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The anti-apoptotic effect of nerve growth factor on propofol-induced neurotoxicity in hippocampal neurons is Rac1 dependent

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Propofol has been considered as a near-ideal anesthetic agent since its introduction 40 years ago. However, the side effects of propofol including bacterial contamination, hyperlipidemia, and neurotoxicity also aroused attention. Nerve growth factor (NGF) plays a pivotal role in the development, differentiation, and survival of the neurons of the peripheral and central nervous system. In the present study, we found that NGF alleviated the apoptosis induced by propofol in hippocampal neurons. Furthermore, NGF treatment augmented the protein abundance and mRNA level of Rac1 while silencing Rac1 significantly blunted the effects of NGF upon propofol-induced apoptosis. In conclusion, NGF decreased propofol-induced apoptosis and this effect was Rac1 dependent.

1. Introduction

Propofol, is a quite popular intravenous anesthetic agent due to its rapid effect, short action and few side effects (Lundstrom et al. 2010; Tang and Eckenhoff 2018). Propofol is chemically described as 2,6- diisopropylphenol and functions by activation of the central inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Miao et al. 2010; Jin et al. 2015). Currently, the neurotoxic role of propofol is controversely discussed, especially for children. Some researchers claimed that propofol exerted a neuroprotective role following cerebral ischemia (Ergun et al. 2002; Bayona et al. 2004), while others found that propofol caused significant cerebral vasoconstriction due to a fall in cerebral blood flow, metabolic demand for oxygen and any pre-existing cerebral edema (Vavilala and Lam 2003; Szabo et al. 2009). Current concerns of neuro-apoptosis induced by propofol thus continue to be a focus of research which may guide novel therapeutic strategies.

Prolonged exposure to propofol anesthesia causes caspase-3- and calpain-mediated neuron loss while enhances Nerve Growth Factor (NGF) protein expression in neonatal rat brain (Szabo et al. 2009; Milanovic et al. 2014). The balance between death and survival controlled by propofol needs further investigation, thereby establishing an approach to protect neurons from the toxic effects of anesthesia during the early phase of brain development. NGF plays a pivotal role in the development, differentiation, and survival of the neurons of the peripheral and central nervous system. It exerts a neuroprotective role in childhood hypoxic-ischemic brain injury (Chiaretti et al. 2011) which is frequently associated with poor clinical and neurological outcomes. Moreover, NGF induces rapid activation of Rac1 and suppression of RhoA activity (Yamaguchi et al. 2001). Rac1, also known as Ras-related C3 botulinum toxin substrate 1, is a member of the Rac subfamily of the family Rho and has been shown to be involved in the regulation of neuronal morphology. Concurrently, Rho-kinase inhibitors augment the inhibitory effect of propofol on rat bronchial smooth muscle contraction (Hanazaki et al. 2008). Taken together, we propose that Rac1 might contribute to the neuroprotective role of NGF.

In the present study, we examined the neuroprotective role of NGF in propofol-induced hippocampal neurons apoptosis and delineated the mechanism.

2. Investigations and results

2.1. NGF alleviated propofol-induced apoptosis

Propofol is generally believed to induce cellular apoptosis. To protect neurons from propofol-induced apoptosis, we used NGF to treat the primary hippocampal neurons for 4 h at different concentrations (5, 10, 15, 20, 25 ng/ml). As shown in Fig.1A and B, MTT assay showed that propofol (4 h, 1 μM) induced apoptosis of hippocampal neurons, and this effect was abolished by NGF treatment in a dose-dependent manner (5, 10, 15, 20, 25 ng/ml). NGF did not affect cell viability. We thus chose 25 ng/ml NGF to perform the following experiments. Moreover, western blotting results were consistent with the above finding, showing that propofol enhanced BAX expression, an effect abolished by NGF treatment (Fig. 1C and D).

2.2. NGF enhanced Rac1 expression

To explore the mechanism by which NGF influences apoptosis of hippocampal neurons, we evaluated the mRNA level of Rac1 after NGF treatment (25 ng/ml) for 4 h by RT-PCR and western blotting. As shown in Fig. 2A, B and C, mRNA level and protein expression of Rac1 were significantly increased after NGF administration for 4 h.

2.3. Rho inhibitors abolished the effect of NGF

We then treated neurons with the Rho kinase inhibitors thiazovivin and GSK429286A. Results showed that thiazovivin and GSK429286A abolished the neuroprotective role of NGF (Fig. 3). NGF showed no effects on propofol-induced apoptosis in the presence of thiazovivin (4 h, 1 μM) and GSK429286A (4 h, 1 μM).

2.4. Rac1 silencing inhibited the effect of NGF

To confirm the involvement of Rac1 in NGF signaling, we silenced Rac1. As shown in Fig. 4A, Rac1 silencing inhibited the neuroprotective effects of NGF on Propofol-induced hippocampal neurons apoptosis. Moreover, NGF treatment did not affect propofol-induced BAX expression in siRac1 neurons (Fig. 4B).

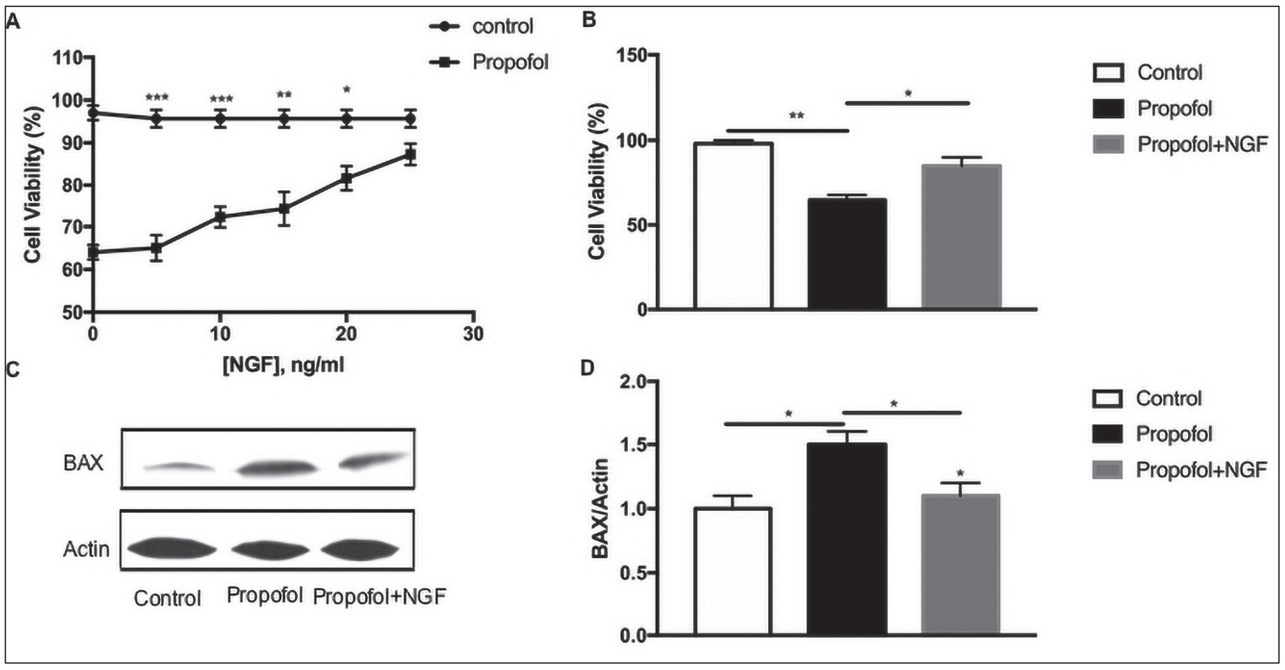


Fig. 1: NGF protects hippocampal neurons from propofol-induced apoptosis.
A. MTT analyze was used to determine cell death. Hippocampal neurons were incubated with different concentrations of NGF (5, 10, 15, 20, 25 ng/ml) for 4 h.
B. Hippocampal neurons were incubated with NGF (25 ng/ml) with the treatment of propofol (1 μM) for 4 h. Data are means±SEM (n = 5).
C. Original western blot showing the level of BAX and as well as respective Actin in neurons with NGF (25 ng/ml) treatment in the presence of propofol (1 μM) for 4 h.
D. Arithmetic means±SEM (n=5) of BAX protein abundance in neurons with NGF (25 ng/ml) treatment in the presence of propofol (1 μM) for 4 h.
* ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) indicate statistically significant difference

3. Discussion

The safety of propofol administration for children remains questionable and needs to be confirmed. Here, we assessed the toxicity of propofol in hippocampal neurons isolated from new-born SD rats and evaluated the neuroprotective role of NGF in propofol-induced neurons apoptosis. Propofol induced neuron apoptosis and increased apoptosis regulator BAX expression, which could be reversed by NGF. Moreover, the neuroprotective effect of NGF was abolished by Rho kinase inhibitors and Rac1 silencing. Western blot further showed that NGF enhanced Rac1 expression. We thus

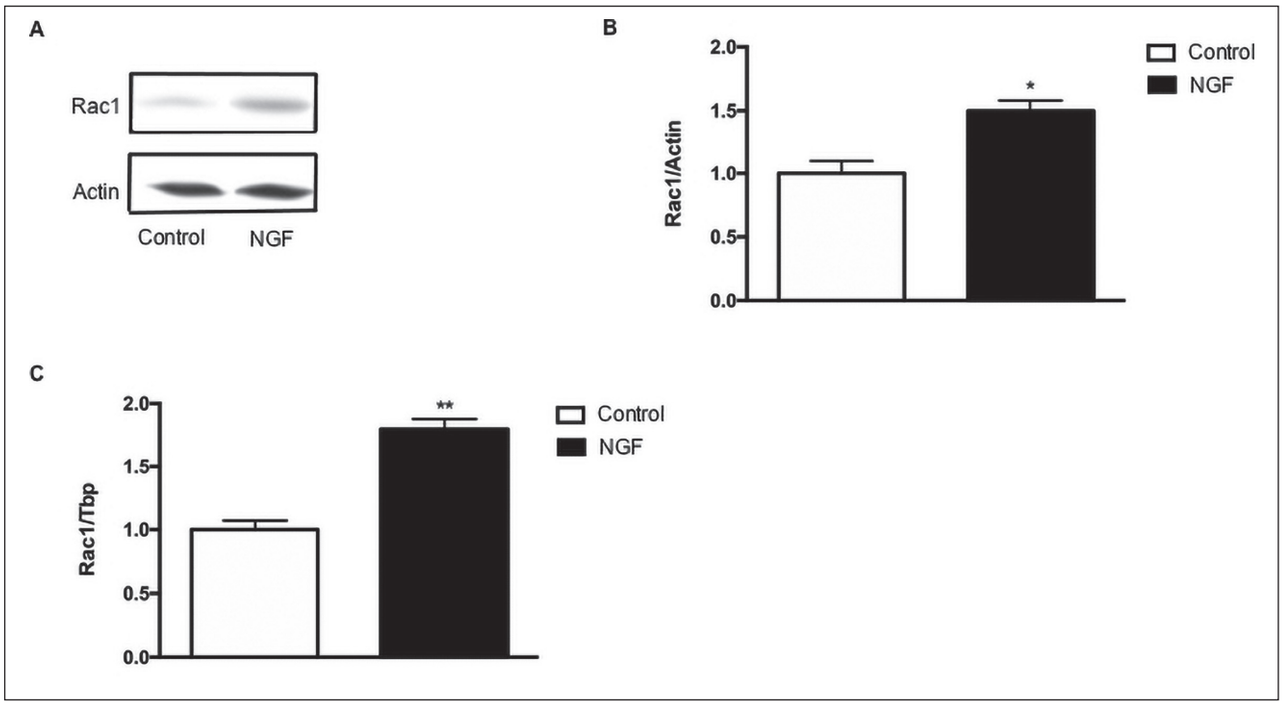


Fig. 2: NGF increased the expression of Rac1
A. Original western blot showing the level of Rac1 and as well as respective Actin in neurons with NGF (25 ng/ml) treatment for 4 h.
B. Arithmetic means±SEM (n=5) of Rac1 protein abundance in neurons with NGF (25 ng/ml) treatment for 4 h.
C. Arithmetic means±SEM (n=5) of mRNA level of Rac1 in neurons with NGF (25 ng/ml) treatment for 4 h.
* ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) indicate statistically significant difference

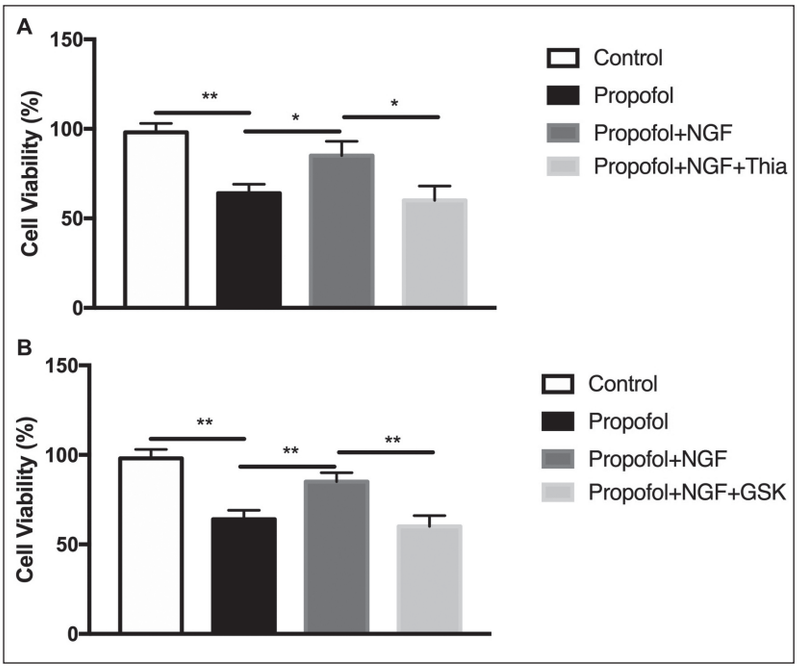


Fig. 3: The neuroprotective role of NGF is inhibited by Rho kinase inhibitor
A. MTT analyze was used to determine cell death. Thiazovivin (1 μM) was applied to NGF (25 ng/ml) and propofol-treated hippocampal neurons. Data are means±SEM (n=5).
B. MTT analyze was used to determine cell death. GSK429286A (1 μM) was applied to NGF (25 ng/ml) and propofol-treated hippocampal neurons. Data are means±SEM (n=5).
* ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) indicate statistically significant difference

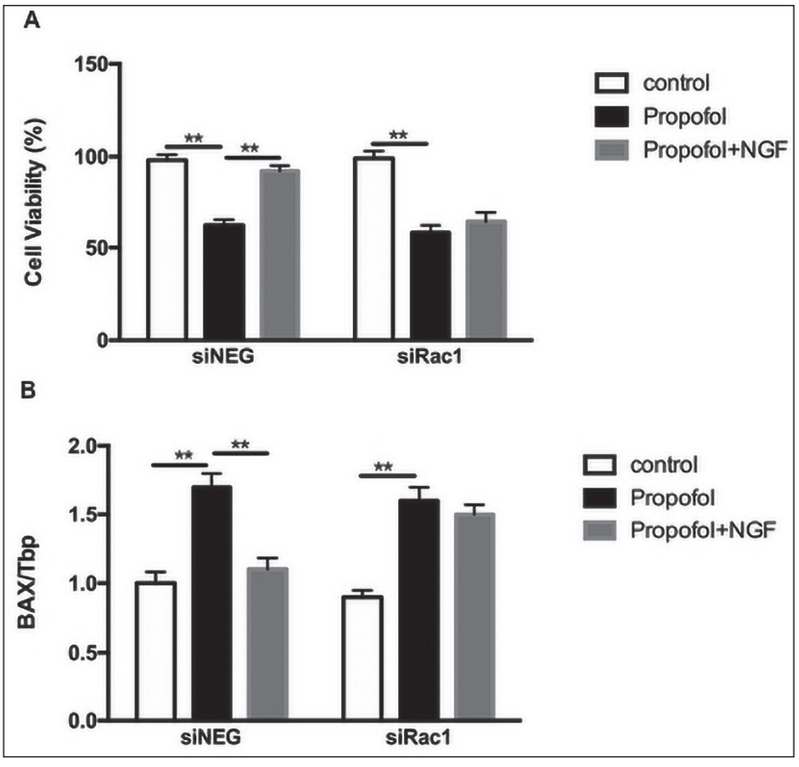
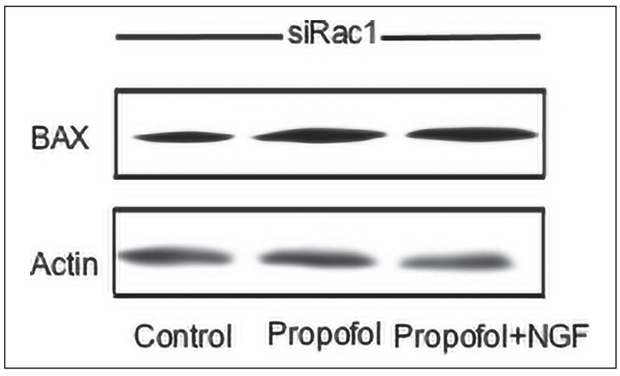


Fig. 4: Rac1 silencing abolishes the effects of NGF
A. MTT analyze was used to determine cell death. Neurons viability was analyzed in siRac1 and siNEG hippocampal neurons incubated with NGF (25 ng/ml) in the presence of propofol. Data are means±SEM (n=5).
B. Arithmetic means±SEM (n=5) of BAX mRNA level in siRac1 and siNEG hippocampal neurons with NGF (25 ng/ml) treatment in the presence of propofol (1 μM) for 4 h.
* ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) indicate statistically significant difference

propose that NGF could rescue propofol-induced hippocampal neurons apoptosis by regulating Rac1.

Propofol is widely known to be an effective intravenous anesthetic agent with many pharmacological advantages such as rapid effect, short action and fewer side effects (Chidambaran et al. 2015), but its safety and side effects in children remains unclear (Borgeat et al. 1990; Smith et al. 2012). Propofol metabolism is related to hepatic clearance (Hiraoka et al. 2004) and hepatic enzymes (Al-Jahdari et al. 2006), but childhood, which is a period of physiological maturation, undergoes pharmacokinetics and metabolism changes including hepatic and renal function, as well as cerebral cortical effects (Constant and Rigouzzo 2010). Therefore, finding a therapeutic strategy to alleviate the toxicity of propofol in children is necessary.

NGF is a neurotrophic factor and is involved in the regulation of growth, maintenance, proliferation, and survival of neurons (Calamandrei et al. 2000; Stelmashook et al. 2015). NGF upregulation correlates with a better neurologic outcome and could be useful to obtain clinical and prognostic information in children with severe traumatic brain injury (Chiaretti et al. 2009). Herein we



confirmed the neuroprotective effect of NGF on propofol injured hippocampal neurons, providing a promising combination usage of NGF and propofol in children.

Rac1 is a member of the Rac subfamily of the family Rho family of GTPases (Didsbury et al. 1989; Polakis et al. 1989). We found that NGF enhanced the expression of Rac1 and Rho-kinase inhibitors and abolished the neuroprotective effect of NGF, indicating a Rac1-sensitive neuroprotective role of NGF in hippocampal neurons. Furthermore, Rho-kinase inhibitors facilities the impact of propofol (Hanazaki et al. 2008) and attenuated the inhibitory effects of NGF on propofol-induced apoptosis. We propose that Rac1 might probably play a pivotal role in NGF signaling.

In conclusion, we suggest that NGF inhibits propofol-induced apoptosis via the Rac1 signaling pathway.

4. Experimental

4.1. Cell culture and treatments

Primary hippocampal tissue was prepared from the newborn Sprague-Dawley (SD) rats (provided by the laboratory animal center in Hebei province license) as described previously (Mattson et al. 1992; Yan et al. 2016). Briefly, collected neurons were seeded in a concentration of 1×10^6 cells/cm^2. Cells were cultured for 6 d in medium, consisting of Eagle's minimum essential medium containing 10 mm sodium bicarbonate, 1% glucose, 1 mm l-glutamine, 20 mm KCl, 1 mm sodium pyruvate, and 10% (v/v) heat-inactivated fetal bovine serum (Sigma). After 24 h in culture, the culture medium was replaced with Neurobasal medium containing B27 supplements (Invitrogen) in a humidified atmosphere (6% CO_2, 94% room air) at 37 °C. Approximately 12 h later, 5 μM cytosine arabinoside was added to the dishes to prevent the growth of non-neuronal cells.

To examine the effect of NGF on the neurotoxicity of propofol (Sigma, United States), neurons were treated with propofol (4 h, 1 μM) (Sigma, United States) in the presence of NGF (25 ng/ml) (Sigma, United States). Rho inhibitors thiazovivin (Park et al. 2015) (4 h, 1 μM) (Medchemexpress, United States) and GSK429286A (Schenkelaars et al. 2016) (4 h, 1 μM) (Medchemexpress, United States) were applied in NGF and propofol-treated neurons.

4.2 MTT assay

Neurons were grown in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were incubated in a stable environment with 5% CO_2 at 37 °C in a humidified incubator and medium was replaced every 24 hs. Cells were grown to about 80% confluence prior to treatment. Each group of cells was seeded in 96-well microtiter plates and incubated for 24 h. At different points, 20 μl of MTT were added to each well followed by 4 h incubation. The medium was discarded, and 150 μl of DMSO were added to each well and incubated for 20 min. The OD (optical density) 492 nm was assessed. The proliferation inhibition rate was calculated as: (1-the OD of the experimental group/the OD of the control group) ×100%. Each experiment was repeated five times.

4.3 Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted from DC cells according to the manufacturer's instructions. After DNAse digestion reverse transcription of total RNA was performed using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, United States). Real-time polymerase chain reaction (RT-PCR) of the respective genes were set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega, United States) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 68 °C for 20 s. For amplification the following primers were used (5`>3`orientation): The following primers were used:

for Tbp:
forward (5'-3'): ACTCCTGCCACACCAGCC;
reverse (5'-3'): GGTCAAGTTTACAGCCAAGATTCA
Rac1
forward (5'-3'): CAATGCGTTCCCTGGAGAGT
reverse (5'-3'): AACACGTCTGTTTGCGGGTA
Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad, United States) and all experiments were done in duplicate. Tbp was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔCT method as described (Feger et al. 2013).

4.4. Western blot analysis

Total protein was prepared as follows. Cells were lysed in RIPA buffer (Beyotime, Shanghai, China) with 1 % phenylmethylsulfonyl fluoride (Beyotime) and 1% protein phosphatase inhibitor (Beyotime) on ice for 30 min. The samples were centrifuged at 14,000 rpm and 4 °C for 20 min. The supernatant was removed and used for Western blotting. Total protein (40-60 μg) was separated by SDS-PAGE, thereafter transferred to PVDF membranes and blocked in 5% non-fat milk/Tris-buffered saline/Tween-20 (TBST) at room temperature for 1 h. Membranes were probed overnight at 4 ºC with polyclonal rabbit anti-BAX (1:1000, Beyotime, Shanghai, China). After incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000, Beyotime, Shanghai, China) for 1 h at room temperature, the bands were visualized with enhanced chemiluminescence reagents (Sigma, United States). Membranes were also probed with Actin (1:1000, Beyotime, Shanghai, China) antibody as loading control. Densitometric analysis was performed using Quantity One software (Abbiotec, United States).

4.5. Silencing

1×10^5 hippocampal neurons were seeded 24 h before the experiment in antibiotic-free medium. Cells were transfected with 5 μl/1000μl ON-TARGETplus Rat Rac1 siRNA (5μM, Thermo Fisher Scientific, USA) and ON-TARGETplus Non-targeting siRNA (5μM, Thermo Fisher Scientific) using the cationic lipid DharmaFECT 1 transfection reagent (0.5 μl/1000μl, Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were then collected in 24 hours after transfection. To confirm the silencing efficiency, transcript levels were quantified by PCR.

4.6. Statistics

Data are provided as means±SEM, n represents the number of independent experiments. All data were tested for significance using one-way ANOVA followed by post hoc Bonferroni test was applied when multiple comparisons between different groups were made. Only results with $p < 0.05$ were considered statistically significant.

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Authors' contributions : XL, ZZ, LH, RK, XL performed experiments and analyzed data, ZD designed the project and drafted the manuscript. All authors corrected and approved the manuscript.

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