7 d post-SCI. However, miR-362-3p agomiR reduced the augment of COX-2, IL-1β, TNF-α and IL-6 levels (Fig. 4A). Similarly, the consistent changes of COX-2, IL-1β, TNF-α and IL-6 levels were indicated via ELISA assay (Fig. 4B). The results showed that miR-362-3p overexpression inhibited the neuronal inflammation in SCI rats.

3.5 PAX2 is verified as the target of miR-362-3p

In order investigate the relationship between miR-362-3p and PAX2, the binding site of miR-362-3p was predicted. Luciferase activity from PAX2 3’ UTR induced by miR-362-3p was reduced. However, that from PAX2 3’ MUT was not affected via luciferase assays (Fig. 5A). Subsequently, PAX2 level was increased in the SCI rats with NC agomiR. Nevertheless, miR-362-3p agomiR suppressed the increase of PAX2 level using quantitative real-time PCR and western blot analysis (Fig. 5B, C). Moreover, the negative correlation between miR-362-3p and PAX2 was analyzed (Fig. 5D). The data suggested that PAX2 was verified as the target of miR-362-3p.
was useful for functional resoration and it can inhibit the neuronal inflammation and neuronal apoptosis. In brief, the data suggest that the potential role of miR-362-3p may function as a suppressor in neuropathic pain following SCI.

In addition to the function of miR-362-3p in SCI rats, the target gene of miR-362-3p was then predicted via bioinformatics technology. We hypothesized that miR-362-3p may act directly on the 3’UTR regions of the PAX2 gene to show its function. It is noteworthy that PAX2, as marker of inhibitory neuron, could control GABAergic differentiation and it was involved in pain procession of central nervous system (Cheng et al., 2004; Gutierrez-Mecinas et al., 2017). Moreover, accumulating evidence demonstrated that miR-497 bind with PAX2 and elevated cell apoptosis (Lin et al., 2016). High PAX2 expression was found to be related with cell apoptosis (Zheng et al., 2015). Like the previous studies, in this study, we demonstrated that miR-362-3p enhanced cell apoptosis, evidenced by elevation of cleaved-caspase-3, cleaved-PARP and Bax protein levels, as well as reduction of Bcl-2 protein level in SCI rats. Importantly, miR-362-3p was proved to bind with PAX2. These results suggest that miR-362-3p promoted cell apoptosis through targeting PAX2. Besides, previous studies showed that SB203580 and PD98059, as p38 and MEK inhibitor, could repress PAX2 expression (Fujita et al., 2010). Interestingly, our study indicated that miR-362-3p not only alleviated the neuralgia, but also suppressed the p-ERK1/2, p-MEK1/2 and p-p38 protein levels by inhibition of PAX2 after SCI. Increasing evidence reported that ERK phosphorylated and then activated CREB, promoting transcription of CREB-dependent and pain-associated genes (Ji et al., 1999). This phosphorylation event elevated the translation of brain-derived neurotrophic factor mRNA in dorsal root ganglion neurons (Moy et al., 2018), inducing translation and transcription of pain-relevant genes. Therefore, these findings indicate that miR-362-3p attenuate neuropathic pain following SCI by targeting PAX2.

However, other genes may be targeted by miR-362-3p. Moreover, the current study does not contribute to the evaluation of clin-
Figure 5. PAX2 is verified as the target of miR-362-3p [A] Luciferase activity was used to measure the relative luciferase activity. (B) and (C) PAX2 level was analyzed using quantitative real-time PCR and western blot analysis. (D) The correlation between miR-362-3p and PAX2 was analyzed. *** indicated $p < 0.001$.

Figure 6. miR-362-3p alleviates the neuralgia by inhibiting PAX2 in SCI rats. (A) and (B) Both quantitative real-time PCR and western blot analysis were conducted to examine the PAX2 level, respectively. (C) The animal locomotor activity was detected through BBB scores. (D) and (E) Quantitative real-time PCR and ELISA assay were used to explore the level of COX-2, IL-1β, TNF-α and IL-6. * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$.

In summary, present study demonstrated that miR-362-3p attenuated neuropathic pain following SCI by targeting PAX2 for the first time, participating in regulation of brain-derived neurotrophic factor. The outcomes of the current study provide a potential target in molecular treatment of SCI in the future.
Figure 7. miR-362-3p reduces the activation of ERK and p38 by inhibition of PAX2 in SCI rats. The ERK1/2, MEK1/2, p38, p-ERK1/2, p-MEK1/2 and p-p38 protein levels were assayed via western blot analysis. ** indicated \( p < 0.01 \), *** indicated \( p < 0.001 \).

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Authors' contributions

YH and QL designed the research study. MZ and YY performed the research. HY and LG provided help and advice on the ELISA experiments.

Conflict of interest

The authors declare no competing interests.

Ethics approval and consent to participate

The animal use protocol listed below has been reviewed and approved by Center for Animal Experimental of Gansu Provincial Hospital. Approval No. IACUC DB-16-025

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