Review Article

Effect of mammalian target of rapamycin signaling pathway on nerve regeneration

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Abstract: Nerve injury is a serious clinical common problem not only caused by violence usually in traffic accident but also non-violence related disease like amyotrophic lateral sclerosis and other motor neuron diseases. All these diseases always come with severe consequences including loss of function and even to paralysis which are associated with significant high mortality. Although many useful treatments have been developed, completely recovery of damaged nerve function is usually hardly obtained. Because successful functional recovery of injured nerve lie in not only axon regeneration, neuronal survival but also reconstructing transmission of myelin-based electric nerve stimulation as well as re-innervation of denervated targets. Recent research has suggested that low intrinsic capacity of neuron may be genuinely responsible for regeneration failure. PI3K/Akt/mammalian target of rapamycin (mTOR) signaling pathway has been found playing crucial roles in the growth of central nervous system axons, and confirmed as a major intracellular signaling axis that control axon regeneration. In nerve dissection animal models, the mTOR activity was suppressed and protein synthesis was impaired, while reactivating this pathway successfully led to axon regeneration. Emerging evidence show that mTOR is required during multiple intricate physiological process in nerve regeneration. This review focuses on recent study which are mTOR related, introducing basic knowledge of mTOR including protein structure and its function in either physiological or pathological process, discussing the therapeutic potential of mTOR-based treatment.

Keywords: Mammalian target of rapamycin (mTOR); nerve injury; axon regeneration; signaling pathway

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Repairment of nerve injury is a thorny challenge in modern medicine, as nerve damage often leads to varying degrees of functional loss and even to paralysis. For example, after optic nerve injury, the patient's visual function is usually permanently lost despite some reports discovering a recovery of a tiny part of visual function in very few patients occasionally (1). Successful functional recovery of damaged nerves depends on both the number of surviving neurons and the regeneration of neuronal axons, as well as successful re-innervation to its effectors (2). It is not difficult to understand that survival of neurons is a prerequisite condition for axon regeneration. However, in absence of particular axon regeneration stimulators, they cannot grow even though the neuron has successfully survived (3). Actually, there are distinct regulatory mechanisms for the survival of neurons and the regeneration of axons. For

example, knocking out p53 in retinal ganglion cells only improves the survival rate of neurons, but produce no effect on axon growth (4). Similarly, consistent activation of GTPase Rheb in injured spinal cord significantly greatly promote neurite outgrowth as well as axon elongation instead of survival rate (5). Several key steps need to be accomplished during a successful axon regeneration, including prolongation of axons, crossing cell debris barrier, reinnervating with the target (6). Interestingly, we found distinct repairing mechanisms exist between central and peripheral nervous system, that is why nerve regeneration in peripheral nervous system is usually much more difficult than in central nervous system. In the early days, this difference was recognized as poor micro-environment in central nervous system where there is a low concentration of neurotrophic factors. Therefore Schwann cells were implanted at nerve crush site as they can secrete a variety of neurotrophic factors, and it is gladly to observe that many axons successfully thread the envelope formed by Schwann cells and further stretch into the distance (7). In subsequent study, a large number of axon regeneration related inhibitory molecules were identified in the micro-environment of central nervous system. Some of them are named as myelin-associated inhibitors (MAIs), which is thought to be the major ingredients of this inhibitory microenvironment (8). Classic MAIs include myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (Omgp) and Nogo protein (9,10), also newly discovered netrin-1 (11). However, removing of inhibitory molecules only has a slight increasement on axon regeneration. Thus more and more researchers assert it is the intrinsic capacity, a regeneration capacity that diminishes with age, especially for mature neurons of which mitotic capacity completely lost, that accounts for regeneration failure (12,13).

Regeneration-associated transcription factors (TFs) play key roles in axon regeneration

Recently a large number of researches have proved that activating some intracellular signaling pathways in neurons can robustly promote axon regeneration (14). The mechanisms under which are quite complex, but they can be basically summarized as following process: activation of relevant signaling pathways in neuronal somatic bodies; up-regulation of regeneration-associated TFs; synthesis of growth-associated proteins such as GAP-43 and SPRR1A; finally promotion of axon regeneration (15). The importance of TFs can be proved by the following experiment. First, it was found the expression of TFs in central nervous system was significantly lower than that in peripheral nervous system. Subsequently, overexpression of VP16-Stat3CA cross-linkers (the role of VP16 here is just to recruit transcriptional co-factors to the DNA-binding domain to enhance the transcriptional activity of Stat3CA) in both RGCs and cerebral cortex neurons showed much more prominent promotion effect on axon growth than Stat3CA overexpression alone (16).

Identification of TOR, structure and function

Mammalian target of rapamycin (mTOR) has been found an important signal transduction pathway that promotes nerve regeneration. In fact, mTOR has long been known for its extensive biological and physiological roles in various cell types (17,18). It controls cell proliferation, cell growth as well as other important life activities. As early as the 1970s, a group of macrolide antibiotics with anti-fungal effects, namely rapamycin, was found in the soil of Easter Island (19). Further research found that the antibiotics can inhibit the proliferation of eukaryotic differentiation and have an immunosuppressive effect. The TOR receptor was then identified in the subsequent pharmacological mechanism research of rapamycin (20). TOR receptors are widely expressed in eukaryotic cells, and all eukaryotic genomes contain the *TOR* gene (21). For mammals, mTOR is quite conserved in biological evolution. Under stress condition, cells can maintain energy metabolism balance by adjusting mTOR (22-24). Some study has found that mTOR signaling disorder may lead to obesity, diabetes (25), some deadly malignant tumor (26). Now the structure of mTOR is clear, mTOR belongs to the family of phosphatidylinositol kinase-related kinase (PIKK), which has a serine/threonine kinase activity domain at its carboxy terminus and is complexed with rapamycin-FKBP12 body-bound domains. mTOR contains two different protein complexes, mTORC1 and mTORC2, and both have own distinct regulating mechanism. The mTORC1 complex contains mTOR, proteins Raptor, PRAS40, Deptor and GβL, and it is sensitive to rapamycin (27,28). Rapamycin inhibits the catalytic activity of mTORC1 by forming a complex with FKBP12 as described above. mTORC1 is mainly involved in cell cycle-dependent regulation of cell proliferation (29,30). In addition to Deptor, GβL, mTORC2 also contains specific Rictor, mSIN1 and Protor-1 (27). The most remarkable difference between

mTORC1 and mTORC2 is their sensitivity to rapamycin, although it has also been reported that long-term rapamycin treatment also can inhibit its activity recently (31). mTORC2 plays an important regulatory role in neuronal morphology and synapse formation by regulating actin cytoskeleton (32). It was found that mTORC2 can promote the formation of long-term memory (LTM) and take part in the establishment of cognitive function (33).

A classic pathway PI3K-Akt-TSC1/TSC2-mTOR

A variety of extracellular signals can participate in physical processes such as ribosome biosynthesis, protein synthesis, energy metabolism, apoptosis, autophagy through the mTOR (34). These extracellular stimuluses are widely distributed, including amino acids, ATP/AMP, growth factors, hormones, neurotransmitters, etc. (35). The TSC1/2 complex is a key node through which many molecules activate or inhibit mTOR. An important pathway called PI3K-Akt-TSC1/TSC2-mTOR can be initiated by extracellular signals including fibroin, BDNF, insulin, etc. (18). Phosphorylation of PI3K is the main step of this signaling pathway, it phosphorylates TSC2 by phosphorylated Akt, which forms a GAPase-interacting protein GAP with TSC1. GAP binds to Rheb and thus activates mTORC1 to exert its major physical effects (36-38). However, it is intriguing to note that recent research has found that Rheb-GTP can inhibit mTORC2 as well (39). Activated mTOR turn on transcription and translation by activating downstream proteins. The more studied are pS6K and 4E-BP1/2. The kinase activity of S6K requires phosphorylation at two key sites, Thr389 on the hydrophobic motif and Thr229 on the T loop. While activated S6K further activates 40s ribosomal protein S6, which encodes multiple transcription-related factors by up-regulating a group of mRNAs known as TOP (5' tract of oligopyrimidine) (21). Dephosphorylated 4E-BP1 is its activated form, while mTORC1 phosphorylates 4E-BP1, then the latter is released from the binding of eIF4E to initiate the translation of the protein (40). In addition, other pathways, such as the Ras pathway, also phosphorylate TSC2 by activating MAPK, finally contribute to activation of mTOR (41). When cells lack energy, AMPK will be activated by intracellular high concentration of AMP or decrease of rate of ATP/AMP. Activated AMPK can slow down the cellular protein synthesis rate by inhibiting mTOR, so as to ensure the minimum energy requirement of cells (42). Hypoxia can also directly activate TSC through RTP801 to inhibit mTOR. Interestingly, in addition to acting directly on mTOR, the above AMPK pathway can also inhibit mTOR by upregulating RTP801, thus achieve multiple and complicated inhibition effect (43). Recently, it has also been found that activated mTORC2 can phosphorylate the downstream PKB/Akt, PKCα, at the same time, phosphorylated Akt can also affect the expression of mTORC1 through PRAS40, therefore the two complexes of mTOR do not function independently (44).

The mTOR signaling pathway plays a crucial role in the development and physical activity in nervous system, such as the development of the nervous system, synaptic plasticity and axon growth process (34). In the early development of neurons, mTOR is involved in the regulation of axon formation and directional growth, dendritic development and dendritic spine formation. In mature neurons, mTOR take part in several pathways that regulate synaptic plasticity, which is essential to the formation and development of memory and cognition. Several groups demonstrated the important role of TSC2-mTOR in the development of central neurons such as targeting of retinal ganglion cells, synthesis of motor neurons growth cone, neuron axon differentiation, and neuronal cells body polarization (45-49). In the peripheral nervous system, TSC2-mTOR signaling pathway also regulate axon targeting and bifurcation. Significant defects in sensory distribution of the skin and the axon distal over-bifurcation as well as other anomalies were found in the TSC2KO mouse model (50). Therefore, it is not surprising to know that mTOR signaling pathways are directly related to multiple neurological diseases, such as brain tumor formation, tuberous sclerosis, cortical dysplasia and neurodegenerative diseases such as Parkinson's disease, Huntington's disease and Alzheimer's disease (51,52).

mTOR regulate axon regeneration in central nervous system

In a central nervous system study, mTOR was first found have an important role in axon regeneration. In 2008, using RGCs injured mice model, Park *et al.* found that PTEN (phosphatase and tensin homolog) knocked out RGCs have a strong ability to regenerate axons and prolong even across the rupture site. The longest optic nerve fibers can reach almost 4.0 mm. PTEN, as one of the negative regulators of mTOR signaling, converts PIP3 (phosphatidylinositol 3,4,5 triphosphate) into PIP2 (phosphatidylinositol 4,5 diphosphate), while decrease in PIP3 activates Akt and further activates mTOR. Whereas in the control group,

mTOR as one of the PTEN downstream effectors was found to have decreased along with decreased neuronal protein synthesis. At the same time, if another negative regulator of the mTOR, TSC1 (tuberous sclerosis complex 1) is knocked out, the enhanced ability of axon regeneration can be significantly observed again (4). In fact, in a study of axonal growth and development, it has been already confirmed that activated mTOR can recruit SAD kinases to synthesize particular proteins which axon formation need, thereby allowing shift from neurites to axons (48). It is easy to see activation of mTOR signaling pathway has the ability to enhance axonal regeneration, and shortly thereafter, similar role of mTOR was found in spinal cord injury. Liu *et al.* successfully regenerate the corticospinal tract in damaged spinal cord by silencing PTEN. They found that silencing PTEN not only allow uninjured axons to penetrate into axonal buds hence exert compensatory functions, but also regenerate damaged axons and even traversed across spinal cord injuries. In previous studies, there was no effective ways to get damaged axons across the crush sites (53). Sun *et al.* attempted to knock out both PTEN and SOCS3 in RGCs (SOCS3, an inhibitor of the JAK/STAT pathway, down regulation SOCS3 enhances axonal regeneration), enhanced and sustained regeneration was more obvious than knocking out PTEN/SOCS3 alone. Interestingly, a more detailed gene analysis reports that these two originally independent pathways eventually work synergistically (54). Another team used both zymosan and CPT-cAMP in PTEN knocked out mice, as a result, they pleasantly found a small fraction of the optic nerve fibers succeeded in reaching the lateral knee of the thalamus after passing through the optic chiasm body, more surprisingly some visual function recovered (55). However, complete visual function recovery is still a hard challenge, the main difficult lie in axon regeneration failure of optic nerve and the formation of abnormal synapsis connections (56). Du *et al.* found that activation of mTOR can even regenerate the axons of rat corticospinal tract one year after injury (57). Wyatt *et al.* also demonstrated that the activation of mTOR promotes neurite regeneration on human embryonic stem cell-derived neuronal precursor cells (58).

mTOR regulate axon regeneration in peripheral nervous system

In the peripheral nervous system, does mTOR still play a decisive role in axon regeneration? Christie *et al.* found that silencing PTEN-mRNA increase the axon regeneration of peripheral sensory neurons, and PTEN-specific inhibitors acquired consistent results (59). Eickholt *et al.* found that in the p110δ PI3K-inactivated transgenic mice, axon regeneration of DRGs was significantly weaker than that of the control group. At the same time, the expression of Sprr1A, a gene associated with regeneration, was detected in transgenic mice (60). Saijilafu *et al.* hypothesize that PI3K may regulate the transcription of regeneration-related genes via GSK3, a downstream molecule of PI3K (61). In subsequent experiments, knockdown of PTEN in sciatic nerve injury models also enhanced axon regeneration of peripheral neurons. However, this enhancement does not depend on mTOR, but on GSK3 signaling. Not only that, they also found that GSK3 promotes axonal regeneration via the TF Smad1. However, it is noteworthy that in Saijilafu's experiment, electroporation of Smad1 plasmid only restored the regenerative capacity of no more than 50% of dnPI3K neurons, whereas in the control group, overexpression of Smad1 did not enhance axon regeneration (14). This suggests that there are other regulatory pathways downstream of PI3K. In fact, when Christie silenced rat PTEN, they also believe that this increase in axonal growth does not depend on mTOR (59). However, in the experiment of Abe *et al.*, sciatic nerve injury in mice significantly enhanced the axon regeneration capacity of DRG neurons, at the same time, activating mTOR signaling pathway was detected in DRG neurons (50). mTOR regulates protein synthesis through the activation of downstream effectors. One of the possible ways to promote the regenerative enhancement of axon regeneration is through the up-regulation of GAP-43 expression, since overexpression of GAP-43 and overexpression of mTOR do not show the exactly same effect (62). In another word, there are other downstream effectors that participate in mTOR signaling.

mTOR regulate glial cells after nerve injury

Glial scar tissue not only constitutes a physical barrier but also secrete chemical components that inhibit nerve regeneration. mTOR signaling pathway also plays an important role in glial cells. During central nervous development, oligodendrocytes eventually participate in the formation of myelin after multiple transitional states. Oligodendrocyte differentiation and myelination are dependent on the activation of specific cellular signaling pathways. Akt and mTOR are important regulatory pathways in this process. Intact myelination of

oligodendrocytes depends largely on mTORC1, a small part on mTORC2 (63). Deletion of Raptor (a component of mTORC1) in oligodendrocytes will result in abnormal formation of myelin (64). Similarly, in the development of the peripheral nervous system, if knocking out mTOR in Schwann cells, the myelin formation of Schwann cells drastically decreases both in length and thickness. The defective myelin can also significantly slow down the speed of nerve signal transmission (65). Knocking out Rheb1 in the Rheb1f/f mouse brain, which is required in mTOR mediated myelination, greatly inhibited myelination (66). Likewise, the formation of scar tissue also depends on the activation of the PI3K/Akt/mTOR signaling pathway. It was observed astrocytes are involved in the formation of scars in spinal cord injury only after mTOR activation (67). At the same time, activated astrocytes can also secrete chondroitin sulfate proteoglycans (CSPGs), an important neuro-repressor, and this secretion per se depends on the activation of mTOR (68). Another inhibitory molecule secreted by astrocyte, interleukin-6 (IL-6), not only regulates the formation of scar tissue but also has the ability to promote axon regeneration. The secretion of IL-6 is also regulated by the mTOR signaling pathway (69). Astrocytes can also secrete ciliary neurotrophic factor (CNTF) to promote axon regeneration (70,71), which is also regulated by mTOR (72). Recently, it has been reported that CNTF can stimulate the proliferation of RGCs and rapamycin can significantly reduce the growth of RGCs in the presence of myelin. However, in absence of these inhibitory molecules, rapamycin cannot affect the growth and regeneration of RGCs. More interestingly, exposure to inhibitory molecules such as myelin or neurocan could also decrease the mTOR activity of RGCs. This study suggests that mTOR activity may also affect RGCs' sensitivity to inhibitory molecules (73). In addition, mTORC1 can increase the sensitivity of RGCs to myelin-associated inhibitory molecules by up-regulating neurotrophic factor, which in turn induces RIP of p75NTR complex, then growth cone will not be recognized for regeneration-related signals (7,73,74).

mTOR as a new potential therapeutic target

In the current clinical treatment to nerve injury, methods are very limited to surgery, hormone therapy, neurotrophic medications and so on and all these treatments are mainly to reduce pressure and edema so as to alleviate symptoms (1,75). To days there is not any treatment aimed at improving the intrinsic regenerative ability of neuron itself. Since PTEN-PI3K-mTOR signaling pathway can directly enhance the regenerative capacity of neurons, up regulating intracellular protein synthesis may be a promising therapeutic treatment. However, some dilemmas need to be overwhelmed. One of the main reasons is that mTOR has multiple function in living organisms. In a CNS injury model, inhibition of mTOR not only failed to produce negative effect on the regeneration and nerve repair, but also attenuated the damage response and finally promoted functional recovery (76). An interesting phenomenon that inhibition of mTOR also showed a promotion to function recover, which may be explained to mTOR inhibitors can promote astrocyte proliferation, attenuate inflammatory reaction at injury site, reduce inflammatory cytokines released by macrophages, such as microglial IL-1β, TNFα and so on. Researchers also found inhibition of mTOR suppress NO synthase and regulates oligodendrocyte differentiation while the latter is involved in the formation of scars of nerve injury (77). In fact, inhibition of mTOR activates damaging neuron autophagy, which can block apoptosis by accelerating the clearance of mitochondria, decreasing cytochrome C and downstream caspase activity (78). As adequate blood supply is the footstone of nerve growth in vivo, mTOR also regulates the formation of newborn blood vessels through angiogenic factors such as vascular endothelial growth factor, NO, angiogenin and so on (79). Nevertheless, activating mTOR may appear to have encouraging results, is necessary to note that overexpression of mTOR may increase the incidence of malignant disease (80,81). Overexpression of mTOR may result in protein translation errors and deformities in development of nervous system, for instance, epilepsy, cognitive disorders such as autism, dementia, inflammation in brain trauma are all associated with overexpression of mTOR (82). Therefore, efforts should be devoted to eradicate the adverse effects during medical treatment. Recently, gene therapy has attracted much attention, since direct intervention of mTOR signaling pathway in neuron may be more efficient than merely drug administrating, moreover gene therapy can avoid the side effects of drugs. Therefore, some people conceive knockout of PTEN may be a useful treatment. However it is sadly to see PTEN as a tumor suppressor gene, the mutation can induce a variety of human malignant tumors such as skin cancer, breast cancer, etc., hence activation of mTOR by silencing PTEN may not be a safe way (83). In a long-term mouse PTEN knockout trial which enduring up to 18 months, no

other abnormal features were found except for neuronal hypertrophy (84).

Conclusions

Our understanding of the role of mTOR in nerve regeneration is presently dramatically increasing. Considerable progress has been achieved about mTOR-mediated regeneration after nerve injury. However, we are still far from understanding how modifications in mTOR activity contribute to regeneration progress. Due to mTOR plays important roles in various physiological processes and functions in different cell types, precise regulation of mTOR both in time and space may be the direction solving the problem completely.

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Footnote

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