



Tug1 Acts on ERK12 Signaling Pathway to Aggravate Neuronal Damage after Acute Ischemic Stroke

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ABSTRACT

Approximately 85% of stroke patients suffer from ischemic stroke, which has a high incidence and difficult prognosis. It has become one of the leading causes of death in middle-aged and elderly people and seriously threatens human health. This study mainly considers the role of lncRNA tug 1 on the ERK 12 signaling pathway to enhance neuronal damage after acute ischemic stroke. In the experiment, the middle cerebral artery occlusion (MCAO) model was constructed using the thread embolization method. The real-time quantitative RT-CR method was used to detect the relative transcriptional activity of TG1, GAS5 and SM22a genes in tissues. The relative expression level of SM22a protein in tissues was detected by the immune-histochemical method. Twenty-four hours after cerebral infarction, the nerve function, cerebral infarction area and ERK1/2 protein expression level of cerebral cortex on the side of cerebral infarction were detected in each group. The experimental results showed that the successful animal behavior scores of the MCAO model in the normal saline control group and Pepstatin A interference group were 1 point 25, 2 points 17 and 3 points 18. The results show that lncRNA tug1 can enhance the neuronal damage of the ERK12 signaling pathway after acute ischemic stroke. lncRNATUG1 plays an important role after OGD/RX and can accelerate cell apoptosis. If the expression of lncRNATUG1 is inhibited, the number of apoptosis is significantly reduced.

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Introduction

The incidence of ischemic stroke is increasing year by year with the aging of the social population. Patients with ischemic stroke require hospitalization, long-term care and rehabilitation training, which impose a great burden on society and family members (1). The onset of AIS is mainly caused by local cerebral ischemia caused by blood clots or other embolisms. Among them, about one-third to one-half of patients suffer from internal carotid or middle cerebral artery occlusion (2). Once you get sick, nerve cells will disappear at a rate of 190 million per minute, causing irreversible brain damage. The main purpose of the treatment of ischemic stroke is to promote the recanalization of blood vessels, reduce the death of nerve cells, and reduce the damage of ischemic penumbra (3, 4). If the ischemia persists, it will develop into permanent damage due to hypoxia and energy disorders (5). The research results of various serological indicators after acute ischemia-induced brain tissue injury

provide clinicians with very important serological indicators such as early state judgment and prognostic evaluation of patients with acute ischemic stroke (6). Therefore, it is very important to explore the role of lncRNA tug1 and ERK12 signal transmission pathways in acute ischemic stroke in reducing ischemic preconditioning in ischemic brain injury.

lncRNA plays an important role in various biological processes. For example, cell growth, cell differentiation and disease states. The abnormal regulation of lncRNA TUG1 is related to the occurrence of many neurological diseases. Chen et al. believe that endothelial cell (EC) apoptosis is a key process in the development of atherosclerosis. According to reports, salvianol can protect the vascular endothelium and reduce the formation of atherosclerosis (7, 8). However, the potential molecular mechanism of the protective effect of tanshinol in atherosclerosis needs further research (9). He fed ApoE mice with a high-fat diet and treated

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them with salvia to detect the effect of salvia on endothelial cell apoptosis by TUNEL staining (10). He performed qRT-PCR and Western blot to detect the expression of TUG1 and miR-26a in endothelial cells (11). He performed an RNA-binding protein immunoprecipitation test to verify the relationship between TUG1 and miR-26a (12, 13). Their experiments have shown that salvianol reduces the aortic atherosclerotic lesions of the entire aorta and aortic sinus in a concentration-dependent manner, and inhibits endothelial cell apoptosis in ApoE mice (14, 15). They further found that salvianol reduced the mRNA level of TUG1 in endothelial cells and increased the expression of miR-26a. In addition, TUG1 down-regulated the expression of miR-26a in ECV304 cells (16).

ERK12 exists in the neurovascular unit and plays an important role in regulating the survival of the neurovascular unit. Xu et al. believe that the Ras/Raf/ERK12 signaling pathway plays an important role in central and peripheral neurons, such as dendritic formation, neuronal polarity, and axon assembly (17, 18). However, emerging evidence also suggests that up-regulation of this signaling pathway may lead to the development of spinal cord injury. Their research aims to determine the effect of Ras/Raf/ERK12 signaling pathway inhibition on the neuronal characteristics of spinal cord injury (19). First, he collected neurons from C57BL/6J mouse pups with spinal cord injury and sham-operated C57BL/6J mouse pups. Then, he studied the effect of Ras/Raf/ERK12 signaling pathway inhibition on spinal cord injury neurons through immunofluorescence, western blot, cell adhesion and cell migration analysis, and DiI labeling (20, 21). The immunofluorescence results of synapse formation showed that the experimental spinal cord injury model was successfully established (22). Western blotting confirmed the up-regulation of Erk phosphorylation in neurons with spinal cord injury and also showed that U0126 inhibited Erk phosphorylation, which is a downstream kinase in the Ras/Raf signaling pathway (23, 24). In addition, cell migration and adhesion are significantly increased in neurons with spinal cord injury. DiI labeling results also showed that after inhibiting Ras/Raf/ERK12 signaling, the formation of mature spines increased (25, 26).

In this study, the effect of lncratug1 on the ERK12 signal transmission pathway of nerve injury deterioration after acute ischemic stroke was studied. The animal model of ischemic stroke was established by cerebral artery embolization in rats. The effects of lncratug1 and erk12 signal transmission pathway on brain neurons, the mechanism of ERK1/2 in acute ischemic stroke and the effect of ischemic stroke on lncratug1 in the body were detected.

Materials and methods

Subject

Fifteen adult healthy male SD rats, 250-300 grams, were used in the present study. They were avoided from strong light, noise and ventilation. Day and night room temperature was 21-24 °C, 12 hours light and dark cycle.

Model Establishment

(A) The experimental mice were fasted for 12 hours and hydrated for 4 hours. After anesthesia, they maintained 3.5% chloral hydrate (45mg/kg) and maintained the pad temperature ($36.5\pm 0.5^{\circ}\text{C}$).

(B) The rats were placed in the supine position, and two ear sticks were inserted into the external auditory canal for fixation. We observed the scale of the double ear stick, then we gently twisted the fixing screw on the double ear stick to make the head of the rat center; the front teeth were fixed on the front teeth hook, and then tighten them gently after pressing the nose ring (the fastening of the nose ring and ear rod is good). Finally, the plane of the anterior teeth hook was 0.5mm lower than that of the intra-auricular line, and the protrusion was adjusted to approximately the same level.

(C) A 1.5cm long skin incision was cut longitudinally from the top of the head. The soft tissue under the skin was slowly separated by cotton stick and forceps stained with normal saline. After the skull was fully exposed, the periosteum was opened. At this time, the front door, herringbone and arrow-like spaces were clearly seen. 6% rose-red solution (RB stock solution dissolved in 1% normal saline, diluted by 6%, stored at low temperature after 0.45 μm ultrafiltration) was slowly injected for less than 2 min.

(D) After the irradiation, local iodine disinfection, suture the muscle, soft tissue and epidermis in layers.

The control group was injected with 1% normal saline instead of 6% rose bengal solution in the same way. During the operation, the spontaneous breathing of the mouse was ensured. After the operation, the mouse was taken out and returned to the cage in the lateral position.

Experimental Reagents and Instruments

The main reagents and instruments used in the experiment are shown in Table 1 and Table 2.

Table 1. Main reagents and their models that are used in this experiment

Serial number	Reagent name	Model
1	The red blood cell lysate	Solarbio life sciences
2	Cell lysis buffer	Thermo fisher scientificInc
3	Phenylmethylsulfonyl fluoride	Solarbio
4	4× protein loading buffer	TaKaRa
5	BCA protein concentration determination kit	ThermoFisher scientificInc
6	Bovine serum albumin standard reagent	CWBIO
7	30% acrylamide	BIO-RAD

Table 2. Main instruments and their Manufacturers

Serial number	Equipment name	Company
1	CO ₂ incubator	Thermo
2	High-pressure steam sterilizer	Thermo
3	Deionized water preparation equipment	Millipore
4	Dissecting microscope	Prima nano
5	Cryostat	Leica
6	Upright fluorescence microscope	Nikon
7	Ice maker	Grant

Primary cortical neuron culture

Cortical neurons were separated from SD mice. The first-generation cells were first cultured in DEME/F12 medium supplemented with 10% fetal bovine serum for 8 hours, using a serum-free Neurobasal culture dish with 3% B27, and liquid exchange treatment was performed twice in 2 days. SH-SY5Y cells were cultured in DEME supplemented with 8% fetal bovine serum, plus penicillin 90U/ml and streptomycin 90mg/ml, and liquid exchange treatment was performed once every 2 days. After stirring, an equal volume of nerve cell culture medium containing B27 was added to neutralize pancreatic enzymes, centrifuged for 2 minutes, and discarded the supernatant. The

leukocytes were gently stirred with fresh culture base 13 times, the tissue suspension was filtered into a new centrifuge tube with a 70µm nylon filter, they were centrifuged for 8 minutes and discarded the supernatant to obtain a neuronal pellet, which was suspended in 1%B27 and 0.4mM L-glutamate on a neuronal base culture dish. The petri dish was packaged with 40µg/mL Polariin at room temperature. After discarding the Polariin, the petri dish was washed with PBS to inoculate the cells at a certain density. Neurons were cultured at 35°C in boxes containing 2% carbon dioxide, and the culture was changed every 2-3 days. Neurons were subjected to other experiments on the 9th day after inoculation.

TTC dyeing

The experiment was divided into a 24-hour control group and a normal saline control group, and a 48-hour Peptachin as an intervention group. Eight rats were randomly selected within 48 hours: intraperitoneal injection of vinyl chloride hydrate was used for ischemia-reperfusion, the rats were quickly put to death, and the skull and meninges were carefully dissected. The integrity of the brain tissue was kept and sterilization was used, then, the brain serum surface was rinsed with normal saline and was frozen for 40 minutes at -20°C. After removing the hard tissues, the head was thawed for continuous sectioning (sections need to be made quickly, and the product is easily dissolved in brain tissue sections and loses consistency). Five brain slices were taken. Three slices were soaked in phosphate buffer solution. They were kept in a constant temperature water bath at 37°C for about 30 minutes and then rotated for 10 minutes to uniform staining. After the cerebral infarction visible to the eyes was completed, uniformly white, red, and normal brain tissue would appear. The stained brain tissue was fixed with 3% formaldehyde solution for 12 hours to harden the brain tissue and a photo was taken to calculate the size of the infarction.

Western blot method to detect the expression of ERK12 and lncRNA *tug1* in brain tissue

The cerebral cortex was put on the side of cerebral infarction and 2ml of RIPA lysate was dissolved. PMSF1ml was added a few minutes before use to

make the final concentration of PMSF 3M. It was put in a blender and stirred for half an hour and then centrifuged.

A polymer gel cloth was put in the electrophoresis tank and the comb was removed carefully. Then the electrophoresis buffer solution was added to the sample vessel. After electrophoresis, according to the pre-staining instructions for protein labeling, the appropriate target protein and internal reference protein bands were cut into the conveying tank. Next, the PVDF membrane was cut and soaked in methanol for 10 seconds and was put in a container together with the gel strip. The electrophoresis vessel was placed on the constant flow membrane in ice water.

Measurement of microvessel density

After perfusion, the lungs, liver, and muscles of the whole body became hardened, and the rats were successfully irrigated. The head was immediately cut off, the brain tissue was drained, the cerebellum was removed, and the central part was fixed overnight in a 3% formaldehyde fixative prepared in advance. After color development, they were washed with distilled water, decomposed with 0.3% hydrochloric acid, and washed with tap water. PBS returned to blue after washing. Microvessel density was counted according to the microscopic counting method. Using the cell counting method, four non-repetitive high-power fields were randomly selected in the positive cell distribution area, the number of stained blood vessels was calculated in each field, and the average value was used.

Immunofluorescence staining

Anesthesia was intraperitoneally injected with 12% water and chloroform. The abdominal cavity was opened, the incision upwards was extended, the ribs were removed on the left side of the sternum, and the coating on the surface of the heart to fully expose the heart was passivated and separated. The immunofluorescence staining method was used to stain endothelial cells and astrocyte markers molecules.

Flow cytometry

The single-cell suspension was stained with a specific fluorescent dye, and then entered the fluid

chamber filled with the sheath fluid, and lined up in a line covered by the sheath fluid, and formed a cell fluid column through the nozzle. Then, the cells emitted different colors of fluorescence under the excitation of different colors of laser and entered the channels of different colors under the action of voltage to perform qualitative or quantitative analysis on the sample cells.

Statistical Methods

Data statistics were performed by SPSS23.0. The measurement data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). In the case of data that satisfies the normal distribution, the measured data were compared by the T-test, the corresponding T-test was used before and after the intervention, and the independent sample T-test was used between the groups. A non-parametric test was used to disperse data or grade data, and χ^2 test was used to compare count data. All hypothesis tests used two-sided tests. $P < 0.05$ and $P < 0.01$ considered the probability level of differences.

Results and discussion

Analysis of Neuronal Damage after Acute Ischemic Stroke

The test results of the absorbance value of each group stained with Aristotle Red are shown in Table 3 and Figure 1. The BM-SMSC was used as the control group, and the BM-SMSC transmitted in the lncRNA-TUG1 interference virus was used as the test group. After 9Gy gamma radiation treatment, bone formation was induced on 14 days, and then the formation of calcium nodules stained with Alexandrin Red was detected. In order to detect bone formation-related genes, the RNA of each group was stained for 14 days. Compared with the non-radiation group, the results of Arison Lin Red staining showed that the bone formation and differentiation of the radiation group were significantly inhibited, and the calcium nodules after 14 days of induction were significantly reduced; compared with the control group, the interference of lncRNA-TUG1 significantly promoted bone The formation of differentiation, 14 days after induction, the calcium nodules were greatly reduced and increased; compared with the control group after irradiation, the interference of lncRNA-TUG1

partially restored the formation of calcium nodules in BM-MSC osteogenic differentiation.

Table 3. Absorbance test results of each group stained with Alizarin Red

Group	EV	SI	R-EV	R-SI
lncRNA-TUG1 group	2.51	3.14	2.16	2.78
Empty vector virus group	3.05	2.68	2.12	4.22
Interference lncRNA-TUG1 group	3.01	2.11	4.28	2.33

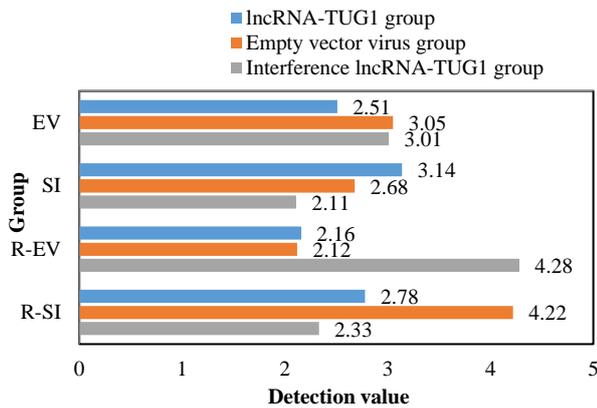


Figure 1. Test results of absorbance values of each group

The comparison of the two groups of MoCA points is shown in Figure 2. The MoCA scores of the traditional Chinese medicine group and the control group were compared by the t-test, the independent sample t-test was used between the groups, and the corresponding t-test was used within the group. There was no significant difference in MoCA scores between the traditional Chinese medicine group and the control group before treatment ($P > 0.05$), they were the same. There was no significant difference in MoCA scores between the two groups after treatment ($P > 0.05$). However, after one month, the MoCA score of the traditional Chinese medicine group was higher than that of the control group, and the difference between the two was statistically significant ($P < 0.05$). The comparison of NIHSS scores between the traditional Chinese medicine group and the comparison group before treatment ($P > 0.05$) showed little statistical difference, and the curative effect was the same. Compared with before treatment, the NIHSS scores of the two groups were reduced ($P < 0.01$), but the improvement of the traditional Chinese medicine group was more obvious than that of the comparison group, and the difference was statistically significant ($P < 0.05$), which can be considered the neurological symptoms of the two groups improved.

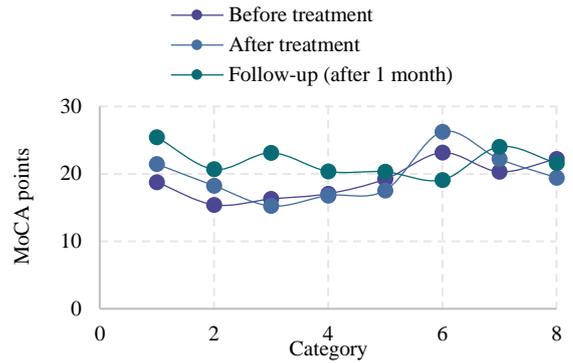


Figure 2. Comparison of two groups of MoCA points

Effect of lncRNA Tug1 on Neurons after Acute Ischemic Stroke

The comparison of neurological deficit assessment at different time points is shown in Figure 3. After waking up from anesthesia, 12 rats in the sham operation group had normal basic activities, without obvious symptoms and signs of nervous system diseases, such as exercise, reaction, drinking water and diet, with a score of 0; the rats in the saline control group and pepsin inhibitor an interference group woke up after anesthesia, with slow reaction and drowsiness, and reduced activity due to reduced or stopped eating. During the examination, it was found that the right forelimb of the mice was weak and could not stand upright. The successful ethical results of the MCAO model in the normal saline control group and pepstatin an interference group were 1:25, 2:17 and 3:18 respectively. Meanwhile, the evaluation of neural function defect in pepstatin an interference group was significantly lower than that in the normal saline control group ($P < 0.05$), indicating that CATD aprotinin an inhibitor can improve the evaluation of neural function and has a neuroprotective function.

Using OGD/RX to simulate the ischemia-reperfusion damage of AS, it was found that the expression of lncRNATUG1 increased at OGD/IIX, and as the time of reoxygenation and re-sera prolonged, its expression level further increased, indicating that TUG1 was involved in the C process. After OGD/RX, the LDH concentration in the cell increased significantly, but when TUG1 was inhibited, the increase in LDH concentration was limited, indicating that TUG1 participated in the cell damage after OGD/RX. The flow cytometry test further shows

that when the cells undergo OGD/RX, the cell apoptosis rate is significantly increased, but when TUG1 interferes, the cell apoptosis rate is significantly reduced. This shows that TUG1 as a lncRNA mediates cell apoptosis in CRI.

lncRNA is a type of non-code transfer RNA with a length of more than 200 nucleotides. Without a completely open lead frame, it cannot encode proteins. Using OGD/RX to simulate the ischemia-reperfusion injury of AS, the appearance of lncRNATUG1 increases OGD/RX, and the longer the reoxidation and re-serum time, the more its performance level increases. After OGD/RX, the LDH concentration in the cell increased significantly. When TUG1 was blocked, the increase of LDH concentration was limited. The cell damage after OGD/RX was related to TUG1. The cell test also showed that if the cells are subjected to OGD/RX, the cell death rate will be greatly increased, but if TUG1 is disturbed, the cell death rate will be greatly reduced. This indicates that as a lncRNA, TUG1 regulates the apoptosis of CRI.

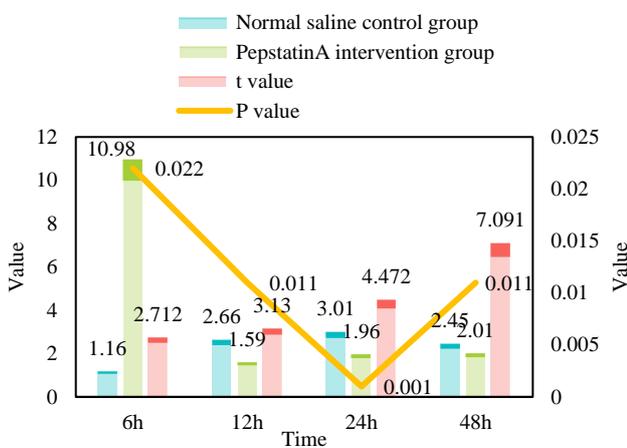


Figure 3. Comparison of neurological deficit scores at different time points

Effect of Erk12 Signaling Pathway on Neurons after Acute Ischemic Stroke

The onset changes of ERK12 in each group after acute ischemic stroke are shown in Figure 4. ERK1/2 is mainly activated by micro-derived stimuli, which then leads to the activation of a series of transfer factors, which regulate cell proliferation and differentiation. Through the phosphorylation pathway, ERK1/2 is dually regulated by the specific kinase MEK1/2, and phosphorylation of erk1/2 plays a role

in the pathophysiological process. In terms of cerebral ischemia, JNK and p38MAPK of the MAPKs family mainly regulate the level of inflammation through inflammatory response, thereby enhancing ischemic brain injury, inhibiting the activation of JNK and p38MAPK, and reducing ischemic brain injury. ERK1/2 harms brain tissue by discovering the deformation process (MMP-9) and acrylate tissue inhibitor (TIMP-1). Cerebral ischemia-reperfusion injury activates the MAPK signal pathway, increases the appearance of inflammatory factors, and damages the cerebral infarct area and peripheral nerve cells. The ERK1/2 signal pathway promotes the appearance of inflammatory factors in the ischemic field after cerebral ischemia through IL-1 β . ERK1/2 acts on the target gene of the anti-inflammatory factor IL-1 β to increase the appearance of IL-L β and worsen cerebral ischemia. In the permanent cerebral ischemia model, 24 hours after cerebral infarction, the level of phosphate ERK1/2 in the cerebral infarction side cortex increased significantly, indicating that P-ERK1/2 can promote nerve cell damage. Ischemia itself induces the discovery of Bax and Bad and the activation of caspase-3, but the phosphorylation levels of Bcl-2 and CREB remain unchanged. Caspase participates in the death cascade, implying that it does not participate in endogenous survival and repair mechanisms. The slight activation of ERK1/2 may involve signal transmission pathways related to these deaths, but other signal transmission pathways are also likely to involve cell death. Cerebral ischemia, as an external stimulus signal, activates ERK1/2. The activation of ERK1/2 from cerebral ischemia not only exists in neurons but also exists in the Griffin cells and vascular endothelial cells adjacent to neurons and participates in cerebral infarction. Control of the neurovascular unit happened in the posterior brain.

After ischemic stroke, the supply of cerebral artery blood flow is greatly reduced and interrupted. The ischemic central region and peripheral ischemic region are formed by cerebral ischemia, and the peripheral ischemic area is the ischemic penumbra. In this case, hemoperfusion is mainly supplied by the lateral collateral circulation, and the areas without vascular necrosis mainly rely on the lateral collateral circulation. Therefore, if we stop the water effect of cAMP signal imbalance caused by ATP consumption in ischemic stroke, we can prevent and reduce the

nerve damage caused by ischemia and hypoxia, and save the nerve cells around the ischemia, which is helpful to the recovery of neural function.

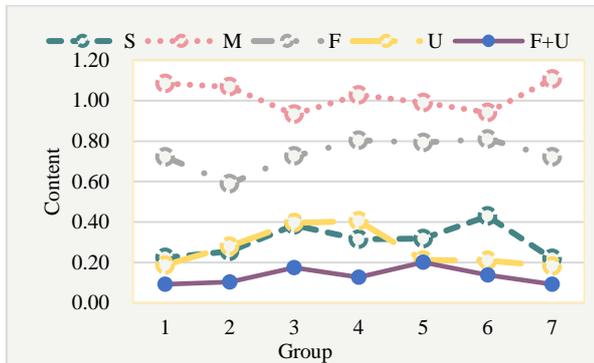


Figure 4. Changes in the expression of ERK12 in each group after acute ischemic stroke

LncRNA *tug1* acts on the *erk12* signal transmission pathway, which worsens neurological disorders after acute ischemic stroke. The excess expression of *lncnatug1* is closely related to the gray of brain cells. *Lncrna tug1* combines with YB-1 to promote phosphorylation and promote the proliferation, metastasis and invasion of cancer cells. Therefore, *lncRNA tug1* has become a serum marker for HCC diagnosis. *Lncnatug1* is associated with hepatitis B virus (HBV) - induced HCC. The increase of *lncnatug1* may indicate poor prognosis by binding with EZH2 and inhibiting the occurrence of tumor suppressor genes p15 and p16.

MEK was activated by *erk12* downstream of phosphoric acid. Activated *erk12* enters the nucleus, activates *elk-1* and camp reactor binding protein (CREB) as the downstream matrix, regulates the activity of transfer factor, induces the appearance of initial reaction gene and late reaction gene, completes extracellular signal to nucleus and regulates the functional state of cells. Extracellular signals are transmitted to cells, and the biological basis for ensuring the accuracy of cell response is the high selectivity of signal transmission.

Acknowledgments

None

Conflict interest

The authors declare no conflict of interest.

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