1 **Ginsenoside Rg1 defenses PC-12 cells against hydrogen peroxide-caused damage**
2 **via up-regulation of miR-216a-5p**

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4 **Running title:** Protective mechanism of ginsenoside Rg1

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6 Guangkun Yi¹, Li Liu², Chaoliang Lv¹, Yanchun Wei¹, Tingzhen Yan^{1,*}

7 ¹Department of Spine Surgery, Jining No.1 People's Hospital, Jining 272011,
8 Shandong, China

9 ²Department of Cardiology, Jining No.1 People's Hospital, Jining 272011, Shandong,
10 China

11 ***Address Correspondence to:** Tingzhen Yan, Department of Spine Surgery, Jining
12 No.1 People's Hospital, No.6, Jiankang Road, Jining 272011, Shandong, China

13 E-mail: tingzhen67ya@163.com

14 Guangkun Yi: zejing47481632@126.com

15 Li Liu: zhaoban9037448@126.com

16 Chaoliang Lv: xini028495@126.com

17 Yanchun Wei: aikai50162218@126.com

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19 **Abstract**

20 **Background:** Spinal cord injury (SCI) is a destructive trauma accompanying with
 21 local injury, half of which cause chronic paralysis. Ginsenoside Rg1 exerts
 22 anti-apoptosis and anti-autophagy properties. Therefore, our goal was to study the
 23 protective mechanism of Rg1 in attenuating cell injury.

24 **Methods:** MiR-216a-5p inhibitor was transfected into PC-12 cells, then cells were
 25 pre-treated by Rg1 and treated with 300 μ M hydrogen peroxide (H_2O_2) for 24 h.
 26 CCK-8 and apoptosis experiments were done to test cell activity and apoptosis
 27 respectively. Expression of miR-216a-5p and cell damage relative factors was tested
 28 via qRT-PCR and western blot experiments, respectively.

29 **Results:** H_2O_2 induced cell activity suppression, apoptosis and autophagy well at the
 30 concentration of 300 μ M, leading cell injury. Rg1 could attenuate cell injury induced
 31 by H_2O_2 at the working concentration of 200 μ M that it elevated cell activity,
 32 attenuated apoptosis and autophagy and activated PI3K/AKT and AMPK signal
 33 pathways. Further, miR-216a-5p was up-regulated by Rg1. Rg1 played its role in
 34 relieving cell injury by positively regulating miR-216a-5p.

35 **Conclusion:** Our study demonstrated that Rg1 attenuated H_2O_2 -caused cell injury
 36 through positively regulated miR-216a-5p.

37 **Key words:** ginsenoside Rg1, miR-216a-5p, cell injury

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39

40 **Introduction**

41 Spinal cord injury (SCI), a common and destructive trauma (Hyun and Kim, 2010), is
 42 mainly caused by external forces such as lateral bending, excessive stretching,
 43 rotation, axial load and excessive bending, resulting in motor dysfunction, paralysis
 44 and other symptoms (van den Berg et al., 2010). Because of the limited therapy
 45 selection, the administration and care of SCI patients places a heavy burden on
 46 patients and caregivers. Of particular note, more than 60% of damages occur at the
 47 cervical level (Cripps et al., 2011), and the lifetime care costs are about at \$1.1-\$4.2
 48 million per patient (Krueger et al., 2013). So, the precaution, curing and recovery of
 49 SCI are a major topic in the medical area. SCI relates two different stages of tissue
 50 injury, called primary and secondary hurt (Badner et al., 2019). Local tissue injury is
 51 caused by SCI and is important in secondary hurt in SCI (Fu et al., 2018), leading to
 52 apoptosis with loss of neurological roles. So, mechanism study of local injury after
 53 SCI is highly significant for curing SCI.

54 Ginsenosides, considering as one of the main pharmacological active ingredients of
 55 ginseng, is a steroid compound (Xiang et al., 2008). Ginsenoside contains the
 56 Panaxatriol (Rg1, Rg2, Re and Rf) and Panaxadiol (Rb1, Rb2, Rc and Rd) classes
 57 (Zhang et al., 2012a). Many beneficial effects of Rg1 have been proved in disorders
 58 such as hypertension (Chen et al., 2012), hypoxia/reoxygenation (Zhang et al., 2012b),
 59 Alzheimer's disease (Huang et al., 2012) etc. Importantly, it has been reported that
 60 Rg1 exerts roles in inhibiting cell apoptosis, thereby exhibiting notable
 61 cardioprotective effects against I/R damage through a variety of mechanisms (Lee and
 62 Kim, 2014). Besides, Rg1 counteracts the aging of endothelial progenitor cells (Shi et
 63 al., 2011) and human fibroblasts (Zhou et al., 2012) and exerts a notable influence in
 64 suppressing cardiomyocytes and renal tubular cells' autophagy (Mao et al., 2016). The
 65 influence of Rg1 in local injury after SCI still has been unknown yet.

66 MicroRNAs (miRNAs), short (22 nucleotides in length) non-coding RNAs, involve
 67 in many biological processes (Jiang and Chen, 2012), such as differentiation of
 68 ordinary tissues and are important in the pathogenesis of lots of human cancers
 69 (Taucher et al., 2016). MiR-216a-5p, known as an oncogene, involved in the
 70 progression of many cancer subtypes (Chen et al., 2018). Chen *et al.* has proved that
 71 miR-216a-5p elevates cell proliferation, activity and motility, and inhibits apoptosis
 72 (Chen et al., 2018). This finding demonstrates that miR-216a-5p has a positive effect
 73 on cell viability and anti-apoptosis. So it could be interesting to investigate if exerts

regulation relation of miR-216a-5p and Rg1 in cell injury after SCI. Based on the above questions and guesses, we probed mechanism of Rg1 against H₂O₂-caused cell damage in PC-12 cells.

Materials and Methods

Cell

PC-12 cells were bought from Kunming Institute of Zoology (Kunming, China) in this whole study. Seed cells at a denseness of 1×10^4 cells/ml in Dulbecco's Modified Eagle Medium (DMEM)/F-12 medium (Gibco, Carlsbad, CA, USA) adding with 10% fetal bovine serum (FBS, Gibco), 100 µg/ml streptomycin and 100 U/ml penicillin (Gibco). Cells were kept in a wet incubator carried 5% CO₂ and 95% air at 37°C. Change fresh medium every day. Ginsenoside Rg1 (analysis level of 97% pureness) was bought from Sigma-Aldrich (St. Louis, MO, USA), solubled in ethanol and stored at -20°C. Pre-treatment of cells with Rg1 for 1 h, and then were treated with a series of consistences of hydrogen peroxide (H₂O₂) for 24 h.

CCK-8 experiment

A Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA) was to test cell activity. Seed cells in 96-well plate with 5000 cells/well, and then add CCK-8 solution, keep cells in a wet environment carried 95% air and 5% CO₂ for 1 h at 37°C. Absorbance was tested at 450 nm via a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis experiment

Apoptosis analysis was done through propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining (BD Pharmingen, San Diego, CA, USA). Cells were cleaned in phosphatebuffered saline (PBS) for three times and stained in PI/FITC-Annexin V with 50 µg/ml RNase A (Sigma-Aldrich). Keep cells in dark processing at the room temperature for 1 h. Flow cytometry analysis was made through FACS can (Beckman Coulter, Fullerton, CA, USA). Data was analyzed via FlowJo software (Tree Star Software, San Carlos, California, USA).

Transfection

MiR-216a-5p inhibitor and its relative NC were compounded by Life Technologies Corporation (Carlsbad, CA, USA) and transferred into cells. Transfection was done following the Lipofectamine 3000 reagent (Life Technologies Corporation). 48 h post-transfection was regarded as harvest moment in following assays.

qRT-PCR

Overall RNA was extracted through Trizol reagent (Life Technologies Corporation) and handled with DNaseI (Promega, Madison, WI, USA). Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) were to test miR-216a-5p expression. U6 was taken as inside comparison.

Western Blot

Overall protein was extracted through RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Roche, Basel, Switzerland), and then quantified through BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). A Bio-Rad Bis-Tris Gel system was taken to build up a western blot system. Primary antibodies specific against Bax (ab32503, Abcam, Cambridge, MA, USA), pro-caspase-3 (ab183179), cleaved-caspase-3 (ab49822), pro-PARP (ab32064), cleaved-PARP (ab4830), β -actin (ab8226), beclin-1 (ab62557), p62 (ab56416), LC3-I and LC3-II (ab48394), t-PI3K (ab140307), p-PI3K (ab182651), t-AKT (ab179463), p-AKT (ab38499), t-AMPK (ab131512) and p-AMPK (ab23875) were readied in 5% blocking buffer. Primary antibody was cultured with membrane at 4°C all the night, then washing and incubating with secondary antibody, marking by horseradish peroxidase for 1 h at room temperature. Then the Polyvinylidene Difluoride (PVDF) membrane taken along blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, adding 200 μ l Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) to shroud film surface. At last, semaphores were seized and strength of strip was quantified via Image Lab™ Software (Bio-Rad).

Statistical analysis

All assays were duplicated for 3 times. Our consequences of multiplex assays are revealed as mean $\pm$ SD. Statistical analysis was done via Graphpad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). *P*-values were counted via a one-way analysis of variance (ANOVA). *P*-value of < 0.05 indicated statistical significant data.

Results

Rg1 extenuated H₂O₂-induced cell activity suppression and cell apoptosis

PC-12 cells were treated in various H₂O₂ consistences. From **Figure 1A**, we found that H₂O₂ had notably inhibiting effect on cell viability when the concentration was 100 ($P < 0.05$), 200 ($P < 0.05$), 300 ($P < 0.01$), 400 ($P < 0.01$) and 500 μ M ($P < 0.001$). We chose 300 μ M as the working concentration in the later assays because this was cell viability semi-lethal concentration. Besides, we tested effect of H₂O₂ on cell

apoptosis. We found that apoptosis was notably increased by H_2O_2 ($P < 0.001$, **Figure 1B**). Similarly, apoptosis relative factors (Bax, cleaved-caspase-3 and cleaved-caspase-PARP) were obviously enhanced through H_2O_2 (**Figure 1C**), and standards of these factors were notably raised (all $P < 0.01$, **Figure 1D**). We got that H_2O_2 caused cell activity suppression and apoptosis.

For function of Rg1, following experimental results were clear. As shown in **Figure 1E**, there was no effect on cell viability by Rg1. We found that H_2O_2 could notably reduce cell viability ($P < 0.01$), whereas Rg1 could notably attenuate this reduction at 200, 300 and 400 μM (all $P < 0.05$). We chose 200 μM as the working concentration in the following experiments because this is the concentration when cell viability was half restored. Besides, for cell apoptosis, we found that Rg1 attenuated apoptosis induced by H_2O_2 ($P < 0.01$, **Figure 1G**). Similarly, **Figure 1H** revealed that expression of apoptosis relative factors was weakened by Rg1 compared with H_2O_2 group. Levels of these factors were raised through H_2O_2 ($P < 0.01$, $P < 0.001$ and $P < 0.01$), whereas Rg1 could decrease their levels (all $P < 0.05$, **Figure 1I**). So we got that Rg1 attenuated cell activity suppression and apoptosis induced by H_2O_2 .

Rg1 extenuated autophagy induced by H_2O_2

For autophagy, we tested three autophagy relative factors. Beclin-1 is autophagy gene and its overexpression can stimulate autophagy (Yue et al., 2003). Accumulation of p62 is a notable phenotype of autophagy-deficient tumor cells (Mathew et al., 2009). LC3-II is a marker for mature autophagosomes. Autophagy could be analyzed by testing the conversion of the autophagosome marker LC3-I to LC3-II (Wu et al., 2010). According to our results, **Figure 2A** showed the enhancement of beclin-1 and LC3-II/LC3-I through H_2O_2 , while Rg1 could weaken this enhancement. Expression of p62 was weakened by H_2O_2 , while Rg1 could eliminate this mitigation (**Figure 2A**). Besides, **Figure 2B** revealed the notable addition of beclin-1 and LC3-II/LC3-I through H_2O_2 ($P < 0.01$ and $P < 0.001$), whereas were opposite by adding of Rg1 ($P < 0.05$ and $P < 0.01$). p62 expression was notably weakened through H_2O_2 ($P < 0.05$), whereas was increased by the adding of Rg1 ($P < 0.05$, **Figure 2B**). So we got that Rg1 could attenuate H_2O_2 -induced autophagy.

Rg1 positively regulated miR-216a-5p

From **Figure 3**, qRT-PCR assay indicated that miR-216a-5p was notably down-regulated after H_2O_2 treatment ($P < 0.05$). But, it was specifically up-regulated by adding Rg1 ($P < 0.01$). So we got that Rg1 up-regulated miR-216a-5p.

176 ***Rg1 extenuated cell activity suppression, apoptosis and autophagy induced by H₂O₂***
 177 ***through up-regulating miR-216a-5p***

178 qRT-PCR revealed that miR-216a-5p expression was notably suppressed after
 179 miR-216a-5p inhibitor transfection ($P < 0.01$, **Figure 4A**). Cell viability was notably
 180 aggravated by Rg1 contrast with H₂O₂ set ($P < 0.05$), whereas was notably alleviated
 181 when treated with Rg1 plus miR-216a-5p inhibitor ($P < 0.05$, **Figure 4B**). This result
 182 indicated that Rg1 attenuated H₂O₂-induced cell activity suppression by up-regulating
 183 miR-216a-5p. Besides, cell apoptosis was notably decreased by Rg1 contrast with
 184 H₂O₂ set ($P < 0.01$), whereas was notably increased when treated with Rg1 plus
 185 miR-216a-5p inhibitor ($P < 0.01$, **Figure 4C**). **Figure 4D-E** further indicated that
 186 levels of apoptosis relative factors were notably decreased through Rg1 contrast with
 187 H₂O₂ set ($P < 0.05$, $P < 0.01$ and $P < 0.01$), whereas were raised in Rg1-treated cells
 188 with miR-216a-5p inhibitor ($P < 0.05$, $P < 0.01$ and $P < 0.05$). So we got that Rg1
 189 reduced H₂O₂-caused apoptosis by up-regulating miR-216a-5p. Additionally, **Figure**
 190 **4F-G** revealed the notable attenuation of beclin-1 and LC3-II/LC3-I ($P < 0.01$ and P
 191 < 0.001) by Rg1 and level of p62 was increased ($P < 0.05$) by Rg1 contrast with
 192 H₂O₂ set. However, beclin-1 and LC3-II/LC3-I were notably enhanced (both $P < 0.05$)
 193 and level of p62 was notably decreased when treated with Rg1 plus miR-216a-5p
 194 inhibitor ($P < 0.05$). So we got that Rg1 attenuated H₂O₂-induced autophagy by
 195 up-regulating miR-216a-5p.

196 ***Signal pathway***

197 To further study the mechanism of Rg1, we focused on PI3K/AKT and AMPK signal
 198 pathways. **Figure 5A-B** indicated the notable addition levels of p-PI3K and p-AKT
 199 through Rg1 contrast with H₂O₂ set ($P < 0.01$ and $P < 0.05$), whereas were notably
 200 alleviated in Rg1-treated cells with miR-216a-5p inhibitor ($P < 0.01$ and $P < 0.05$).
 201 Besides, **Figure 5C-D** revealed that level of p-AMPK was notably aggravated by Rg1
 202 compared with H₂O₂ group ($P < 0.01$), whereas was notably alleviated in Rg1-treated
 203 cells with miR-216a-5p inhibitor ($P < 0.01$). These results indicated that Rg1 elevated
 204 PI3K/AKT and AMPK pathways via positively modulating miR-216a-5p.

205 **Discussion**

206 SCI is a life-changing event. Recently, there is no effective treatment method to
 207 resume the functions of SCI patients. The complexity of SCI pathophysiology poses a
 208 huge challenge for researchers and clinicians seeking to develop therapeutic
 209 interventions (Bareyre, 2019). Local injury is the main event of secondary injury in

SCI, eventually leading to apoptosis and ultimately loss of neurological function (Genovese et al., 2009). Therefore, defending cells against local injury or relieving this injury can be an effective method to cure SCI. Rg1, an active component of ginsenosides, has been proved to exert positive effect on anti-apoptosis (Zu et al., 2016). Cell damage has been reported to be induced by H₂O₂ in cardiomyocytes (Zeng et al., 2019) and neural cells (Chen et al., 2016). Therefore, our study also found that H₂O₂ was a mediator capable of inducing damage in PC-12 cells, including activity suppression, promotion of autophagy and apoptosis. Our study firstly researched the attenuated mechanism of Rg1 in H₂O₂-caused damage in PC-12 cells. Rg1 was effective in defending PC-12 cells against H₂O₂-caused damage. Rg1 could extenuate H₂O₂-induced cell activity suppression, apoptosis and autophagy, and active PI3K/AKT and AMPK pathways via positively regulating miR-216a-5p. Our results indicated that Rg1 may be an effective treatment of curing SCI.

Ginsenoside Rg1, the main bioactive ingredient in ginseng, has been proved to exert low toxicity that there was no change on cell viability and proliferation (Li et al., 2017). Much evidence indicates that Rg1 exerts beneficial effects, like anti-aging properties (Zhu et al., 2014). As we all know, Bax may control mitochondrial permeability transition and promote releasing cytochrome c, ultimately triggering the activation of caspases, leading to apoptosis (Li et al., 2017). Consistently, in our study, treatment of Rg1 reduced the level of Bax, at the same time, cleaved-caspase-3 and cleaved-caspase-PARP were weakened. These observations verified the anti-apoptosis function of Rg1. Additionally, autophagy is an important cellular process where cytoplasmic components are digested by lysosomes to keep cell homeostasis and energy production (Ravikumar et al., 2010). Rg1 has a notable pharmacological influence in suppressing autophagy (Mao et al., 2016). Our study were consistent with the report that Rg1 strongly inhibited autophagic factor (beclin-1 and LC3-II/LC3-I), and counteracted attenuation of p62 induced by H₂O₂, leading to inhibiting H₂O₂-induced autophagy in PC-12 cells. Consistently, our study indicated that Rg1 could counteract inhibition of cell activity induced by H₂O₂ and attenuate cell apoptosis and autophagy, suggesting the anti-oxidant and anti-autophagy functions of Rg1 in SCI.

To further study the mechanism of Rg1, we turn our attention to miRNA. MiRNA is important in cell growth, like proliferation and apoptosis (Ameres and Zamore, 2013). MiR-216a-5p, acknowledged as an oncogenic gene, is involved in tumorigenesis and

development of human cancers (Liu et al., 2018). MiR-216a-5p significantly elevated cell activity and reduced apoptosis in H₂O₂-caused 16 HBE cells of Asthma, suggesting that miR-216a-5p could regulate H₂O₂-caused damage (Chaoyang et al., 2019). Besides, of interest, beclin-1 was the latent mark of miR-216-5p, which could inhibit ox-LDL-induced autophagy in human umbilical vein endothelial cells (HUVECs) through modulating levels of intracellular beclin-1 (Menghini et al., 2014). These reports indicate that miR-216a-5p not only elevates activity, suppresses apoptosis, but also inhibits autophagy, suggesting that miR-216a-5p may reduce H₂O₂-caused cell damage. Also, functions of miR-216a-5p are similar to those of Rgl. So it is worth to investigate if exerts a relation of Rgl and miR-216a-5p. For the first time, our study found the regulation relationship between Rgl and miR-216a-5p. We got that Rgl could up-regulate miR-216a-5p to attenuate cell injury induced by H₂O₂. This finding is a major discovery in SCI research.

Furthermore, the biological process is inseparable from the regulation of signal pathways. It has been proved that AMPK/PI3K/AKT pathways are key coordinator protecting cells from oxidative and inflammatory damage (Lv et al., 2017). PI3K was reported to be related to many cellular functions, like proliferation and apoptosis (Rasul et al., 2012). AKT is a key downstream effector of PI3K and exhibits anti-apoptotic effects (Zheng et al., 2015). AMPK is present in metabolically related organs. Cellular metabolism stimulation like cell stress can active it (Zheng et al., 2015). Moreover, Lin *et al.* found that the formation of autophagosomes was accompanied by inhibition of the PI3K/AKT and AMPK signal pathways. This finding indicated that there was negative regulation between these two pathways and autophagy. Therefore, the above findings suggested that there might be positive effects of these two pathways on H₂O₂-caused cell damage. It is worth noting that our study verified this suppose. We firstly build up the relation among Rgl, miR-216a-5p and AMPK/PI3K/AKT pathways that Rgl activated PI3K/AKT and AMPK signal pathways through positively modulating miR-216a-5p to reduce cell damage. This regulation mechanism provides a theoretical basis for attenuating cell injury after SCI.

273 **Conclusion**

Our study firstly reported the underlying effects and mechanism of Rgl in cell injury of SCI. We demonstrated that Rgl could up-regulate miR-216a-5p, attenuate cell activity suppression, apoptosis and autophagy, and active PI3K/AKT and AMPK signal pathways to decrease H₂O₂-caused damage in PC-12 cells. Because local cell

278 injury significantly aggravates SCI, we propose that Rg1, an effective biological
279 macromolecular, may supply a novel therapeutic approach for curing SCI.

280 **Conflict of Interest statement**

281 The authors declare that there are no conflicts of interest.

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287 **Availability of data and materials**

288 The dataset(s) supporting the conclusions of this article is(are) included within the
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290

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Figure legends

Figure 1 Influence of Rg1 in cell activity and apoptosis caused by H₂O₂ in PC-12 cell.

(A) Cell viability was tested under diverse consistences of H₂O₂ (0, 100, 200, 300, 400 and 500 μ M). 300 μ M was chose in the following experiments. (B) Cell apoptosis treated by H₂O₂ was tested via flow cytometry. (C) Apoptosis relative elements expression was tested via western blot. (D) Level of apoptosis relative factors was tested via western blot quantitative. (E) Cell viability was tested via CCK-8 by Rg1. (F) Rg1 attenuated H₂O₂-induced suppression of cell viability. (G) Rg1 attenuated H₂O₂-induced cell apoptosis. (H) Expression of apoptosis relative factors was tested via western blot. (I) Apoptosis relative elements standards were detected via western blot quantitative. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ contrast with control and the indicated set.

Figure 2 Influence of Rg1 in autophagy caused by H₂O₂. (A) Standards of autophagy relative factors were tested via western blot. (B) Standards of autophagy relative factors were tested via western blot quantitative. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ contrast with indicated set.

Figure 3 MiR-216a-5p was up-regulated by Rg1. mRNA standard of miR-216a-5p was tested via qRT-PCR. * $P < 0.05$ and ** $P < 0.01$ contrast with indicated set.

Figure 4 Protective effects of Rg1 via up-regulating miR-216a-5p after transfected with Rg1 plus miR-216a-5p and relative NC in PC-12 cells. (A) mRNA standard of miR-216a-5p was tested via qRT-PCR after miR-216a-5p inhibitor transfection. (B) Cell activity was tested via CCK-8. (C) Apoptosis was tested via flow cytometry. (D) Expression of apoptosis relative factors was tested via western blot. (E) Level of apoptosis relative factors was tested via western blot quantitative. (F) Standards of autophagy relative factors were tested via western blot. (G) Standards of autophagy relative factors were tested via western blot quantitative. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ contrast with indicated set.

Figure 5 Rg1 elevated PI3K/AKT and AMPK signal pathways through positively regulating miR-216a-5p after transfected with Rg1 plus miR-216a-5p and relative NC in PC-12 cells. (A) Expression of PI3K/AKT pathway relative factors was tested via

421 western blot. (B) Standards of relative proteins were tested via western blot
422 quantitative. (C) Expression of AMPK pathway relative factors was tested via western
423 blot. (D) Level of AMPK pathway related proteins were detected via western blot
424 quantitative. * $P < 0.05$ and ** $P < 0.01$ contrast with indicated set.

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A **B**

miR-216a-5p