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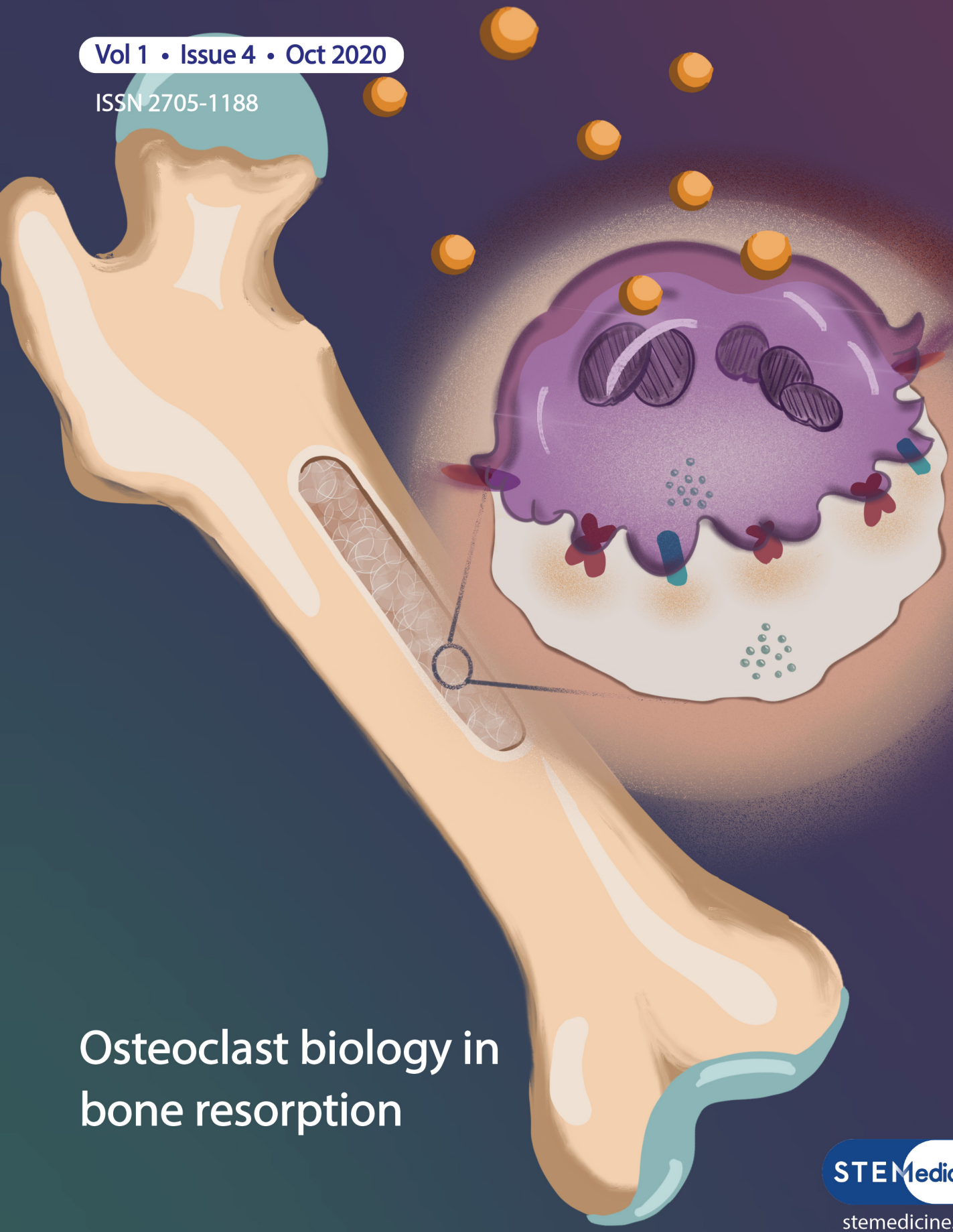

Osteoclast biology in bone resorption

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REVIEW ARTICLE

Osteoclast biology in bone resorption: a review

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ABSTRACT

What we know about bone resorption has changed a lot in the last few decades. The osteoclast is the only cell to nibble and break down the bone, and in the formation and resorption of bone tissue, osteoclasts play an important role. Once the balance of bone formation and bone loss is out of control, diseases like osteopetrosis and osteoporosis occur. Bone resorption is a unique function of osteoblasts, which are multinucleated cells formed by the fusion of mononuclear progenitor cells of the monocyte/macrophage family. In the formation of osteoclasts, there are two main factors affecting this process, macrophage colony-stimulating factor (M-CSF) and ligand-activated receptor (RANKL) of nuclear factor kappa B (NF-κB). The identification of RANK-RANKL signaling and other classic signaling pathways such as Wnt and Notch, as the major signaling regulation in osteoclast differentiation, was a significant breakthrough in the field of osteoclastogenesis. In this review, we briefly describe the latest knowledge of osteoclast-induced bone resorption and cellular factors that regulate the activity of osteoclasts and cell fusion, for the purpose of understanding osteoclastogenesis and the development of drugs that enhance bone resorption to improve pathological bone diseases.
Keywords: Osteoclast · Bone resorption · M-CSF · RANKL pathway · Cell fusion

Introduction

Bone is the hardest organ in the body that makes up the internal bones of vertebrates. Its function is moving, supporting and protecting the body and storing minerals. One of the components of the bone is mineralized skeletal tissue with a solid honeycomb-like 3D structure inside, and there are tissues include bone marrow, periosteum, nerves, blood vessels and cartilage in the bone.

Osteoclasts are described as the only cells to be capable of destroying the bone tissue. They express markers that are thought to be specific to skeletal tissues, such as avb3 integrin, cathepsin K (CTSK), calcitonin receptor, and tartrate-resistant acid phosphatase (TRAP) (1). The osteoclast is essential for the two basic processes of bone biology, the first is bone modeling, which forms bone elements and ensures the correct shape and density of the bone. The second process is bone remodeling, and the mechanism of bone remodeling ensures bone tissue renewal and adapting to the environment. The balance of bone remodeling and resorption is essential for health. When the functions of osteoclasts and osteoblasts are disordered, it will cause bone diseases. The exacerbated bone resorption is associated with osteoporosis, rheumatoid arthritis and periodontal disease, while decreased bone resorption leads to osteopetrosis, a rare genetic disease. On the other hand, except for the function of bone resorption, osteoclasts are able to regulate cells in the bone marrow (2). We give this concise summary of previous work to better understand the osteoclast differentiation and identification of potential therapeutic targets (3).

The differentiation of osteoclasts

There are two basic groups in the blood cells which are differentiated by hematopoietic stem cells (HSCs): lymphoid lineage and myeloid lineage, such as macrophages. Osteoclasts are derived from the hematopoietic system (4). In the bone marrow, HSCs experience several differentiation and self-renewal, each subtype of the cell has a specific surface marker. There are four stages in the osteoclast differentiation: 1. HSCs differentiate into multipotential progenitor cells (c-Fms-,

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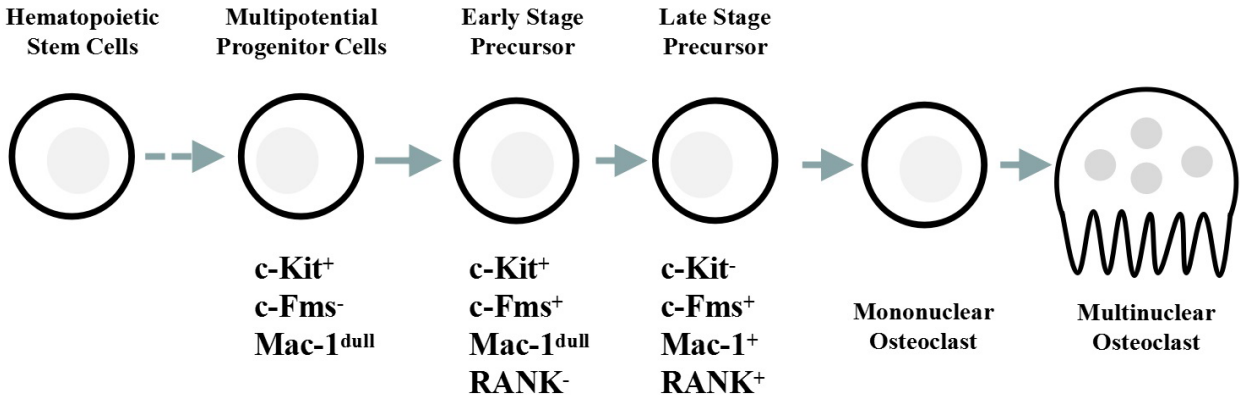

Figure 1. Stages in osteoclast differentiation. Phenotypic changes during the osteoclast differentiation and regulatory cytokines in the maturation and differentiation of osteoclasts.

c-Kit+, Mac-1dull); 2. multipotential progenitor cells differentiate into early-stage precursor (c-Fms+, c-Kit+, Mac-1dull, RANK-) and late-stage precursor (c-Fms+, c-Kit+, Mac-1+, RANK+); 3. precursors differentiate into mononuclear osteoclasts; 4. mononuclear osteoclasts differentiate into multinuclear osteoclasts (**Figure 1**) (5). D. G. Walker found that the capacity of resorbing bone was restored in osteopetrosis mice with intravenous administration of normal spleen and bone marrow cells (6). Interestingly, the dendritic cells are undergoing trans-differentiation into functional osteoclasts by stimulated with the microbe and the presence of receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL). Furthermore, The activation of dendritic cell-derived osteoclasts can be inhibited by aspirin which downregulates the expression of NFATc1 via the NF-κB pathway (7).

The differentiation of osteoclasts is mainly regulated by two critical cytokines, RANKL and macrophage-colony stimulating factor (M-CSF). PU.1 is a domain transcription factor of myeloid, B-lymphoid cells also regulate the transcription of c-fms and CD11b/CD18 which controls the osteoclast phenotype (8).

Mature osteoclasts are large whose size is up to 100 μm, multinucleated and polarized, firmly adhering to the surface of the bone. In the process of bone resorption, there are four different cell structures of osteoclast: 1. sealing zone, isolates the resorptive area from the extracellular environment; 2. ruffled board, facing the bone matrix, is composed of the plasma membrane to absorb the bone matrix; 3. basolateral membrane, facing the vascular compartment, is participating in bone resorption, which contributes to transporting the bone degradation products; 4. functional secreted domain.

Factors and pathways which regulate the formation of osteoclasts

M-CSF is critical for osteoclastogenesis (5). It is well known that the differentiation of osteoclasts needs two key molecules: NF-κB ligand RANKL and M-CSF (9). Osteoclasts are derived from the monocyte/macrophage cell line, and PU.1 controls the switch of activation of stem cell precursor. PU.1 drives a positive regulation of the M-CSF receptor, called c-fms. In the next step, the precursor becomes an osteoclast lineage by M-CSF and RANKL. On the other hand, macrophage or B lymphocyte can also trans-differentiate into an osteoclast. C-fms supports the progenitors to survive and give rise to osteoclastogenesis by inducing the expression of RANK. Malt1-/- mice induced M-CSF production which played an important role in osteoclastogenesis and decreased osteoprotegerin (OPG) production while in the presence of inflammatory stimuli (10). GM-CSF can induce bone marrow-derived macrophages to differentiate to dendritic cells (11). M-CSF knockout mice (op/op mice) causes osteopetrosis by inducing the numbers of osteoclasts (12). RANKL is one member of the tumor necrosis factor superfamily and belongs to type II transmembrane protein. It is crucial for bone metabolism. It is a membrane protein (also known as CD254) on the osteoblast membrane that can activate the osteoclast and accelerate the formation of osteoclast and bone loss, which is important for bone regeneration. When RANKL on the osteoblast membrane activates the RANK protein on the osteoblast membrane, osteogenesis will begin (13). RANK is the critical receptor that mediates the function of osteoclasts on bone resorption and remodeling (14). Study shows that, when RAW264.7 type CRL-2278 cell line was cultured in the hydroxyapatite surface, the hydroxyapatite induces autocrine of RANKL and RANK by monocyte/macrophage cells to differentiate itself into osteoclasts (15). NF-κB is an essential factor for the differentiation of osteoclasts (16). The overexpression of OPG results in osteopetrosis by decreasing the differentiation of osteoclasts (**Figure 2**) (17).

Several kinds of research have shown that the RANK/RANKL/OPG pathway is a key signal pathway in bone metabolism and bone diseases resulting in imbalances in bone formation and resorption. RANK-/- mice (18), RANKL-/- mice (14), and the overexpression of OPG in rat (19) have shown symptoms of osteopetrosis. On the other hand, adolescent and adult OPG-/- mice developed osteoporosis and arterial calcification (20). The RANK/RANKL/OPG pathway tightly and precisely controls the balance between bone formation and resorption. Some

bone diseases in human patients such as familial expansile osteolysis (FEO) and the familial form of early-onset Paget's disease of bone (PDB2) occur when this process is out of control. In 2010, an investigation has found an extremely rare bone disease called dysosteosclerosis in a 3-year-old girl, the "Osteoclast-Poor" symptoms are similar to osteopetrosis caused by the deficiency of osteoclasts, but there are no mutations in the genes that encode RANK, RANKL, OPTG or M-CSF (21). How the lack of bone resorption occurs in this disease is still unclear.

Recent research has found that the salt-inducible kinase (SIK) signaling pathway takes part in the checkpoints of controlling the osteoclast formation. SIK inhibitors may denote a potential new treatment for preventing bone erosion (22). The SIKs are a serine/threonine kinase subfamily which belongs to the AMP-activated protein kinase (AMPK) family (23). SIK inhibitors can reduce the expression of osteoclast differentiation markers, such as TRAP and CTSK. Furthermore, the levels of c-Fos and NFATc1 proteins have an extreme downregulation when SIK inhibitors are present (22). Pkn3 bound to c-Src is critical for the activity of bone resorption by osteoclasts, while in the Wnt5a-Ror2 signal pathway, Pkn3 bound to c-Src can enhance the activityof osteoclasts (24). The Wnt signaling pathway has been extensively reported in osteoblasts lineage, but less known in osteoclasts. Recent studies found that early Wnt3a treatment inhibited the activation of NFATc1, which was also activated during macrophage differentiation into osteoclasts (25). Moreover, injection of microRNA-410 or downregulated Wnt-11 inhibited osteoclast function in osteonecrosis of the femoral head (26). On the other hand, the Notch signaling pathway plays a different role in osteoclastogenesis: in the osteoclast precursor cells, the stimulation of the Notch signal leads to large osteoclast with numerous nuclei and the activity of resorption but depresses the small osteoclast resorptive activity (27).

Osteoclastic bone resorption

The cytoskeleton of osteoclasts is unique because the polarization forms different sections to comply with different functions. The ruffled border is the isolated structure that shapes an actin-ring or sealing zone to segregate the absorption microenvironment from the outer space of the cell. This process needs the presence of αvβ3 integrin. αvβ3 integrin combined with the M-CSF receptor c-Fms to activate a signaling pathway constituted with Vav3, Syk, Dap12, c-Src, Slp76 and Rac which switches on the formation of actin-ring (28). β3 gene knockout mice show that β3-/- cells are still multinucleated and express TRAP but are not able to form normal actin-ring, indicating osteoclast cytoskeleton dysfunction (29). In the contacting area between osteoclasts and bone surface, matured osteoclasts release enzymes like CTSK and TRAP to destroy the minerals. In healthy physical condition, there is high expression of TRAP by osteoclasts, activated macrophages and neurons (30). Under some pathological circumstances, TRAP expression is increased. These

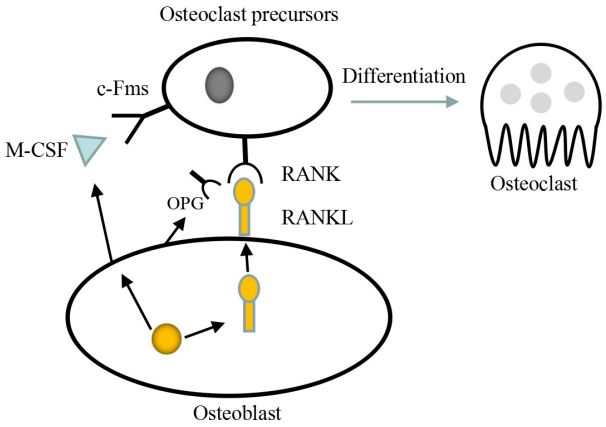

Figure 2. Regulation of osteoclast differentiation by osteoblasts through M-CSF and RANKL.RANKL and M-CSF expressed by osteoblasts are essential for osteoclast differentiation and fusion into multinucleated cells. On the other hand, OPG inhibits osteoclast differentiation by blocking the combination of RANKL and RANK.

diseases include osteoclastoma and osteoporosis, as well as metabolic bone disease. CTSK is expressed mainly in osteoclasts, which is a protease that performs the function bone resorption by catabolizing elastin, collagen and gelatin to break down bone tissues (**Figure 3**) (31).

Recently, some evidence has shown that the inhibitors of CTSK like 2-(3-(2-fluoro-4methoxyphenyl)-6-oxo-1(6H)-pyridazinyl)-N-1H-indol-5-ylacetamide is a potential therapeutic drug for osteoporosis (32). Kent et al using the specific marker and time-lapse found that the resorption model of osteoclasts was not only the formation of a round pit, more importantly and more aggressively, there were long trenches that osteoclasts resorbed while moving (33).

To develop the capability of resorbing bone matrix, polarization is required. Through an area named sealing zone, osteoclasts can communicate with the bone and form an isolated zone with the bone matrix. This process requires the participation of molecules like c-Src, vitronectin receptor, carbonic anhydrase and CTSK (34). The osteoclasts secret protons through the ruffled border membrane to acidify the bone surface. In this process, carbonic anhydrase enzyme transports protons by V-ATPase. Thus, the creation of pH 4.5 in isolated area decomposes bone mineral components and exposes the organic material such as type I collagen, which can be decreased by CTSK (35).

Cellular fusion in osteoclastogenesis

The fusion of plasma membranes is a common phenomenon in almost every cell, such as the fusion of intracellular membranes which makes hormone secretion and neurotransmission possible. On the other hand, less is known about the mechanisms by which intercellular fusion occurs during osteoclastogenesis. Cell-cell fusion is a fundamental process in the generation of osteoclasts and multinucleated giant cells (36). The progenitor cells of osteoclast move to some specific location of hard tissues through the blood vessel, then these cells gradually form a

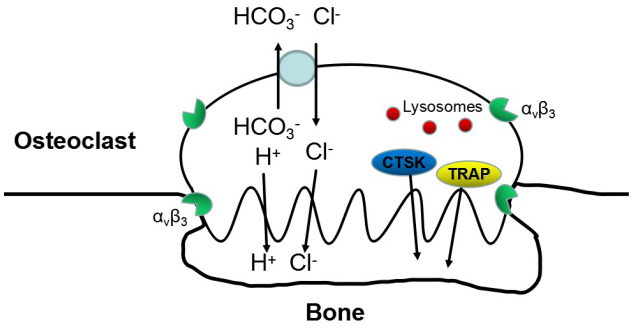

Figure 3. Mechanism of bone resorption. Osteoclasts release acid to the bone surface. Under acidic conditions, the inorganic minerals of bone are dissolved in the area of absorbing microenvironment. Osteoclasts degrade collagen by secreting several lysosomal enzymes, particularly CTSK and TRAP..

cluster and fuse with each other, and in the end become a mature osteoclast (37). Masaru put forward that cell fusion was the key process of osteoclast formation and regulating this process would provide potential therapeutic targets in bone diseases (38).

The cell fusion process in osteoclastogenesis involves many molecules, including dendritic cell-specific transmembrane protein (DC-STAMP) (39, 40), dendritic cell-specific protein, integrin, src family kinases, and integrin and metalloproteinase family proteins (41). Fusion regulatory protein FRP-1 is also in charge of cell fusion. The antibody of FRP-1 enhanced the generation of multinucleated giant cells and induced the generation of functional osteoclasts (42).

The process of cell fusion depends on DC-STAMP including the presence of phosphatidylserine. At the surface of the fusion area, molecules like Anx A5 and Syn-1 play an important role in forming protein scaffold structure to link the phosphatidylserine with S100A4 in cell surface to regulate the fusion process (43). On the other hand, the activation of G protein-coupled receptor 119 can downregulate DC-STAMP to suppress preosteoclast fusion (44). Researchers find that actin binding LIM 1 (abLIM1) plays the role of negative control in cell fusion. Overexpression of abLIM1 suppresses osteoclast differentiation and formation. But when the expression of abLIM1 was downregulated by small interfering RNA, the formation and the marker genes of osteoclasts increased (45).

Conclusion

In summary, as a key cell in the bone metabolism system, studies on the mechanisms of osteoclast formation and activation have further deepened our understanding of osteoclasts. Research in bone immunology suggests that osteoclasts are involved in the regulation of the bone immune system, and the discovery of new signaling pathways, such as Semaphorin, may provide a new perspective on the mechanism of bone metabolic balance (46). With further research, especially in bone immunology (47, 48) and the RANK/RANKL pathway, it is expected to provide new ideas for the biological mechanism of osteoclasts and new directions for drug development for bone diseases.

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Conflict of interest
The authors declare that they have no conflict of interest.

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REVIEW ARTICLE

The physiological functions of IKKs-selective substrate identification and their critical roles in diseases

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ABSTRACT

The nuclear factor κB (NF-κB) transcription factors exert central hub functions in multiple physiological processes including immune response, cell survival, proliferation and cytokine production, which has naturally become the core of research almost in all aspects of biomedical science over 30 years. Since both the activation and termination of NF-κB pathway are tightly regulated, little alteration can lead to excessive inflammatory responses and even result in tissue damage and severe diseases. The inhibitor of nuclear factor kappa-B (IκB) kinase (IKK) complex is the main regulator of the NF-κB signaling pathway, they mediate and deliver signals through phosphorylating certain substrates. In recent years, increased proteins have been identified to be targeted by IKK members and the particular modification mechanism becomes clear with the development of detecting techniques and structural biology. In this review, we summarize the known substrates of IKK family members either relevant or irrelevant to NF-κB signaling, their structures and phosphorylation patterns, and the related physiologic and/or pathologic responses. Understanding the regulation of IKKs on their substrates may be helpful to connect IKKs with specific signaling pathways or physiological phenomena, and is essential for targeting IKKs in clinical research.

Keywords: NF-κB · IKKs · Substrates · Immune response · Disease

Brief introduction of NF-κB pathway and IKK families

The nuclear factor κB (NF-κB) family of transcription factors are structurally homologues that include NF-κB1 p50, NF-κB2 p52, RELA p65, RELB and c-REL, which exist inactive in the cytoplasm in a complex combined with members of the inhibitor of κB (IκB) family (1, 2). In response to multiple stimuli including numerous mediators in immune systems like tumor necrosis factor (TNF), lipopolysaccharide (LPS), interleukin-1 (IL-1) or T cell activators (3), IκB kinase (IKK) complex mediates NF-κB activating process by directly phosphorylating IκB, targeting it for proteasomal degradation (4). When released into the nucleus, NF-κB proteins regulate the expression of a large spectrum of genes encoding transcription factors, cytokines, adhesion molecules and immunoreceptors, thus to be essential for cell proliferation and survival, innate and adapt immune response and inflammation (5).

Signal-induced activation of NF-κB pathway demands specific phosphorylation of IκB proteins at Ser32 and Ser36 through the canonical IKK-dependent pathway (6). This IKK complex consists of two catalytic subunits IKKα and IKKβ, together with the regulatory subunit NF-κB essential modulator NEMO (also called IKKγ) (7, 8). The 85 kDa IKKα (previously known as CHUK), containing a protein kinase domain at its N-terminal half, and an elongated α-helical scaffold/dimerization domain (SDD) and a NEMO binding domain (NBD) at its C-terminal, is identified first by yeast two-hybrid screen for NF-κB-inducing kinase (NIK)-interacting proteins in 1997 (9, 10) (**Figure 1**).

By searching for IKKα-related kinases with an expressed sequence tag (EST) on NCBI database, the second

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component of the IKK complex is cloned and designated IKKβ (11). Overall, the two subunits are 52% identical, while their kinase domain exhibit 65% identity (12). Both of their kinase domains have two serine residues (Ser176, Ser180 for IKKα and Ser177, Ser181 for IKKβ), whose phosphorylation are required for the kinase functions (13, 14). A great structure difference between IKKα and IKKβ locates in their ubiquitin-like domain. IKKβ contains a key leucine at position 353 which is critical for IKKβ-induced NF-κB activation, while deleting the equivalent region on IKKα has no impact on its activities (15). Moreover, there is a putative nuclear localization signal on IKKα but not IKKβ, which possibly contributes to the reported functions of IKKα in the nucleus (16). Recent years, diverse combinations of the IKK components have gradually been observed, IKKα or IKKβ can respectively form homodimers either with or without the scaffold NEMO, however the predominant IKK complexes remain to be the 1:1:2 ratio combination of IKKα-IKKβ-NEMO (4, 17).

In addition to IKKα and IKKβ, it has been reported that there exist two other non-canonical IKK-related kinases, TNF receptor-associated factor (TRAF) family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKKε (IKKi) (18). TBK1 (also called NF-κB-activating kinase NAK), was initially amplified by PCR with primers containing the same sequences to IKKα and IKKβ, and was first identified by virtue of its interaction with TANK in a yeast two-hybrid screening (19, 20). IKKε, which shares 64% homology to TBK1, was found by searching for proteins similar to IKKα and IKKβ in a database, and then isolated by using suppression subtractive hybridization technique in 1999 (21). These two kinases contain the same trimodular structure to IKKβ and the N-terminal catalytic domain of IKKε has approximate 30% similarity with that of IKKβ (22) (**Figure 1**). In contrast to the constitutive expression of IKKα and IKKβ in almost all cell types, the IKKε protein is primarily observed in immune cells, with the highest expression in the spleen (21). TBK1 and IKKε also form homodimers and heterodimers adopting the similar assembly of the canonical IKK subunits, which require a scaffold protein to efficiently target their substrates, whereas TANK, (NAK-associated protein 1) NAP1 and SINTBAD (similar to NAP1 TBK1 adaptor) seem to fulfill this function (23, 24). Using phosphopeptide mapping and site-specific mutagenesis, it has been found that phosphorylation on Ser172 in the kinase activation loop of TBK1 and IKKε should be necessary for the kinase activity (25).

In spite of the sequence similarities and presence in common complexes, IKKα and IKKβ or IKKε and TBK1 maintain distinct substrate specificities and largely nonoverlapping functions. Pro-inflammatory stimuli like TNFα and IL-1, or Toll-like receptor (TLR) ligands like LPS mainly activate IKKβ and NEMO for canonical NF-κB pathway and innate immunity, whereas IKKα plays an ineffectual supporting role. In contrast, in response to CD40-ligand or B cell-activating factor (BAFF), IKKα primarily targets p100 for processing and exhibits its kinase activity and function in adaptive immunity and lymphoid organ development (26, 27).

IKKε and TBK1 were initially described as NF-κB- and IKK-activating kinases, whereas it is already clear that they are also important mediators of antiviral responses by activating two other transcription factors, interferon regulatory factor 3 and 7 (IRF3 and IRF7) (28-30). Additionally, TBK1 is reported to be involved in neuroinflammation and autoimmunity, and to be more essential than IKKε in the innate immune response (31).

In recent years, numerous substrates in addition to IκBs and various NF-κB-independent functions of the IKK family have been discovered. These substrates and functions encompass signaling pathways regulating tumorigenesis, inflammation and cell cycle, and provide a bridge for crosstalk between IKK-related signaling cascades with critical diseases. This review focuses on the kinase activities of four IKK members, highlights some of the major substrates targeted by IKKs either NF-κB pathway-dependent or independent with the emphasis on the exact phosphorylation patterns and regulatory mechanisms. Understanding these post-translation modifications may shed light on exploitative capabilities of IKKs as effective therapeutic targets.

Substrates identification by IKKs and its physiological functions

IKKα/IKKβ mainly regulates canonical and alternative pathways of NF-κB

The pivotal function of the IKK complex is to phosphorylate IκBs, including classical IκBα, IκBβ and IκBε, and NF-κB precursors p105 and p100 (17) (**Figure 2**). These IκB proteins share a tandem helical repeat motif called ankyrin repeat domain (ARD), which can functionally bind to the NF-κB dimers (2). A structurally flexible PEST domain exists between their C-terminal and the ARD, and it is abundant in proline (P), glutamic acid (E), serine (S) and threonine (T) (32). Besides, two critical serine residues on the N-terminal of IκBα, β and ε serve as phospho-acceptor sites during canonical NF-κB pathway activation (the exact sites are Ser32 and Ser36 on IκBα, Ser19 and Ser23 on IκBβ, and Ser18 and Ser22 on IκBε) (8, 27, 33). Via adaptors like TRAFs, a variety of membrane receptors activate the canonical NF-κB pathway. Once phosphorylated by IKKβ, IκBs are recognized and ubiquitinated by SCF/β-TrCP E3 ubiquitin ligases, and then go through polyubiquitination and degradation (34, 35). Although IKKβ phosphorylates unbound IκBs as well (36), NF-κB-bound IκBs are better substrates for phosphorylation (37). The degradation of IκBs releases the restraint of NF-κBs, the free NF-κBs thus go through nuclear transport and bind promoters of a wide range of target genes to function in numbers of NF-κB-related cellular regulations.

As a non-classical IκB molecule, p105 is phosphorylated with the same mechanism as IκBα. But in contrast to the rapidly phosphorylation of IκBα, IKKβ phosphorylates p105 with slow kinetics (38). In cells stimulated with NF-κB-activating agents, IKKβ directly phosphorylates Ser927

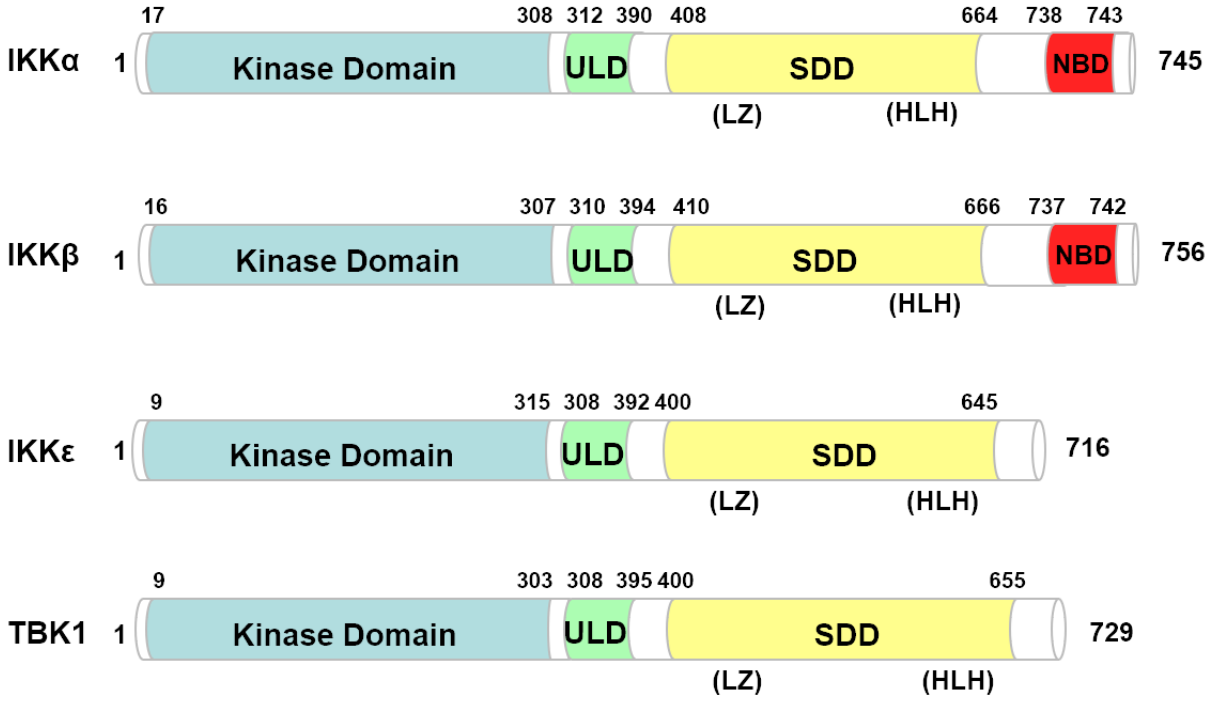

Figure 1. The structural comparison of the IKK family members. The principal structural motifs of the four members of IκB kinase (IKK) family are shown, together with amino acid numbers corresponding to the human proteins. ULD, ubiquitin-like domain; SDD, scaffold dimerization domain; LZ, leucine-zipper; HLH, helix-loop-helix; NBD, NEMO-binding domain.

in the PEST domain of p105 causing degradation of p105 via the ubiquitin-dependent proteasome pathway, however not affecting the p105 processing (39, 40). Furthermore, a functional p105 death domain is reported necessary for recruitment of IKK, with an additional requirement of Ser932 phosphorylation on p105 proteolysis (41, 42). In addition, p105 is also found to form a complex with the MEK kinase TPL2 in some inactivated cells. Following the degradation of p105, the released TPL2 activates the pro-proliferative mitogen-activated protein kinase (MAPK) signaling pathway (43). On the other hand, during the non-canonical NF-κB pathway activation, processing of p100 to generate p52 depends only on the IKKα, neither IKKβ nor NEMO (44, 45). The processing pathway is triggered by different ligands, such as LTβ or BAFF, and further activated IKKα can phosphorylate Ser99, 108, 115, 123, 866 and 870 of p100, which leads to ubiquitination of p100 by SCF/β-TrCP and then partial degradation of its ARD by proteasome (46, 47). Intriguingly, due to the slow activation kinetics of kinase NIK/IKKα, the degradation of p100 is also a relatively slow process. Unlike the classical IκBs which assemble with a single NF-κB dimer, p105 and p100 prefer to integrate two inhibitor proteins with at least two NF-κB molecules (48). Given the situation that p100 and p105 bind over half of cellular RelA, RelB and c-Rel, they could have profound impact on multiple NF-κB-mediated cellular processes (49).

In addition to p105 and p100, the phosphorylation of p65 conducts in the similar signaling pathways. The N-terminal Rel homology region of p65 is responsible for its association with IκBs and NF-κBs, and also determines its nuclear localization and DNA binding, while the transcriptional activation domain (TAD) at C-terminal determines its function as an activator of transcription (2). Several serine residues on the p65 TAD have been identified as phosphorylation sites and are targeted by distinct signaling pathways, the most extensively studied are Ser536, 468 and 276 (50). It has been reported that either induced by LPS or TNF, the inactive p65 in the cytoplasm is phosphorylated on Ser536 by IKKβ, and is dephosphorylated rapidly in the nucleus (51-53). The Ser536 phosphorylation by IKKβ is a key to potentiate p65 transcriptional activity, and it requires prior phosphorylation of IκBα at Ser32 and Ser36 as well as an intact NF-κB/IκBα complex (54). Additionally, more recent reports have shown that Ser468 in TAD2 of p65 is also phosphorylated by IKKβ, and moreover, IKKα is found capable of phosphorylating p65 and c-Rel, which shows an opposite function compared to IKKβ. Directly phosphorylated by IKKα on C-terminal, p65 and c-Rel go through accelerated turnover followed by quick detachment from target genes, resulting in the termination of NF-κB-associated transcription of critical genes (55). These discoveries reveal that selective inhibition of IKKα, which has little impact on IκBα, has remarkable effect on upregulating innate immunity by preventing the turnover of p65 and c-Rel, raising the novel therapeutic roles for IKKα inhibitors in the treatment of intricate infections.

Although no catalytic function of NEMO has been reported, this crucial regulatory component of the IKK complex is indispensable for signal-dependent activation of IKKβ (56). NEMO is comprised of a C-terminal zinc-finger region, a leucine-zipper and two coiled-coil domains. The N-terminal region of NEMO interacts with

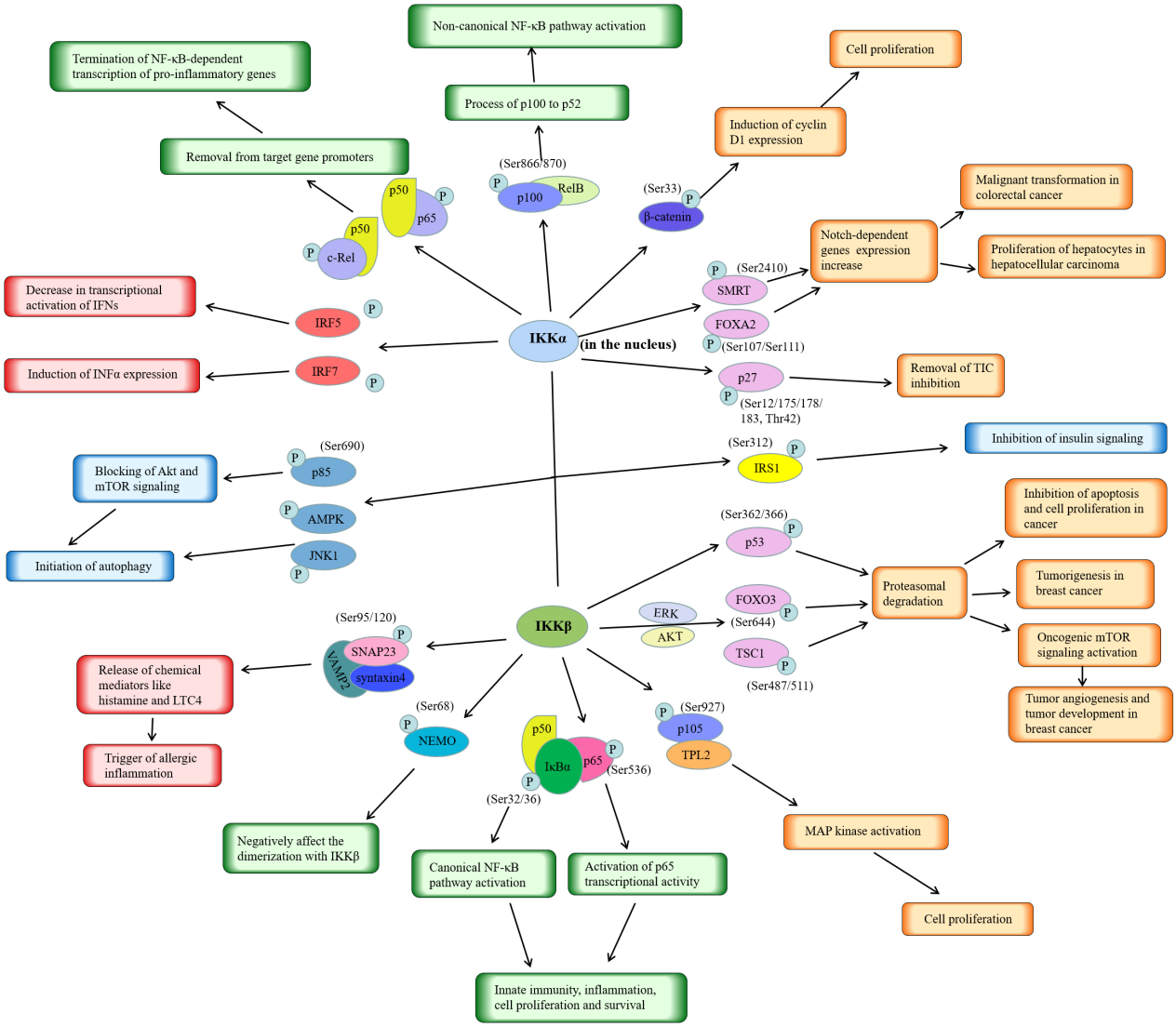

Figure 2. The substrates of IKKα and IKKβ and the related functions. In addition to activation of the canonical and non-canonical NF-κB pathways through phosphorylation of the IκBs, IKKα and IKKβ are now thought to phosphorylate a number of other substrates. And in turn, other than immune responses, IKKα and IKKβ are also important signaling proteins for critical cellular processes associated with several diseases like cancer. For more information see text.

the IKK's NBD which is essential for IKK activation (17). Multiple phosphorylation sites have been identified on NEMO, however the precise model and the underlying molecular mechanism contributing to IKK activation are missing until the phosphorylation of NEMO by IKKβ is found (57). *In vitro*, IKKβ phosphorylates NEMO at Ser43, Ser68 and Ser85 within IKK binding domain, while only Ser68 phosphorylation exhibits physiological functions *in vivo*. The NEMO phosphorylation at position Ser68, which is located in the interacting region with IKKβ, has negative effect on NEMO's dimerization as well as the NEMO-IKKβ interaction, suggesting the negative regulatory function of phosphorylated NEMO upon the activation of the IKK complex and NF-κB (58).

IKKβ phosphorylates tumor suppressors and promotes tumorigenesis

It is worth mentioning that IKKβ is a commonly activated oncogenic kinase in human cancers. It has been reported that IKKβ phosphorylates several tumor suppressor proteins, among which the most remarkable one is p53 (**Figure 2**). p53 contains the same phosphorylation motif (D/A) S (G/L/D/R) (G/D/R) XS in its C-terminal as IκBα does, and indeed, IKKβ phosphorylates p53 at Ser362 and Ser366 within this motif. This DNA-damaging reagent-induced phosphorylation is recognized by β-TrCP-mediated ubiquitination and leads to proteasomal degradation of p53 (59). The resulting loss of p53 causes enhanced IKKβ activity and impairs the glucocorticoids-dependent repression of NF-κB target gene transcription, and increases the rate of aerobic glycolysis (60, 61). Together, these findings suggest a positive-feedback loop exists during the IKK-NF-κB activation driven by glycolysis and provides evidence for the mechanism that tumor cells may damage normal inflammatory regulation in the tumor microenvironment.

Another two tumor suppressor proteins, Forkhead O transcription factor 3 (FOXO3) and tuberous sclerosis complex 1 (TSC1) are also identified as substrates of IKKβ. We have known that, FOXO3 can suppress tumor growth and tumor size in breast cancer (62). IKKβ, together with AKT/protein kinase B and extracellular regulated protein kinase (ERK) which are commonly activated in human cancers, can phosphorylate FOXO3a in response to insulin stimulation and growth factor. Phosphorylation of FOXO3a by IKKβ occurs at Ser644, which triggers ubiquitination and subsequent proteolysis of FOXO3a. Such downregulation of FOXO3a in breast results in cell proliferation and tumorigenesis (63, 64).

A similarly interesting story is that, the TSC1/TSC2 complex which acts as an inhibitor upstream of mammalian target of rapamycin (mTOR) pathway is also inactivated under the control of IKKβ, AKT and ERK. In response to TNF, IL-1β or a cardiovascular homeostasis controller angiotensin II (Ang II), IKKβ mediates Ser487/Ser511 phosphorylation of TSC1, accompanied by the phosphorylation of TSC2 by AKT and ERK. The phosphorylation results in the destruction of the TSC1/TSC2 complex, and thus induces the oncogenic mTOR signaling, promotes tumor angiogenesis and ultimately leads to tumor development (65, 66). Furthermore, Ser511 phosphorylation of TSC1 is reported in multiple tumor types associated with vascular endothelial growth factor production, and is closely related to poor clinical outcomes in breast cancer (65).

Notably, in addition to IKKβ, substrates regulated by IKKα also have crucial functions in a variety of inflammation-mediated tumorigenesis. The oncoprotein β-catenin and IκBs are both phosphorylated at the similar N-terminal serines, and in normal situation regulated by IKKβ, β-catenin is subsequently targeted for ubiquitination and followed proteasomal degradation like IκB (67). However, once phosphorylated by IKKα, β-catenin abundance increases. IKKα could directly phosphorylate β-catenin at Ser33 to stabilize β-catenin expression and modulate function of Wnt/ β-catenin signaling, which stimulates the pro-proliferative cyclin D1 promoter. Knockdown of IKKα leads to downregulation of β-catenin expression, therefore results in multiple myeloma cell growth inhibition (68).

The observation of IKKα in the nucleus provides possibilities for its novel functions, the related substrates direct various mechanisms in inflammation and cancers. When it translocates into the nucleus, IKKα phosphorylates Ser2410 of SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and releases this repressor protein from Notch1 target gene, resulting in increased expression of Notch-dependent genes like *herp2/hrt1* and *hes1*, and the malignant transformation in colorectal cancer (69). Additionally, IKKα phosphorylates FOXA2 at Ser107/Ser111 in the nucleus to suppress its transactivation activity, leading to decreased expression of downstream targets such as NUMB, which act as Notch1 inhibitors, therefore help the proliferation of hepatocytes and tumorigenesis in hepatocellular carcinoma (70). p27/Kip1, the cyclin-dependent kinase inhibitor and a negative regulator during cell cycle G1-S transition, which has been reported involved in ErbB2-induced mammary tumorigenesis, is also phosphorylated by IKKα at Ser12, Thr42, Ser175, Ser178 and Ser183. Intriguingly, the adjacent sequences are similar to known IKK phosphorylation sites and seem to be conserved in mammals. Phosphorylated p27 goes through nuclear export or exclusion, thus loses the suppressing effect on tumor-initiating cells (71). Actually according to current literature, the role of IKKα in tumor development differs within tissues. In colorectal, hepatocellular, breast and prostate cancers, IKKα acts as promoter of tumorigenesis, but inversely to be tumor suppressor in skin cancer and lung carcinomas (72).

Herein, dissection of the crosstalk between IKKα/IKKβ and such tumor-related proteins provide new understanding of inflammation-related tumorigenesis. In all, inhibitors of IKKα/IKKβ, AKT and ERK may work to reactivate tumor suppressors like FOXOs, TSC and p53, therefore developing therapeutic agents of the above multitarget may provide an efficient strategy for anti-tumor treatment.

IKKα and IKKβ play essential roles in immune response and immunological disorders

Type 1 interferon (IFNs) are a group of polypeptides constituting the first line of host immune defense, and infection by virus or double-strand RNA (dsRNA) leads to the activation of transcription factors of IRF family IRF3, IRF5 and IRF7, key activators of IFN genes and chemokine genes (73). IRF proteins consist of two major domains, a C-terminal activating domain along with an N-terminal DNA binding domain. The C-terminal autoinhibitory region is responsible for their activation in the cytoplasm, phosphorylation of critical serine and threonine residues stimulates the dimerization and nuclear transport, as well as the initiation of transcription (74).

IKKα has been reported critically involved in the TLR-MyD88-TRAF6 pathway-induced IFN production, and IRF5 and IRF7 are direct substrates of IKKα (**Figure 2**). Intriguingly, phosphorylation of IRF5 by IKKα attenuates transcriptional activation of IFNs (75), while in contrast, phosphorylated IRF7 serves as a positive regulator to manipulate TLR-induced IFN production (76). Multiple C-terminal serine residues of IRF5 are suggested to be phosphorylated by IKKα, phosphorylation then inhibits K63 linked ubiquitination of IRF5 and suppresses transcriptional activation of IFN genes (77). On the other hand, activation of *ifnα* gene expression via IKKα-phosphorylating IRF7 suggests that, IKKα could be a potential therapeutic target for certain autoimmune disorders whose IFN-α production is elevated, such as systemic lupus erythematosus (76).

In early-phase allergic response, IKKβ participates in and regulates immune response independent of NF-κB activation. Immunoglobulin E-activated IKKβ phosphorylates substrate SNAP23 at Ser120 and Ser95, which in turn initiates membrane fusion and exocytosis of

the SNARE complex of SNAP23/syntaxin4/VAMP2 for degranulation of mast cells (78, 79). The following release of LTC4, histamine and multiple biochemical mediators all triggers allergic inflammation which may lead to allergic diseases, for instance asthma, atopic rhinitis and dermatitis. NF-κB activation is not responsible for mast cell degranulation, however, NF-κB-dependent IKKβ kinase activity induces proinflammatory cytokine secretion which in turn promotes late-phase allergic reactions (79). All of the above findings indicate the central regulator role IKKβ plays in allergic reactions.

IKKα and IKKβ are double-edged swords in autophagy

In response to sub-lethal stress like cellular starvation, cells may utilize autophagy, a fundamental cellular process contributing to numbers of physiological functions in eukaryotic cells, to survive from nutrient depletion and maintain basal homeostasis (80). IKK is known to mediate amino acid starvation-induced autophagy via a mechanism independent of NF-κB by phosphorylating the phosphatidylinositol 3-kinase (PI3K) regulatory subunit p85 (**Figure 2**). When suffering nutrient deprivation, IKK phosphorylates p85 Ser690 at the conserved Src-homology 2 (SH2) domain, which results in reduced SH2-phosphotyrosine interaction and subsequent blocking of Akt and mTOR signaling. The inhibition of mTORC1 is reported conducive to the initiation of autophagy (81). Another research suggests that c-Jun-N-terminal kinase 1 and AMP-activated protein kinase are also IKK substrates and phosphorylation on their specific serine/threonine residues could mediate starvation-induced autophagy. Interestingly, this pathway requires mTOR inhibition as well (82). Yet, studies of PTEN-inactive prostate cancer cells demonstrate that IKKα suppresses autophagy via mediating mTOR activation (83). Meanwhile, starvation also upregulates anti-apoptotic gene expression like Birc3 through activation of the NF-κB signaling (84).

Altogether, these studies show the dual opposite roles of IKK against cellular stress through separate pathways: on the one hand, it could initiate or suppress autophagy in the absence of NF-κB; on the other hand, IKK regulates anti-apoptotic gene transcription in a NF-κB-dependent manner.

IKKs mediate insulin resistance in diabetes

Insulin resistance contributes importantly to type 2 diabetes mellitus, while many related factors like free fatty acids or TNFα, are also activators of IKK complex (85). Thus there rises the crosstalk between metabolism and inflammatory signaling in the development of insulin resistance. IKK complex phosphorylates Ser312 on insulin receptor substrate 1 (IRS1), thereby blocking out the IRS1 ability (86) (**Figure 2**). As we know, the metabolic actions of insulin are predominantly mediated by signaling involving the IRS family proteins (87). Furthermore, Ser307 phosphorylation of rat IRS1 has been reported to inhibit insulin stimulation of the MAPK or the PI3K cascades, suggesting an equivalent function of human IRS1 Ser312 phosphorylation (88). Besides, the probable existence of extra IKK phosphorylation sites on the IRS family proteins implies that they may represent a novel group of substrates for IKK kinases (89).

Furthermore, insulin receptor (IR) is also a direct substrate of TBK1. It has been shown that TBK1 can phosphorylate IR on Ser994 to impair the activity of the receptor, and may negatively regulate insulin signaling and result in insulin resistance (90).

Taken together, these findings place the inhibition of such kinases as promising targets in the treatment of type 2 diabetes and related disorders.

TBK1/IKKε in NF-κB regulation

Like traditional IKKα/β/γ, IKKε can be induced in response to proinflammatory cytokines such as TNFα, LPS, and phorbol myristate acetate, and Ser172 of IKKε is essential for phosphorylation (21). However, IKKε has different substrate specificity and kinetics from IKKα and IKKβ (91). Activated IKKε specifically phosphorylates Ser36 of IκBα, but not Ser32, therefore it seems that phosphorylation of Ser36 may predispose IκBα towards Ser32 phosphorylation and subsequent degradation (21) (**Figure 3**). Mouse embryonic fibroblasts lacking IKKε or TBK1 exhibit normal NF-κB activity while they are incapable of inducing numbers of well-characterized NF-κB target genes, whereas phosphorylation of p65 is proposed to explain these defects (92). Quite a few serine residues on p65 have been identified as phosphorylation sites and are targeted by distinct signaling pathways. Besides IKKα and IKKβ, upon IL-1 and T cell co-stimulation, IKKε also phosphorylates Ser468 and 536 of p65 to enhance its transcriptional activity. Ser536-phosphorylated p65 is mainly found in the cytosol while Ser468 phosphorylation occurs predominantly in the nucleus (93, 94). A series of NF-κB regulated genes are largely dependent on IKKε-mediated p65 phosphorylation, such as *Il6*, *Vcam1*, *IP10*, *Saa3* and *Cox2* (95). In addition, as an oncogene expressed in numerous types of cancer cells, IKKε controls the basal/constitutive p65 Ser536 phosphorylation and plays vital roles in cancer cell survival and proliferation (96).

TBK1 and IKKε initiate tumorigenesis through phosphorylating Akt and induce transformation

As the crucial downstream effector in PI3K pathway, Akt is essential in normal cellular physiology like proliferation, survival and growth, while pathological Akt activation contributes to many human cancers (97). A common model for Akt activation is that, phosphatidyl inositol-dependent kinase-1 and mTORC2 separately phosphorylate Akt on its activation loop Thr308 and hydrophobic motif Ser473 (98, 99). Here is a report showing that IKKε/TBK1 is sufficient to phosphorylate both the activation loop and hydrophobic motif of Akt protein, and such activation can be induced by several growth factors, for instance platelet derived growth factor and epidermal growth factor (**Figure 3**). Additionally, PI3K signaling is required in this activation process although the IKKε/TBK1 activity is irrelevant to PI3K.

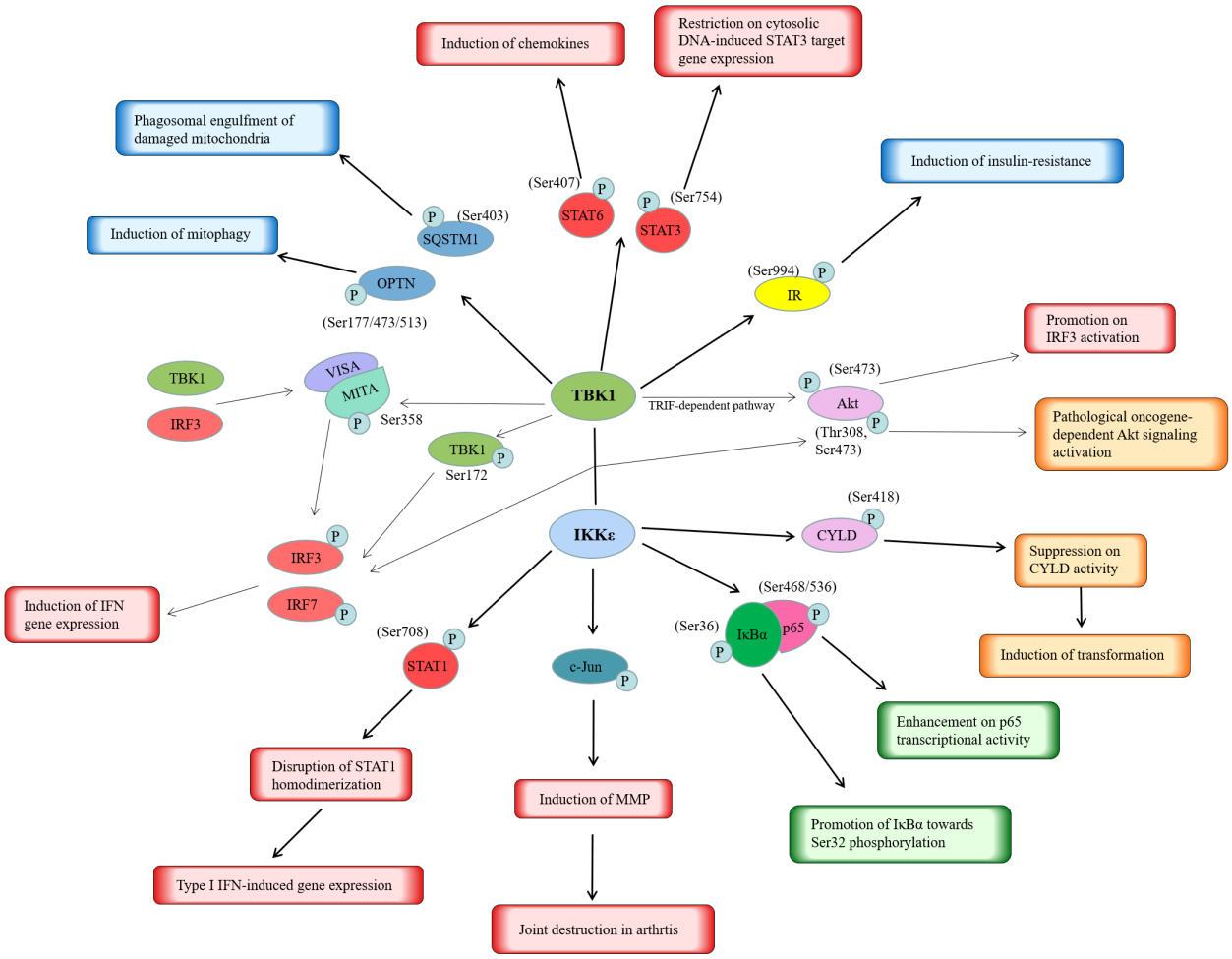

Figure 3. The substrates of TBK1 and IKKε and the related functions. Activated IKKε and TANK-binding kinase 1 (TBK1) modify numerous substrates and function in many biological processes, such as immune response, cell proliferation and survival, insulin signaling, and autophagy, some of which result in immune disorders and cancers.

This phosphorylation is required to sustain pathological oncogene-dependent Akt signaling and contributes to primary tumor initiation and development (100). Intriguingly, Akt activation diverges from PI3K in the TRIF (TIR domain-containing adaptor inducing IFN-β)-dependent signaling, a cascade responsible for TLR4-dependent IRF3 activation. In response to ligand stimulation, TBK1 cooperates with Akt to enhance Ser473 phosphorylation of Akt. In this context, Akt serves as a downstream component of the TRIF/TBK1 pathway to promote activation of IRF3 (101, 102). These discoveries indicate that pathological activation of TBK1 or IKKε promotes tumorigenesis by activating AKT, at least partially so.

Ubiquitin carboxyl-terminal hydrolase CYLD, a member of the deubiquitinating enzyme family specific for K63-linked poly-ubiquitins, acts as a tumor suppressor in familial cylindromatosis (103). CYLD suppresses a large number of inflammatory mediators including TRAF2, TRAF6, and NEMO by removing their poly-ubiquitins (104). However, this disruption disappears once CYLD Ser418 phosphorylation is induced by activated IKKs in a NEMO-dependent way, and the efficiency is comparable to the IκB phosphorylation by IKKs. Phosphorylated CYLD then inactivates its TRAF2 de-ubiquitination activity, promoting expression (105). Not long after, IKKε is also identified to phosphorylate CYLD on Ser418 and seems much more efficient than IKKα or IKKβ. Same as IKKs, IKKε-mediated phosphorylation of CYLD suppresses CYLD activity and increases NF-κB activation, and moreover, is necessary for IKKε to fully induce transformation (106). These findings connect the oncogene IKKε and the tumor suppressor CYLD, spell out the regulation of NF-κB on cell transformation and lead to an increased understanding of how they function in oncogenesis.

TBK1 and IKKε regulate innate immunity by regulating IRF3 and IRF7 activity

In addition to IKKα, IKKε/TBK1 is also involved in IFN-β induction through activation of IRF3 and IRF7 by direct phosphorylation on their C-terminal regulatory domain (107) (**Figure 3**). In the first step, TLR3 or TLR4 ligands like LPS and dsRNA recruit TRIF, which then connects to the TBK1/IKKε complex through TRAF3 for subsequent IRF3 phosphorylation (28). However, TBK1/

IKKε do not seem to be required in TLR7-, TLR8- or TLR9-related IRF7 phosphorylation (108). There are three clusters of phospho-acceptor sites on IRF3, Ser385/Ser386 (cluster1), Ser396/Ser398 (cluster2), and Ser402/Ser404/Ser405 (cluster3). It has been shown that cluster2 and cluster3 are the first sites targeted by IKKε or TBK1 and then prime the targeting of cluster1, sequential phosphorylation is indispensable for the complete unfolding and full activation of IRF3 (109).

Besides TLR-dependent pathway, innate immune responses to viral pathogens or dsDNA separately rely on the melanoma differentiation-associated gene 5 (MDA5) and retinoid acid-inducible gene I (RIG-I) or DNA-dependent activator of IFN-regulatory factors to trigger TBK1/IKKε-mediated IRF3 and IRF7 phosphorylation and IFN gene expression (110). The two caspase recruitment domains (CARDs) on N-terminal of both MDA5 and RIG-I are required for signal transmitting to the downstream CARD-containing adaptor protein VISA (111), while VISA has been demonstrated to be responsible for the activation of IRF3 (112). Researchers designate an uncharacterized protein as MITA, who is phosphorylated on Ser358 by TBK1 during viral infection, TBK1 and IRF3 are then recruited to VISA through this activated scaffold protein. Therefore, TBK1-mediated phosphorylation of MITA is closely associated to virus-triggered IRF3 activation (113). Intriguingly during dsDNA stimulation, endoplasmic reticulum-resident adaptor protein STING acts as another scaffold to recruit IRF3 for TBK1-mediated IRF3 activation (114).

In addition to extra substrates, crystal structure reveals a potential trans-autoactivation domain of TBK1 existing to support the fully autoactivation and maintain the normal function of TBK1 (115). In this situation, glycogen synthase kinase 3β (GSK3β) physically associated with TBK1, promoting the prerequisite dimerization or oligomerization of TBK1 for its Ser172 autophosphorylation, and leading to virus-triggered induction of IRF3, IFN-β as well as antiviral response (116).

TBK1 and IKKε-mediating STAT family phosphorylation crosslinks innate immunity and oncogenic pathways

Type I IFNs, like many other cytokines, utilize the canonical JAK (Janus kinase) - STAT (signal transducers and activators of transcription) pathway to function in innate immunity (117). Phosphorylation of STAT1 at Ser708 by IKKε has been reported to disrupt STAT1 homodimerization, thereby enlarging the STAT1 pool for optimally assembling the crucial IFN-I transcription factor complex ISGF3, which subsequently results in the shift of GAS-driven type I IFN-induced gene expression to ISRE-driven gene expression (**Figure 3**). This well-characterized process implies a role of IKKε in manipulating and balancing the IFN-I and IFN-II signaling pathways (118).

In addition, viral infections or cytosolic nucleic acids activate TBK1 to phosphorylate STAT6 at Ser407 or STAT3 at Ser754 in the transactivation domain, in a STING-dependent manner. However, phosphorylated STAT6 induces chemokines for recruiting immune cells to defend against viral infection, such as CCL2, CCL20 and CCL26, whereas phosphorylated STAT3 restricts its activity to respond to cytosolic DNA for target gene expression (119, 120). Given the well-established roles of STAT3 and STING in carcinogenesis and disease progression, these findings may shed light on the crosstalk between STAT-driven oncogenic signaling cascade and innate immune responses.

Phosphorylation of receptors by TBK1 contributes to selective autophagy

Canonical IKK members have been previously shown to control autophagy initiation, here it is reported that autophagic process is controlled by TBK1 (121). Autophagy receptors such as SQSTM1 (p62), optineurin (OPTN), NDP52 and NBR1, are involved in selective autophagy, whose function is to link ubiquitin cargoes to autophagosomal membranes as the ubiquitin signaling decoders (122). As the most closely related protein to NEMO, OPTN was identified as a substrate of TBK1 in two-hybrid screens (123) (**Figure 3**). TBK1 binds and phosphorylates OPTN on Ser177, Ser473 and Ser513. Ser177 phosphorylation in OPTN is known for ATG8 recruitment, while the dual phosphorylation of Ser473 and Ser513 activates polyubiquitin chain binding of OPTN, followed by the facilitated activation of TBK1 in vivo, as well as OPTN retention on damaged mitochondria, and mitophagy (124, 125). Within a positive feedback loop, ubiquitinated *Salmonella* or mitochondria recruits TBK1 and facilitates its clustering and activation, which in turn phosphorylates OPTN (126).

Earlier studies have suggested that SQSTM1 is recruited to mitochondria clusters to induce autophagosome of damaged mitochondria (127). However, subsequent observations have indicated that SQSTM1 mediates the aggregation of dysfunctional mitochondria through polymerization, but not for itself (128). Activated TBK1 phosphorylates SQSTM1 on Ser403, which is indispensable for its role in autophagic clearance and autophagosomal engulfment of polyubiquitinated mitochondria (129). Altogether, TBK1-mediated phosphorylation of autophagy receptors profoundly affects the regulation of TBK1 and selective autophagy pathway via a self-reinforcing positive feedback mechanism.

Due to its essential contribution to the suppression of excessive reactive oxygen species accumulation(130) and its participation in cellular death and senescence(131), autophagy is assumed to be a potent tumor-suppressive mechanism. It is worth further investigation that whether the IKK-dependent autophagy contributes to their tumor-suppressive function.

IKKε phosphorylates c-Jun to promote rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic autoimmune disease which is closely related to the transcription factor

NF-κB (132). Surprisingly in fibroblast-like synoviocytes, c-Jun, a well-known regulator of matrix metalloproteinase (MMP) gene and activator of antiviral program (133), is identified as an efficient downstream target of IKKε (**Figure 3**). Upon stimulation of IL-1 and TNFα, IKKε immunoprecipitates rapidly and efficiently phosphorylate c-Jun, although the specific residues are still missing. The induction of MMP is significantly increased, which plays a key role in joint destruction in arthrtis (134). These intriguing data suggest IKKε as a novel and potential therapeutic target in RA which links innate immunity, extracellular matrix destruction and cell recruitment.

Conclusions and perspectives

During the past three decades, research has gained great insight into the composition, activation, regulation and function of IKK members. The substrate spectrum of the IKKs exhibits far more than IκBs and precursors, which includes some other components in the NF-κB pathway and lots of extraneous proteins. While some excellent reviews focus on the activation and NF-κB-independent functions of IKK members (135), and non-conventional roles of specific NF-κB elements and the connection with other signaling pathways (84), in this review we have revised some of the well characterized substrates regulated by IKKs and emphasized the exact phosphorylation sites and modification patterns as well as their related function *in vivo*.

Despite the increasing identification of IKK-related substrates with diverse biological functions mediated by their phosphorylation, there still remains an important unanswered question: how substrate specificity is exactly achieved. *Hoffmann* et al. have demonstrated that NEMO functions as a scaffold targeting IKK to IκBs specifically and reduces alternate substrate phosphorylation within the inflammatory pathway (136). It offers us the idea that there must exist other scaffolds that may target IKKs towards alternate substrates and functions. Such scaffolds may totally replace NEMO and represent a new pathway determinant, or they may connect to IKKs together with NEMO and provide the crosstalk of inflammation with the other pleiotropic functions of IKKs. On the other hand, proteomic and immunofluorescence studies imply that TBK1 may use localization as a key specificity determinant, since its interactions with various adaptor proteins lead to different subcellular localizations and direct TBK1 towards diverse downstream pathways (137). To this end, co-recruitment and co-localization of kinases and substrates both offer to drive IKK substrate selection, and understanding the mechanism of IKKs' substrate specificity in different signaling axes may be critical for targeting IKKs in numerous diseases.

As our understanding of IKKs in mammalian physiology and pathophysiology is continuously developing, the IKK members involved human diseases like cancers and immune disorders also increase, however actually the pharmacological intervention targeting IKKs is still limited in clinical application. IKKβ has been by far the major focus in developing selective inhibitors for therapy, and in fact inhibitors targeting IKKβ and NEMO have already been developed and put into preclinical studies, and inhibitors of TBK1 and IKKε also have been tested in several cell studies and animal models. However, due to the unwanted toxicity by interfering with systemic NF-κB activities, none of them receive clinical approval (138), which get us thinking what kind of IKK inhibitors could make ideal drugs. The most important point is that it should only specifically intervene the given diseases or cancers with minimal effects on fundamental NF-κB functions in normal tissues. The numerous roles of IKKs in multiple pathways bring most of the complexity, because in different cells requiring NF-κB with diverse purposes, the outcomes vary. Secondly, the wanted inhibitors should be able to specifically and efficiently recognize its targeted IKK member, since the catalytic domains of four IKKs have significant similarity. Also, the dose, delivery method and delivery schedule which are related to clinical trials should all be taken into consideration. As for designing small molecule inhibitors, the structural differences on catalytic domains of IKKs and the exact phosphorylation sites of non-overlapping substrates targeted by different IKK members which are highlighted in this review may give a clue. Moreover, through comparing the effects of kinase inhibitors versus small molecule inhibitors, we could make it clear which functions are kinase-dependent, and the others perhaps are kinase-independent like scaffolding roles.

Regardless of the difficulties in the development of effective inhibitors targeting IKK members, the notable roles of IKKs in multiple signaling pathways and pathological processes we have presented in this review still indicate that these proteins are potential therapeutic targets for various diseases and malignancies. Additionally, with the developing structural and biophysical techniques, combined with the newly arisen single-cell computational studies, the latest research may shed light on the unsolved problems mentioned above.

Conflict of interest

The authors declare that they have no conflict of interest.

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REVIEW ARTICLE

3D Carbon-based scaffolds for brain models and tissue engineering

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ABSTRACT

Tissue regeneration is probably the most ambitious aim for the tissue engineering research field. Even more difficult it becomes when we attempt to regenerate a complex organ that we do not fully understand, such as the brain. That is why in recent years we have observed an increased number of approaches that strive to create functional brain or networks *in vitro* in order to study their properties and develop platforms that can be used for biomedical applications. In this review, we will describe how carbon-based materials took over all the other materials as the most interesting and promising platform not only in the electronic industry but also to create 3D functional models of the brain *in vitro*.

Keywords: Carbon-based material · Graphene · Three-dimensional scaffolds · 3D brain models · Tissue engineering platforms

Introduction

Understanding the interaction between nanomaterials and biosystems has become crucial in recent years. This growing interest has generated novel nanostructures such as nanoparticles (1), nanowires (2), nanotubes (3) and nanofibers (4) that can be applied for biomedical applications. In neuroscience, for example, magnetic or semiconductors materials have been used to study cell migration, particle internalization and electrophysiological properties of neurons (5–8). Conductive polymers, on the other hand, could have not only the electrical properties of metals or semiconductors but also the suitable mechanical properties (i.e. low stiffness) that are necessary to improve neural attachment and growth (9). Recently, new material-based models that can mimic the central nervous system (CNS) have been produced and described. Their physical, mechanical, chemical and electrical properties are carefully characterized to understand if they are suitable to be applied as successful platforms for applications in neuroscience (10,11). Among all, carbon-based materials (CBMs) are considered one of the most interesting to study and apply in biomedicine (12). When used alone, in combination with other CBMs or by making hybrids with natural or synthetic polymers, they have been demonstrated to promote electrical and mechanical interactions with the nervous system. Finally, carbon-based three-dimensional (3D) scaffolds are state-of-the-art in the tissue engineering field, having not only the outstanding conductive properties but also a third dimension that allows to better mimic the natural environment of the cells *in vivo*. In this review, we will describe these findings to convince the reader that these technologies can be further expanded to develop new *in vitro* models for the nervous system and new platforms for tissue engineering.

Graphene 3D scaffolds to model the brain in vitro

Since 40 years ago, when the first attempt of a 3D culture system was published demonstrating the possibility to maintain the cellular differentiation and organization by using a floating collagen gel (13), an overwhelming number of biomaterials have been discovered or synthesized to recapitulate the "closer to *in vivo*" behavior

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of cells *in vitro*. CBMs have emerged from the herd since the discovery of the fullerenes, and now with the discovery of graphene, their applications in material science, medicine and biology have increased even more.

Graphene is theoretically known since 1960 as a two-dimensional (2D) single-layer sheet of sp^2 hybridized carbon atoms organized in a hexagonal (honeycomb) arrangement. However, it was obtained only in 2004 from Andre Geim and Kostya Novoselov (14). This was the first 2D atomic crystal available to humankind and for their discovery, Geim and Novoselov were awarded the Nobel Prize in Physics in 2010. There are many reasons why graphene is such an attractive material. First of all, graphene has incredible mechanical and physical properties (with a stiffness of 150,000,000 psi and thermal conductivity of around 5000 W/mK). Then it has high electrical conductivity (with a carrier mobility of 15,000 $cm^2V^{-1}s^{-1}$ and a resistance of 10 ohms) and the possibility to be chemically functionalized (15–17). This is why graphene is in the limelight not only for industrial applications but also for a breakthrough in the biomedical, tissue engineering and neuroscience fields.

The biocompatibility of graphene materials with brain cells was shown by N.Li et al. in 2011. They showed that mouse primary hippocampal neurons were able to be kept in culture over a graphene film support allowing neurites sprouting and outgrowth (18). Graphene-based materials were shown to be inert to neurons that were able to preserve their physiological activity, as well as to adhere and grow without any coating with adhesion molecules (19). Graphene films were able to enhance the activity of neural stem cell (NSC)-derived neuronal network (20), and a more recent paper reported the unknown ability of graphene to regulate the extracellular ion distribution, by trapping ions that control the neuronal excitability, and in turn, affecting the neural network activity (21).

How can we move from these 2D graphene-based systems to a 3D graphene-based scaffold to better model and study the brain?

Chemical vapor deposition (CVD) is one of the methods to obtain graphene (17), which allows the synthesis of a 3D graphene-based scaffold using a nickel template that is then removed by etching. CVD was used, for instance, to create an oriented 3D structure of graphene bricks. This novel graphene structure can be customized based on the application by adjusting the pore size, from 10 to 50 μm, and the angle of the bricks across layers using 45° or 90° (22). They have an electrical conductivity value of 60-80 Scm^{-1} and a density of 3.6 $mgcm^{-3}$ showing properties as a supercapacitor electrode and flexible conductor. Another 3D scaffold was made by using a nickel foam as a template. This resulted in a hollow, tubular structure made of graphene that is interconnected in three dimensions called 3D graphene foams (3D-GFs) (23) (**Figure 1A**, left). Ning Li and collaborators used adult NSCs derived from the mouse hippocampus and conducted proof-of-concept studies on the application of 3D-GFs as a conductive substrate for cell electrical stimulation. They demonstrated not only the ability of these cells to adhere and grow within the scaffold but also their capability to differentiate into functional neurons, astrocytes and oligodendrocytes that could be electrically stimulated directly passing a current through the 3D-GFs (24). The same group reported, in a paper led by Qing Song, a reduced inflammatory response of NSC-derived microglia growth on the 3D-GFs upon insult with lipopolysaccharide (LPS) (25) demonstrating the anti-inflammatory effect of this cell-material interaction. Ulloa Severino and collaborators for the first time were able to culture primary rat hippocampal cells on the 3D-GFs and demonstrated that, using calcium imaging techniques and theoretical models, in 3D there are different neural networks properties (26). They reported that a small-world network model with frustrated synchrony could recapitulate the activity of the neuronal network growth on 3D-GFs that showed local and global network activity, as well as the existence of neuronal assemblies with a correlated activity that varies in space and time. These phenomena were similar to the one found *in vivo* making the 3D-GF hippocampal cultures a better *in vitro* system to study the brain. They showed that these results were not only due to the interactions between brain cells and graphene but also to the development of many more processes and connections along the third dimension, as well as to morphological changes in the shape of astrocytes (26), another important component of brain networks. The limitation of this scaffold was that the cells, although sometimes crossing the pores of the GF (**Figure 1A**, right), were adhering and growing on a 2D surface developed in 3D. How can these scaffolds be improved to have a real 3D culture system? What are the proper modifications and improvements that need to be made?

Hybrid carbon-based scaffolds for 3D culture systems

Graphene is obviously not the only CBM used for biomedical applications in neurology (12). Indeed, the most studied CBM before graphene was carbon nanotubes (CNTs). CNTs are cylindrical hallow graphene structures that can exist in the form of single-wall CNT (SWCNT) or multi-wall CNT (MWCNT). As can be inferred by the names, the difference is in the number of graphene sheets which form the tubular shape and in turn determine the diameter (from approximately 1 nm for SWCNTs to 100 nm for MWCNTs) (27). CNTs present good thermal and chemical stability as well as high mechanical strength. Moreover, thanks to their electron-rich properties, CNTs are well suited to interface electrically active tissues such as the heart and the brain. For biological applications, different substrates have been used to deposit 2D CNT-based bricks such as forests of vertically aligned CNTs (28), films of CNT building blocks (29) as well as extended 2D meshes of CNT (30), but the limitation was that cells could not migrate into the deep layers of these CNT assemblies. However, interfacing CNTs with brain tissues was reported to boost the electrical activity, sustain the survival of neurons, modulate neuronal growth as well as promote the functional reconnection of segregated

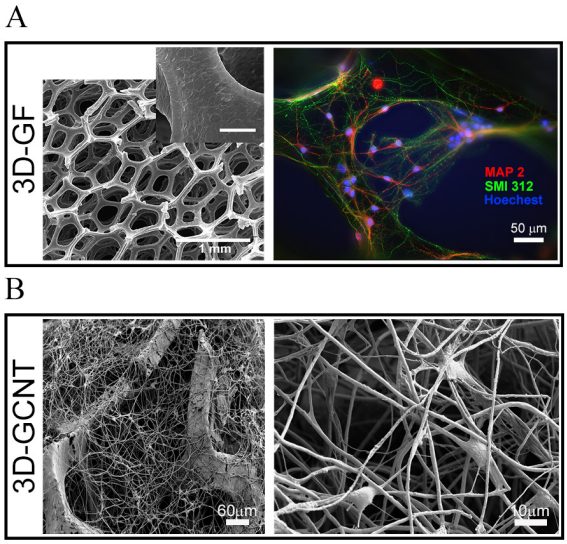

Figure 1. (A) An example of 3D-GF, on the left a scanning electron microscopic image showing the big pore size, and on the right mature neurons growing on the backbone and sometimes able to cross the pore of the structure without support. **(B) An example of 3D-GCNT**, on the left showing the CNT web inside the GF structure, and on the right showing the intricate web of CNT supporting the neuronal cell bodies. Modified from Ulloa Severino et al., 2016 (26).

spinal cord slices (30-33). Aiming to improve the *in vitro* model for the application of CNTs in neuroscience, Bosi et al. fabricated a polydimethylsiloxane (PDMS) 3D porous structure with the inner walls of the pores layered with MWCNTs. In this way, they were able to maintain the mechanical properties of the PDMS structure with the addition of the electrical and physical properties of CNTs (34). These structures were used to culture primary hippocampal cells *in vitro* to show that the network activity, in terms of synchronization and frequency, did not change between 2D and 3D MWCNTs but was anyway higher than both 2D and 3D PDMS substrates alone, in which the third dimension made the difference (34). These same scaffolds were then used to show their ability to functionally reconnect segregated spinal cord slices (35). In the same paper, the authors also showed the effect of the scaffold implant *in vivo*. After 4-8 weeks from the implant in the rat visual cortex, there was no increase in the inflammatory response in the tissue nearby the implant (50 µm from the implant) and a significant reduction at further distance (up to 150 µm from the implant), measured as intensity of glial fibrillary acidic protein (GFAP) and Ionized calcium binding adaptor molecule 1 (Iba1) immunofluorescence as indication of glia reaction after injury. Finally, they demonstrated that neurons were able to infiltrate the scaffold with their processes and soma, demonstrating a good integration of the exogenous material into the tissue as early as 2 weeks post-implant.

The idea of fabricating scaffolds of different materials to integrate their best properties is fascinating and necessary in order to improve the new platforms for brain-material interface and to find new ways of mimicking the brain *in vitro*. This approach was exploited very well by Xiao and collaborators who applied the CVD method to fabricate a 3D-GF with a 3D mesh of CNTs filling the pores of the GF backbone (**Figure 1B**, left), combining the advantages of both materials (36). These 3D graphene CNTs scaffolds (3D-GCNTs) were used to culture rat cortical cells in 3D. What they observed was a functional cortical network that was growing through the whole structure (more than 600 µm in height) made of 3 times more cells than the one counted on the GF. The unexpected observation was that they found cell bodies suspended into the pores with the neurites anchored on the GCNT structure (**Figure 1B**, right). This in vitro 3D cortical co-culture presented an activity dynamic similar to the one observed *in vivo* having an increased frequency and synchronization, even compared to the 3D-GFs, as well as a reduction in synchronization with the maturation of the network. The big step ahead of their approach though was to use this cortical co-culture system as a platform for other studies. They showed that the 3D cortical network can be used to study glioblastoma infiltration in a cell dense *in vitro* brain model using 3D live-cell imaging as well as to screen drugs (36). What they found was that, comparing the bare GCNTs with the ones seeded with the cortical culture, there was a decrease in the speed of infiltration of the glioblastoma cells in the co-culture system. Moreover, the effect of the drugs used to investigate these processes changes between the two conditions. When blebbistatin, a drug used to slow down the infiltration of cancer cells, was used on glioblastoma cells seeded on the 3D cortical network, whose velocity decreased to a lower extent compared to the GCNT scaffold alone. This had a big impact not only as a novel approach that could be compared to another 3D tissue model system but also for the indications that the environment in which researchers screen drugs can affect their functionality, and having better *in vitro* models for these types of experiments is extremely important. Many questions remain to be addressed, but what can we do more? Where is this research field going?

Future approaches for the biomaterial interface

Graphene-based materials have their own characteristics, amount of layers, composition, chemistry surface, purity, defects and oxygen content. Even if graphene oxide (GO) and reduced graphene oxide (rGO) present lower conductivity than graphene, these materials possess better hydrophilic property and versatility than pristine graphene scaffolds, making them more suitable for some different neuroscience applications. In a study published by Serrano and collaborators, rGO scaffolds were built by ice segregation-induced self-assembly technique (37). This material has exceptional mechanical compression properties in the longitudinal and transversal directions. This 3D GO scaffold could be compatible with the nervous tissue mechanical properties (0.3-1.0 kPa) (38). López-Dolado implanted this 3D scaffold made of rGO for the first time in injured rat spinal cord in order to

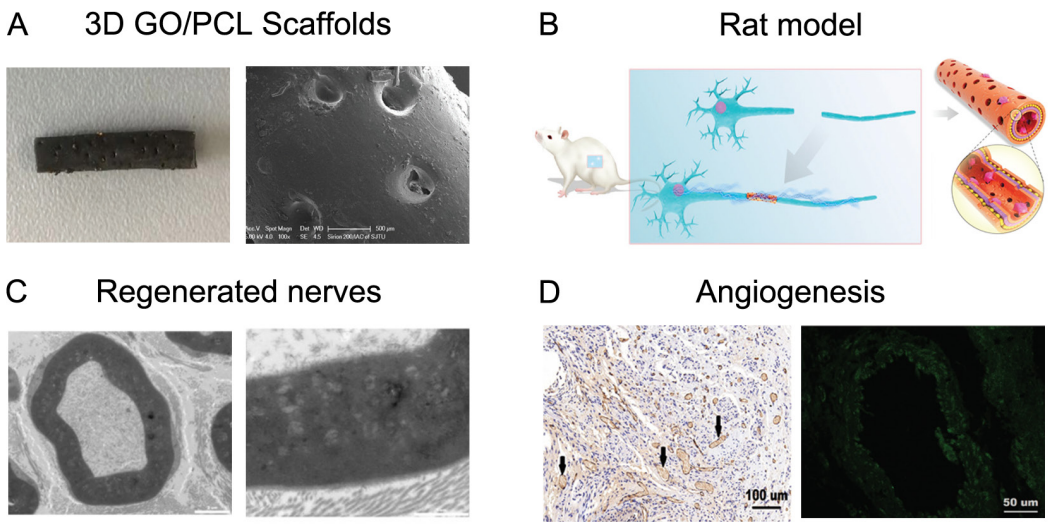

Figure 2. Examples of 3D-GO. (A) Optical (left) and scanning electron microscopic (right) images of GO/PCL scaffold showing the porous structure. (B) Nerve guidance conduits implantation in the rat model and a 3D GO/PCL tubular mold scheme. (C) Transmission electron microscopy images of regenerated nerves (scale left = 2 µm and right = 1 µm) in a GO/PCL conduit. (D) Study of angiogenesis in regenerated nerves by immunohistochemistry (left) of endothelial cells (black arrows) and immunofluorescence (right) staining of an associated vascular tissue protein in a GO/PCL scaffold after 18 weeks post injury (scale bars, black = 100 µm and white = 50 µm). Modified from Qian et al., 2018 (42).

observe regeneration. After 10 days the scaffold was able to facilitate tissue regeneration. The scaffold prevented the extension of the lesion and established a soft interface at the injury site (39). They also investigated the inflammatory effect of this rGO scaffold after 30 days from implantation by studying vimentin^{+} and ED1^{+}. A reduction in the amount of these two molecules caused a reduction of the inflammatory response. Moreover, rGO scaffolds helped to stabilize and seal the injury as well as support angiogenesis. Inside the structure, they were able to observe blood vessels in the proximity of the regenerated neuronal axons (40). One recent study observed excitatory axons growing in rGO foams through the injury site by functional blood microvessels, implying that these scaffolds improved the neuronal recovery after spinal cord injury (41). These findings demonstrated that rGO could be useful for neuronal regeneration. Qian et al. fabricated GO/polycaprolactone (PCL) nano-scaffolds (**Figure 2A**) to study the bases of the process of tissue regeneration, as angiogenesis and nerve regeneration by a Sprague Dawley rat model (**Figure 2B**) (42). Since the scaffolds were fabricated as a multi-layered structure with many pores, the nerve conduit mechanical properties were reinforced. Moreover, the scaffold structure promoted the contact with body fluid as water, oxygen and other nutrients. After 18 weeks of injury GO/PCL repaired a 15 mm sciatic nerve defect. These scaffolds were able to induce functional and morphological recovery in peripheral nerve regeneration. TEM images showed higher area, diameter, thickness, and the number of regenerated nerves and myelinated axons than other controls (**Figure 2C**). The microvessel density was higher using GO/PCL scaffolds after 18 weeks of post-injury. The density was evaluated by immunostaining CD31, an endothelial cell involved in angiogenesis, (shown by arrows in **Figure 2D** left), and CD34, a transmembrane protein associated with vascular tissue. (**Figure 2D** right).

Among other materials used to fabricate scaffolds, hydrogels are getting attention due to their excellent characteristics as low stiffness, porosity and bioactivity. These properties make these materials suitable to mimic human tissues. With the advent of 3D printing technology, the fabrication of 3D-printed hydrogel-based scaffolds became a common approach to design new scaffolds. However, hydrogels present some limitations regarding their low processability and poor mechanical properties. New research is focusing on combining hydrogels with CBMs in order to improve the biocompatibility, processability, mechanical and electrical properties. Since GO is stable in water suspensions, making this material suitable for a combination with hydrogels, one of the most frequently used 3D printing techniques is direct ink writing. Using this technique, Yao Bin et al. were able to make a 3D-printed graphene aerogel (SF-3D GA) electrode presenting the remarkable properties of CBMs (43). Olate-Moya et al. presented a hybrid nanocomposite hydrogel based on alginate crosslinked with genalin, chondroitin sulfate and GO particles as ink. They used a fourth-generation 3D bioplotter to 3D print the scaffolds. These 3D-printed hydrogel-GO scaffolds presented good biocompatibility and excellent cell proliferation, alignment and distribution along the scaffold (44). They used human adipose tissue-derived mesenchymal stem cells to test these scaffolds, showing that they presented all the outstanding properties to be applied in neuroscience. In another recent study, it was used a different approach to make 3D-printed scaffolds. First, they produced graphene using a method that combined bovine serum albumin with wet ball milling. They used a custom setup where the

graphene ink was printed on a Kapton polyimide polymer. They grew neuronal cells (rat dopaminergic, N27) and observed that the platform was able to sense electrical signals (45). These pathways open new possibilities of 3D scaffolds for the study and improvement of neuronal brain models.

Safety of 3D carbon-based scaffolds

3D carbon-based scaffolds are one of the best candidates for the development of functional brain implants. Engineering new scaffolds that can hold the responsibility of helping the regeneration of a damaged nervous system is one major goal of this field. This, however, requires not only to find the most suitable material and the understanding of the cell-material interactions but also to investigate the effects of long-term exposure to these materials and their safety.

The are many routes of exposure to CBMs and we suggest to the reader a thorough review of the effects on human health and on the environment of CBMs published by Fadeel and collaborators (46). Our aim here is to report what is known about the safety of 3D carbon-based scaffolds, a topic that has not been covered very much so far despite its importance. Many of the studies conducted to assess the biosafety of CBMs in the brain are based on injectable CBMs in suspension, such as few layer graphene, CNTs, fullerene and GO (46,47). This is surely a relevant aspect as we have learned that, for instance, SWCNTs show a higher toxicity compared to MWCNTs when injected in the rodent's brain (48,49). However, is this also the case for 3D scaffolds made with CNTs or other CBMs?

The idea that this kind of scaffolds may attenuate the inflammatory response comes from an *in vitro* study conducted by Song and collaborators (25). They evaluated, upon insult with LPS, the production of reactive oxygen species, interleukin-1β, tumor necrosis factor-α and nitric oxide by NSC-derived microglia cultured on 2D and 3D graphene substrates. What they found was promising, the 3D-GFs could reduce the inflammatory response in the presence of LPS compared to the 2D cultures. A recent *in vivo* study reported a reduced inflammatory response when an electro-spun PCL microfiber scaffold coated with self-assembled colloidal graphene was implanted in the striatum or sub-ventricular zone of adult rat brain (50). They observed a reduced infiltration of Iba1+ microglia within the scaffold coated with graphene compared to the uncoated one (bare PCL). In both cases microglia infiltrated only the initial layer of the scaffolds but by the third week, the scaffolds coated with graphene had a reduced distribution and infiltration of microglia compared to the bare PCL. Finally, they reported reduced scar tissue formation around the implant coated with graphene withing 7 weeks from the surgery. Two other studies showed the ability of 3D carbon-based scaffolds to reduce the microglia infiltration and scar tissue formation upon implant, that in turn demonstrated a good integration of the exogenous material within the CNS (30,35). By using a 3D mash of CNTs or a 3D PDMS+CNT structure implanted into the visual cortex of adult rats, it was demonstrated that both scaffolds were able to reduce the inflammatory response as indicated by the reduced presence, at far distance from the implant edge, of astrocytes and microglia over time (up to 8 weeks after implant) and the finding of neurons within the implants.

Finally, the degradation and toxicity of 3D scaffolds are poorly known. Domínguez-Bajo et al, studied the degradation of 3D rGO scaffolds after 4 months of being implanted in the spinal cord (41). Using TEM they showed how these 3D scaffolds were dissociated and degraded without toxicity. They observed how the thickness of the scaffold wall was changing over time and how pieces of rGO were uptaken. The internalization process and how these uptaken pieces of rGO could terminate under the blood stream are still uncertain. They evaluated the effect of rGO in different organs and no damage or toxicity was found in the kidney, liver, lung or spleen after 4 months of the 3D scaffold implantation. They associated this result to their lower dose of rGO (250 μg per rat, 700 μg kg^{-1}) than other studies. More *in vivo* studies and chemical analysis by using Raman spectroscopy, X-ray photoelectron spectroscopy or nuclear magnetic resonance will be necessary to fully understand the degradation and toxicity at longer time points to assess the safety of implanted 3D CBM scaffolds and apply these technologies in the biomedical field.

Conclusion

3D CBM scaffolds were presented as a successful model that could mimic the CNS. These scaffolds showed excellent ability to manipulate neuronal activity and presented exceptional properties for neuronal regeneration. From the neurobiological point of view, they were used as a 3D cell culture model of the brain, recapitulating fundamental processes of neural network formation and function. Their applications spanned from studying electrophysiological properties of neurons to promoting the regeneration after spinal cord injury. We have seen them used to study other cellular mechanisms, like cancer cell infiltration and drug screening. Other open applications could be to use them to study how neuronal network activity changes based on the presence of different ratios of excitatory and inhibitory neurons to investigate their relative contribution within neuronal assemblies and generate new 3D *in vitro* models of epileptic networks. In regenerative medicine we could functionalize their surface with molecules that can promote cell-material interactions as well as vascularization of the scaffolds to have permanent and integrated implants. These are just a few examples of what can be done, we need to investigate the mechanisms by which they can promote regenerative processes. From the material science perspective, however, there is still work to do regarding the understanding of the biophysical mechanisms underlying the cell-material interactions and how to improve them. When designing these scaffolds, it should also be considered to make them small enough

in case they will be used as implants in a complex location, but it should also be large enough to be able to manually handle it. It is still necessary to study the long-term effects of their degradation in a living organism and their potential toxicity. These are a few of the many improvements that can be done to these platforms. We believe that the combination of CBMs with hydrogels or biopolymers presents an excellent potential for the development of new state-of-the-art 3D neuronal system scaffolds that will provide answers to these many questions. These approaches will create a new generation of neuronal model systems that will address these challenges, for the improvement and the study of *in vitro* brain models as well as for the *in vivo* application and tissue regeneration.

Conflict of interest

The authors declare that they have no conflict of interest.

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REVIEW ARTICLE

Misfolded protein aggregation and altered cellular pathways in neurodegenerative diseases

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ABSTRACT

Neurodegenerative diseases are estimated by the World Health Organization to be the second leading cause of human death by 2050. They actually are a group of chronic neurological disorders leading to motor, cognitive and sensory impairments in both human and nonhuman species. Despite different in clinical manifestation, prevalence, risk factors, cell types injured and genes hijacked, neurodegenerative disorders are usually associated with the misfolding and aggregation of a distinct protein that accumulates in diverse cellular locations including the nucleus, cytoplasm, plasma membrane and extracellular space. Here we intend to give an overview of the characteristics and features of several pathogenic protein aggregates in disease brains, and introduce some general signaling pathways involved in protein homeostasis with an emphasis on their puzzling roles under the degenerative conditions.

Keywords: Neurodegenerative diseases · Misfolded protein aggregates · Unfolded protein response · Protein clearance pathways · Insulin/IGF/TOR

1. Introduction

Neurodegenerative diseases (NDs) are chronic neurological disorders including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Creutzfeldt-Jakob disease (CJD), Friedreich's ataxia, spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) in humans, as well as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and several others in nonhuman species (1-7). Although these diseases are initiated predominantly by aggregations of different misfolded proteins, they all result in gradual and progressive loss of nerve cells in the brain, eventually leading to irreversible disability in learning and memory due to impaired motor, sensory and cognitive systems. Pathologic development of neurodegenerative disorders usually is slow but fatal, requiring the accumulation of pathogenic molecules to exceed some critical threshold before neurological dysfunction occurs. Many NDs therefore are not evolutionarily selected and associated with the aging process, which provides time to allow the neurogenic symptoms to manifest (8). It was estimated by the World Health Organization that NDs should replace cancer, becoming the second leading cause of human death by 2050, when senior people aged 65 and above reach 17% of the population and over 152 million people are expected to have these dreaded maladies in their later life (9). The numbers underline the urgent need to develop informative molecular diagnostics and effective medical treatment for the public health problem.

While work in the field of neurodegeneration has been sparked by the prevalence of the world-wide epidemic along with increased life expectancy, yet we are only beginning to understand the underlying genetic and cellular mechanisms, and so far limited steps have been made along the path to promising therapeutics for these age-dependent illnesses. In light of this, the goal of this review is to provide an overview of protein misfolding and aggregation in degenerative brain disorders, and focus on debated knowledge regarding the cellular pathways altered in relevance to protein homeostasis under the

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pathological condition. We hope this review will be helpful to inspire new ideas and new discoveries on NDs.

2 Misfolded pathogenic protein aggregates in NDs

Although distinct in clinical manifestation, prevalence, regions of brain targeted and cell types injured, neurodegenerative disorders, when considered at the molecular level, share many common features, among which the progressive accumulation of misfolded pathogenic protein aggregates is believed to be the key event (**Table 1**). The protein aggregates mentioned here can be small and soluble oligomers, large and amorphous assemblies, or highly ordered fibrillary amyloids. A growing body of evidence indicates that these protein agents, such as amyloid β-protein, tau and α-synuclein, when in native states do not exhibit obvious similarities, and in origin can either come from endogenous gene products, or be seeded by an external infectious process, referred to as prion infection.

2.1 Amyloid β-protein (Aβ)

Aggregation of misfolded amyloid β-protein (Aβ), a secreted peptide derived from an internal domain within the amyloid β-protein precursor (βAPP), is an invariant hallmark of all forms of AD (38, 39). It is well known that the βAPP protein is normally synthesized, secreted and then efficiently degraded when the internal domain for Aβ is cleaved by α-secretase, a protease, to prevent Aβ formation (40, 41). However, *βAPP* in normal brain can also undergo cleavage in the endoplasmic reticulum (ER)-Golgi secretory pathway by β- and γ-secretase instead of α-secretase to release the amyloidogenic fragment, characterized as a 38- to 48-residue peptide (42-45). Among these toxic peptides, Aβ42 is the principal component of amyloid deposits in AD patients as it forms insoluble aggregates much faster than others (46, 47).

It is recognized that the majority of AD cases are sporadic, and only 10% to 20% occur in families (48). Nevertheless, *in vitro* and *in vivo* studies have showed that the underlying genetic factors, whether sporadic or inherited, are aiming to accelerate the accumulation of Aβ neurotoxicity at multiple levels. The first familial mutation discovered was in the *βAPP* gene, near the putative site for γ-secretase cleavage, modifying γ-secretase activity and thereby enhancing only the production of Aβ42 (49-51). After that, more inherited βAPP variants to facilitate Aβ procession were uncovered (52-54). Subsequent genetic analysis by a large number of AD families also identified mutations in presenilin 1 and 2 genes encoding the catalytic subunits of γ-secretase to increase Aβ42 level (55-57). In contrast, apolipoprotein E, a cholesterol transporter binding to Aβ, is the only well-established genetic factor associated with sporadic AD through its function to influence the clearance of Aβ in extracellular space (58-60).

2.2 Tau and tauopathies

Tauopathies are a diverse group of neurodegenerations characterized by neurofibrillary tangles (NFTs) composed of insoluble and hyper-phosphorylated tau proteins in neurons and glia (61). The protein tau, however, naturally is highly soluble and functions as a microtubule (MT)-binding protein to stabilize and promote the assembly of MTs (62). The binding between tau and MT is negatively regulated by the phosphorylation of tau, which is a feature of its pathogenic form (63). In adult human brains, tau is encoded by the *MAPT* gene to generate six isoforms, containing either three or four MT-binding repeats via alternative mRNA splicing (64). It has been proved *in vitro* that the MT-binding repeats are both necessary and sufficient for tau to acquire highly ordered β-sheet structures when it assembles into insoluble NFTs (65). Hence it has been shown that all six isoforms are present and misfolded in disease brains to form a heterogeneous mixture of tau isoforms adopting different conformations, which is probably responsible for the clinical and pathological diversity of tauopathies (66).

As a MT-binding protein, tau is normally considered to function inside a cell, but tau aggregates, likely released from dying or dead neurons, are also detected in the extracellular space where it can be taken up through endocytosis by neighboring cells (34). Once internalized, the small amount of aggregated tau then serves as a seed and transmits a misfolded state specifically to the native tau in healthy cells in a manner similar to prion, which will be discussed later (67). In this way, the disease properties spread from cell to cell along the defined neuroanatomical pathways, causing cellular dysfunctions due to both the physical occupancy of the large tau deposition and the loss of the MT-binding function of tau. Especially the latter, not only disrupts the stabilization of MT cytoskeleton, which is important for the generation and maintenance of neurites, but also suppresses the kinesin-dependent transport of mitochondria, peroxisomes and Golgi-derived vesicles in neurons (68). Shortage of mitochondria and peroxisomes subsequently causes loss of energy production and accumulation of reactive oxygen species, leading to degeneration. In particular, suppression of Golgi-mediated secretion would retain vesicles carrying βAPP in the cell body, allowing an enhanced production of toxic Aβ peptides (69, 70).

2.3 α-synuclein (α-syn)

α-synuclein (α-syn) is a phospholipid-binding protein with a chaperone activity to facilitate presynaptic SNARE-complex assembly and thereby regulate neurotransmitter release in the presynaptic terminals (71). In the presence of negatively charged lipids, normal α-syn folds into amphipathic α-helices through its N-terminal repeat region. Missense mutations located in the N-terminal repeats often lead to the conversion of α-helices into β-sheet-rich structures, which ultimately coalesce into characteristic assemblies called Lewy bodies and Lewy neurites in maladies such as PD and Lewy body disease, as well as into glial cytoplasmic inclusions in multiple system atrophy (20, 72-74).

As in the case of tau protein, the neuropathological process of α-syn lesions is also thought to progress via a

Table 1. Misfolding and aggregation of pathogenic proteins identified from NDs.

Misfolded Protein	Gene	Subcellular locations	Diseases	References
Amyloid β	*βAPP*	ER-Golgi, autophagosome, mitochondria, ES	AD, PD	(10-13)
Androgen receptor	*AR*	cytosol	SBMA	(14)
Atrophin 1	*ATN1*	nucleus, cytosol	DRPLA	(15)
Ataxin 1	*SCA1*	nucleus	SCA	(16)
α-Synuclein	*SNCA*	nucleus, cytosol, ER, mitochondria, PM, ES	DLB, PD	(2, 17-20)
Fused in sarcoma	*FUS*	nucleus, cytosol	ALS, FTD	(21)
Huntingtin	*HTT*	nucleus, cytosol	HD	(22)
Prion protein	*PRNP*	nucleus, cytosol	CJD, Kuru, BSE, CWD, Scrapie	(7, 23-26)
Rhodopsin	*RHO*	ER, PM	ADRP	(27)
Superoxide dismutase 1	*SOD1*	nucleus, cytosol, ER, mitochondria	ALS	(28)
Tau	*MAPT*	nucleus, cytosol, ER, Golgi, lysosome, PM, ES	AD, FTD, Pick's disease	(29-35)
TAR DNA-Binding Protein 43	*TARDBP*	nucleus, cytosol	ALS, FTD	(21, 36, 37)

AD: Alzheimer disease; ADRP: Autosomal dominant retinitis pigmentosa; ALS: Amyotrophic lateral sclerosis; βAPP: amyloid β-protein precursor; BSE: Bovine spongiform encephalopathy; CJD: Creutzfeldt-Jakob disease; CWD: Chronic wasting disease; DLB: Dementia with Lewy bodies; DRPLA: Dentatorubral-pallidoluysian atrophy; ER: endoplasmic reticulum; ES: extracellular space; FTD: Fronto-temporal dementia; HD: Huntington's disease; MAPT: microtubule-associated protein tau; PD: Parkinson's disease; PM: plasma membrane; SBMA: Spinal and bulbar muscular atrophy; SCA: Spinocerebellar ataxia; SNCA: Synuclein Alpha.

seed-induced conversion among cells along anatomically connected structures in the brain, albeit how pathological α-syn exits cells remains elusive (75). Furthermore, compelling evidence has suggested that abnormal α-syn is frequently co-depositing with other pathogenic proteins like Aβ and tau, as hybrid polymers initiated by cross-seeding between different types of protein aggregates have been extensively reported in various NDs (76, 77). As a result, the pathological overlap between disease agents in the same patient raises the question of which one is the predominant cause and complicates the diagnosis and treatment for NDs.

2.4 Prion diseases

Prion diseases, such as CJD and Kuru in humans, as well as scrapie and BSE in animals, can arise sporadically, be inherited, or be acquired by infection under natural conditions. The term "prion", denoting a small proteinaceous infectious particle, was proposed by Stanley Prusiner in 1982 first to describe the scrapie agent that causes a degenerative disorder of the central nervous system in sheep and goats (7). The definition now has been broadened to emphasize the requirement of an unconventional and virus-like protein for infection, which is able to undergo self-replication, similar to nucleic acid molecules, but resistant to procedures with specificity for attacking nucleic acids (78). Hence, it is now widely accepted that the pathogen of prion diseases might not contain any DNA or RNA, unless more sensitive probes are developed.

Although prions are thought to exist in multiple strains composed of different polymeric forms of misfolded proteins to cause phenotypic heterogeneity in various brain disorders, they all arise when normal cellular proteins (PrP-Cellular, or PrP^{C}) misfold and transform into pathogenic prion molecules (conventionally referred to as PrP-Scrapie, or PrP^{Sc}), which are characterized by a high content of β-sheets. Once established in neurons, the disease agent PrP^{Sc} then indefinitely convert more PrP^{C} into the prion form. Mutations in the gene encoding PrP have been identified prone to develop infectivity spontaneously (79, 80). This perhaps hints a genetic origin of prion diseases, but how pathological transformation occurs when PrP^{C} binds to PrP^{Sc} is largely unknown. It is predicated that the efficiency of prion conversion could depend on the homology of the primary and secondary structures between PrP^{C} and PrP^{Sc}, and the architecture of the PrP^{C}-PrP^{Sc} complex (81). According to studies on different prion strains, it is plausible to suspect that environmental factors may also contribute to the conversion of PrP^{C} to PrP^{Sc} as non-host factors, such as surface binding and weathering, which are able to alter strain emergence in vitro in a population of prions (82, 83).

Toxic prions have an enhanced tendency to aggregate and form oligomers or amyloid-like fibrils, disrupting normal cellular functions and eventually spreading within the nervous system mainly through the neural connectome (84). Besides cell-to-cell transmission, person-to-person and even cross-species disseminations are suggested by cumulative evidence as cases were reported that people

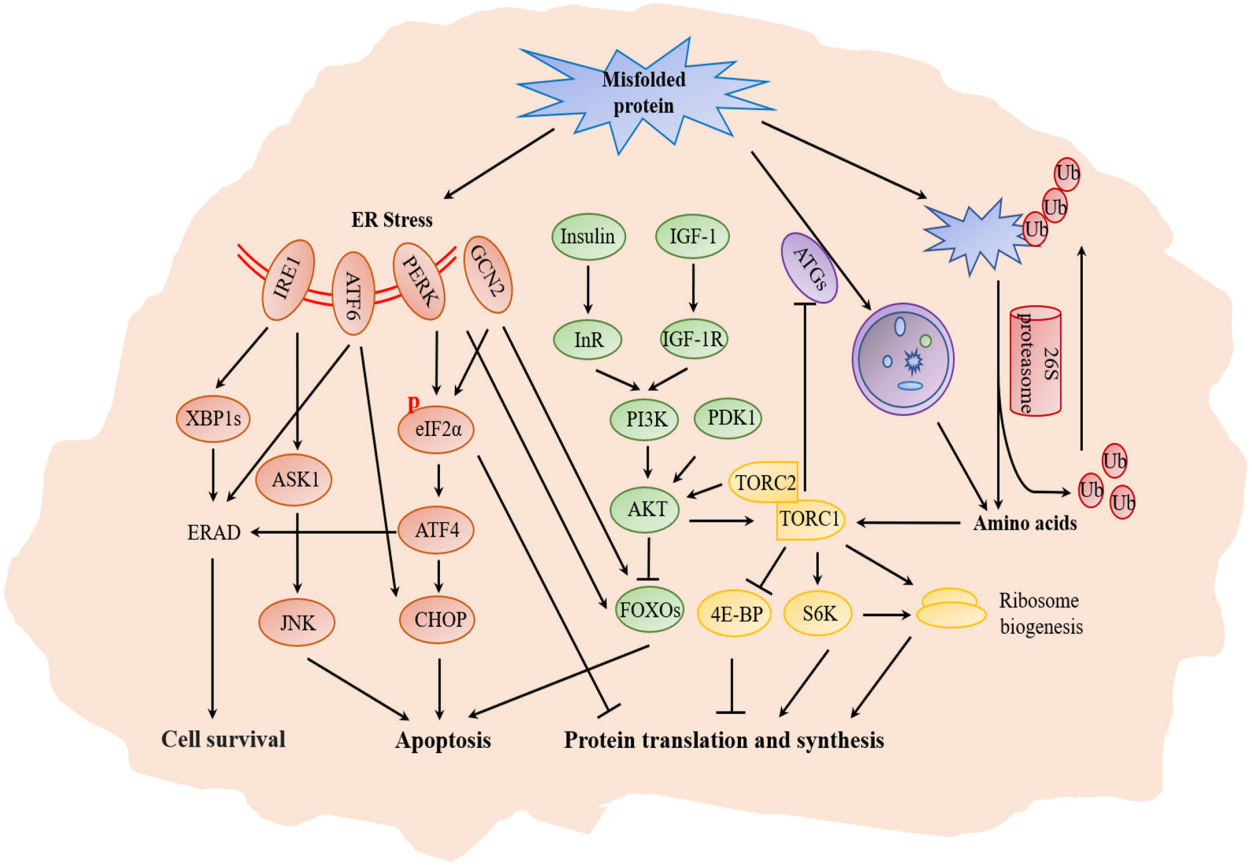

Figure 1. Schematic representation of cellular pathways involved in NDs. Arrows indicate activation, whereas bar-ended lines indicate inhibitory interactions.

with CJD, resulting from consumption of beef prepared from mad cows, transmitted CJD prions to recipients of blood transfusions (23, 85). However, the molecular basis of the intra-species and the inter-species transmissibility of prions remains poorly understood.

2.5 Prion v.s. non-prion

Even though lots of common biological features are shared, we insist to classify prion and non-prion (Aβ, tau, α-syn and others) into two groups in this review based on the transmissibility of associated diseases. After all, there is no clinical evidence for the person-to-person transmission of non-prion NDs under normal circumstances. Nevertheless, studies with Aβ, tau and α-syn have clearly shown that experimental inoculation with brain homogenates from patients or mouse models of these illnesses could lead to disease pathology in recipient animals in laboratory (86-88). As such, it is highly possible that the definition of prion will be further widened when bioassays are well developed so that the transmissibility of non-prion proteins could be fully appreciated. Yet we sincerely hope the infectious property of non-prion diseases is not true as it should challenge the therapeutic strategies and require implementing more precautions in taking care of ND patients. Also, it is serious that to date there are no effective therapies available for prion diseases. Approaches have been explored including small compounds, antibiotics, vaccination, antibodies, peptide aptamer and nucleic acid-based agents, but none have prospects for clinical advancement, owing to either inefficacy against prion after onset of symptoms or inadequate brain distribution. It hereby should be pointed out that a breakthrough from clinical trials can only be achieved with the development of a screening test for the early diagnosis of prion diseases (89).

3 Alteration of signaling pathways in cells of NDs

A key question always concerns how the accumulation of distinct disease proteins contributes to the degenerative process. The mechanisms underlying different neurological disorders probably are not exactly the same, but dysregulation of protein homeostasis linked with abnormal aggregates is an almost universal hallmark of ND pathogenesis. In patients, activities of pathways involved in protein synthesis, protein folding, protein degradation and energy supply for proteostasis are altered in cells of the nervous system (**Figure 1**). However, it is still not completely clear whether these changes play a protective or a toxic role in cell survival.

3.1 ER stress and unfolded protein response

The ER plays a central role in protein quality control to

maintain cellular proteostasis. Membrane and secreted proteins are synthesized, folded and processed in the ER before displayed on the cell surface, or released extracellularly. Misfolded proteins are eliminated via the ER-associated degradation (ERAD) pathways, either the ubiquitin-proteasome system (UPS) or autophagy (also termed as ERAD-I and ERAD-II respectively in some publications), to ensure that only properly folded proteins exit the ER (90). When substrates exhaust the regulatory capacity of ERAD, misfolded proteins accumulate and lead to a stress response called the unfolded protein response (UPR) (91). The UPR is mediated through three principal branches including endoribonuclease IRE1, transcription factor ATF6, and eIF2α kinases PERK and GCN2. The three signal transducers then regulate the expression of tremendous genes to adapt to the stress or to induce cell apoptosis when the stress cannot be mitigated (92).

In most organisms, ER stress-associated UPR is exacerbated during the aging process (93, 94). The capacity of the ER to prevent aberrant protein dramatically decreases in healthy aging, while the burden of unfolded proteins increases instead. In this scenario, the UPR is known to activate its adaptive programs to alleviate the accumulation of misfolded proteins via halting protein translation, stimulating destruction of abnormal proteins by ERAD, and increasing the production of ER chaperons relevant to protein folding. Upon activation, IRE1 is usually acting as an RNase and mediating the removal of an intron from the *XBP1* mRNA to allow the expression of a functional XBP1 transcription factor (95). The activity of XBP1 is linked to various pro-survival events including transcription of genes involved in protein folding and ERAD (96). ER stress also directly modulates gene expression to promote cytoprotection through the transcription factor ATF6 after it is translocated from the ER to Golgi, where ATF6 is activated by a proteolytic cleavage (97). In addition, the protein translation initiation factor eIF2α is phosphorylated upon stress to globally attenuate the cap-dependent mRNA translation and prevent overload of newly synthesized proteins into the already stressed ER lumen (98). In contrast, under eIF2α phosphorylation, translation of a subset of mRNAs, such as transcription factor ATF4 and genes targeted by XBP1 and ATF6, is enhanced to restore homeostasis via upstream open reading frame (99). Phosphorylation of eIF2α in response to ER stress was initially found to be controlled by auto-phosphorylation of the ER-resident PERK kinase, and it is recognized now to be partially contributed by GCN2 as well (100, 101). Although it remains unclear how the cytoplasmic GCN2 kinase senses ER stress, the redundant regulation of the two eIF2α kinases was suggested to occur in a tissue dependent manner (102).

ER stress-triggered UPR has been implicated broadly in neurodegeneration. Previous work in a *Drosophila* model of PD showed that accumulation of wild type or missense mutant α-syn led to the hyper-activation of IRE1, and ectopic overexpression of IRE1 was sufficient to induce neuron death, progressive locomotor impairment and shorter lifespan of flies (103). In brain tissues from both AD and PD patients, a clear increase of PERK and eIF2α phosphorylation levels was also observed when compared to normal elderly controls by antibody staining (104, 105). Interestingly, the same mammalian PD research and others demonstrated that oral administration of a PERK inhibitor had strong neuroprotective effects on many ND models, implicating the potential use of eIF2α phosphorylation as therapeutic targets, even though PERK inhibitor itself was found to have strong undesired side effects (105-107). Finally, ATF6 overexpression has been reported recently to reduce misfolded proteins and restore memory in disease animals albeit less is known about the involvement of ATF6 in neurodegenerative disorders (108).

Taken together, all these studies suggest a complicated scenario where the three parallel arms of the UPR, in comparison to its protective function, turn out to have contrasting and even opposite effects, as sustained ER stress, depending on the disease context, shifts the UPR signaling towards induction of apoptosis. Theoretically, the apoptotic effects are tuned through different downstream networks controlled by the same batch of genes, such as the ASK1 (Apoptotic-Signaling Kinase-1)-JNK pathway mediated by IRE1, pro-apoptotic transcriptional factor CHOP activated by ATF6 and ATF4, as well as apoptosis-related transcription factor FoxO3 (also in section 3.5) phosphorylated by PERK and GCN2 (102, 109-111). However, when and how the UPR converts its dual effect under ND conditions of chronic and irreversible ER stress is still incompletely understood.

3.2 Ubiquitin-proteasome system

Coordinated activities of the UPS and autophagy, the two major protein clearance pathways, can be central to prevent the aggregation and toxicity of misfolded-prone proteins, which manifest in a number of neurological disorders. The UPS is a highly selective and tightly regulated pathway for destruction of soluble, unneeded or potentially toxic polypeptides in most cellular compartments (112). Degradation of a protein via the UPS involves two discrete and consecutive steps named conjugation and degradation: the substrate protein is tagged by covalent attachment of multiple ubiquitin molecules to synthesize a proteolytic signal during the conjugation step; thereafter, the polyubiquitinated substrate is chewed up by the 26S proteasome complex with release of free and reusable ubiquitin, which is the degradation step (113).

Accumulation of ubiquitinated proteins has been reported in NDs, and an age- and disease-related decline of UPS activity has also been reported (114-116). In some cases, malfunctions of the UPS have emerged as a primary cause in the pathogenesis of neurodegenerations. In the past two decades, for instance, a direct link between an aberration in the ubiquitin system and the resulting pathology has been studied in PD.

The gene *Parkin* (or *PARK2*) codes for a ubiquitin ligase that ubiquitinates misfolded proteins targeted for proteasome-dependent degradation (117). Various deletion

and point mutations were found in this gene leading to young-onset PD (118, 119). Since then, a broad array of candidate substrates for Parkin has been identified including α-syn and its interacting protein synphilin-1, which are responsible for Lewy-body formation (120, 121). It should be noticed that by recent findings Parkin also ubiquitinates substrates on the outer membrane of mitochondria and through the UPS participates in the elimination of damaged mitochondria, which contributes to neuronal death as well when Parkin is impaired (122).

Besides, aberrations in the UPS have been implicated as a secondary consequence by disease-associated aggregations in many other cases. Cells engineered to produce or infected with unrelated protein aggregates by different research groups were shown to have the UPS stalled and destroyed (123, 124). Bennett et al., 2005 further found that production of protein aggregates specifically targeted to either the nucleus or cytosol led to global impairment of the UPS function in both compartments (125). Although the molecular mechanisms are undetermined, the observation of severe UPS damage in cellular compartments lacking detectable disease agent suggests UPS disruption could be an indirect phenomenon, arguing the toxic gain-of-function mediated by pathogenic protein aggregates in NDs.

3.3 Autophagy

Autophagy (or macro-autophagy) is a bulk clearance pathway whereby misfolded and proteasome-resistant proteins, macromolecules, and damaged or excess organelles are packaged into double-membraned vacuoles called autophagosomes, and then transported along MTs to the lysosome for degradation (126). Autophagy is normally regulated through a series of protein-coding genes defined as autophagy-related genes (ATGs) to constitutively function at a low level (127). Although autophagy in many organisms is induced primarily in adaption to nutrient deprivation, a tight relationship between autophagy and ER homeostasis is confirmed, given that many terms like “ERAD-II”, “ER-quality control autophagy (ERQC)”, “ER-autophagy (ER-phagy)” and “ER-to-lysosome-associated degradation (ERLAD)” have been proposed to delineate variant ER pathways that intersect with the entire or selective autophagy machinery (90, 128-130).

However, the pathological connection between autophagy and neurodegeneration is not simply restricted to the ER, and is much more complex. Experimental result has supported a role for dysfunctional autophagy as a potential causative factor of NDs, since mice deficient for *Atg5* specifically in neural cells develop progressive motor and behavior deficits, accompanied by the accumulation of cytoplasmic inclusion bodies in neurons (131). The absence of *Atg5* suggests the basal activity of autophagy is already essential for preventing the accumulation of abnormal proteins in the nervous system even without expressing any disease-linked mutant proteins. Not surprising that, in the presence of toxic protein aggregates, increased induction of autophagy is relatively frequent, and substantial benefits to ameliorate neuropathology are often observed with autophagy-inducing agents in a majority of transgenic mouse models of NDs (132, 133).

Yet there are a few exceptions that stimulation of autophagy would become counter-productive when specific stages of autophagy for clearance have been compromised by disease proteins. For example, certain tau isoform has been shown to bind the lysosomal membrane rather than enter the lysosome for degradation (35). In this context, autophagy induction seems to deliver more tau fragments to the lysosome and promote the formation of tau oligomers at the surface of these organelles. Also notably, biochemical experiment implies that Aβ is generated not only in the ER and Golgi compartments but also in autophagosomes, as purified autophagic vacuoles contain both βAPP and highly activated γ-secretase, the protease cleaving βAPP to Aβ (11). Moreover, autophagy is illustrated to influence Aβ secretion *in vivo* in βAPP transgenic mice, where autophagy deficiency reduces extracellular Aβ plaque burden and leads to aberrant intra-neuronal Aβ accumulation, contrary to what may be expected if autophagy only cleaned Aβ (134).

Overall, autophagy responses are generally viewed as neuroprotective, and stimulating the induction of autophagy has therapeutically received great attention. Although consequences of pharmacological modulation of autophagy are still beyond our current knowledge, in specific neurodegenerative disorders where autophagic clearance mechanisms are well-understood, further promotion of autophagy might be the best interventional strategy so far.

3.4 Target of rapamycin (TOR)

The evolutionarily conserved protein kinase TOR has garnered significant attention for its role in neurological diseases. Biochemical purification of TOR-associated proteins has revealed that TOR is present in two complexes, TORC1 and TORC2, with distinct sets of binding partners (135). The two complexes coordinately regulate fundamental cellular behaviors, such as protein synthesis, cytoskeletal organization, cell metabolism, cell proliferation and survival. Compared to TORC1, less is known about TORC2, part of whose function is believed to impact TORC1 through positive and negative feedback mechanisms (136). As such, we will only review the linkage between TORC1 and NDs in this section.

TORC1 and its downstream pathways have been intensively shown to be altered in a variety of neurodegenerations, but the data appear to be extremely conflicting. First of all, TORC1 is a negative regulator of autophagy in response to growth factors, amino acids and cellular energy (137, 138). When TORC1 activity is high, Atg13 undergoes TOR-relied phosphorylation, which blocks autophagosome formation (139). In this circumstance, beneficial effects of removing pathogenic proteins were obtained when using the TOR inhibitor, rapamycin, to induce autophagy in ND models (140). In the case of tauopathies, rapamycin also suppresses TOR-mediated phosphorylation of S6K (ribosomal protein

S6 kinase), and in turn inhibits S6K-catalyzed hyper-phosphorylation of tau, which may foster the conversion of tau into its pathogenic form (141). On the other hand, loss of TORC1 signaling has been implied to impair synaptic plasticity and memory storage in animal models of AD, which can be restored through upregulation of TORC1 activity (142). This is most likely because of the function of TORC1 to modulate protein synthesis required for memory consolidation, given that altered translational control has a vital role in memory and cognitive decline (143). Two well-characterized substrates of TORC1 are involved in the initiation of cap-dependent translation of mRNA: 4E-BP (eIF4E-binding protein) and S6K. Phosphorylation of 4E-BP by TORC1 leads to its dissociation from eIF4E and allows the assembly of the translation preinitiation complex (144). S6K, as mentioned earlier, actually is best known for its ability to phosphorylate 40S ribosomal protein S6 and eIF4B, which enhances the association of eIF4B with the translation preinitiation complex (145). Additionally, TORC1 is also a key mediator of ribosome biogenesis, essential for cell growth and survival (146). Taken together, it is reasonable as well that a decrease in TORC1 activity appears to be harmful and correlate with the progression of ND in clinical patients.

How to explain the discrepancy of TORC1 in degenerative disorders? To some extent, there is a chicken-and-egg scenario here: it is difficult to determine whether alteration of TORC1 signaling emerges first, then contributing to neurodegeneration, or whether activity of TORC1 is adjusted by the cell as a secondary consequence, struggling to survive in response to an existing pathological condition. The two models apparently will lead to opposite outcomes, and current information seems to support both in different physiological contexts of NDs. Alternatively, as speculated from "Norambuena A, et al. 2018" and "Polanco JC and Götz J. 2018", it is where TORC1 is functioning that matters, rather than whether it is up or down (147, 148). In fact, TORC1 has been detected in multiple subcellular compartments, not only in the nucleus, cytoplasm and Golgi, but also located on vacuoles/lysosomes and plasma membrane (149, 150). How the subcellular distribution of TORC1 affects specific cellular responses remains an open question. However, Norambuena A, et al. 2018 found that in the early stages of AD, oligomeric Aβ would abrogate lysosome-localized TORC1 function by an activation of TORC1 at the plasma membrane, where tau is phosphorylated in a TORC1-dependent manner (147). In light of this, subcellular localization may be an important principle used in AD to enact precise spatial and temporal control of TORC1. It will be intriguing to further investigate whether it also holds true for other degenerative diseases.

3.5 Insulin/insulin-like growth factor (IGF) signaling

The mammalian brain has a high demand for energy. Despite representing only 2% of the total body mass, the brain consumes approximately 25% of the glucose and oxygen used by the body (151). As a matter of fact, nearly all neurodegenerations have been corroborated to exhibit a crucial metabolic dysfunction that includes altered glucose uptake/utilization and disrupted mitochondrial activity. The insulin/IGF signaling responsive to systemic hormonal cues is the main regulatory network controlling energy metabolism and longevity in multicellular animals (152, 153). Insulin and IGFs, closely related in terms of biological activity, are primarily secreted from different organs, yet both are also locally synthesized in the brain (154). Insulin resistance takes place when cellular responsiveness to insulin/IGFs is compromised, leading to a disturbance in glucose metabolism and energy balance. Strong evidence has underscored that type 2 diabetes and midlife obesity associated with insulin resistance are risk factors for development of dementia, PD, AD and HD (155-158).

However, contradiction appears in literatures when this comes to the level of molecular and cell biology. While some studies reported reduced expression of insulin, IGFs and their receptors in brains of AD and PD by quantitative RT-PCR, more tried to prove elevated insulin/IGFs in the serum and cerebrospinal fluid of patients with neurological diseases, including AD and PD (159-162). What is more controversial is that positive effects have been observed either by decreasing insulin/IGF signaling or by administration of agonists of insulin and IGF-1 in preclinical models (163, 164). Interestingly, an *in vitro* assay has showed that Aβ in AD is a direct competitive inhibitor on insulin binding to its receptor, indicating insulin resistance perhaps is not simply resulting from the changed amount of pathway components (165). Alternatively, the inconsistency might come from the time point chosen for investigation during the whole disease course. As indicated by a survey based on 3,139 participants for up to 10 years in Rotterdam of Netherlands, the interconnection between insulin metabolism and the clinical manifestation of ND does exist but seems not constant over time (166).

In mammals, both insulin and insulin-like growth factors (IGFs) activate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway through their respective receptors. The protein kinase AKT is recruited to the plasma membrane via phosphatidylinositol-triphosphate (PIP3), which is generated through phosphorylation of PI-4,5-P2 by PI3K. Membrane-recruited AKT then is activated and phosphorylated successively by PDK1 and by TORC2 (153). By monitoring the level of AKT phosphorylation, AKT activity has been implied to be important for neuronal survival and usually is low when cell is insulin resistant (167). Additionally, it has been shown that AKT is able to negatively interact with several pathogenic proteins via different mechanisms, complicating the regulation of AKT in NDs (168). Anyway, a chicken-and-egg analogy could also be used to summarize the interplay between neurodegenerations and AKT, similar to the situations with TORC1.

AKT has a couple of downstream effectors, including TORC1 and FoxOs, the Forkhead box class O transcription factors. Through AKT, TORC1 integrates

information about growth factor signals and nutritional status to adjust cellular proteostasis in conditions such as NDs, which has been discussed in the previous section. In addition to TORC1, AKT also mediates the phosphorylation of FoxO and creates binding sites for 14-3-3 proteins, which promotes the retention of FoxO in the cytoplasm, thereby lowering its activity in the nucleus (169). The evolutionarily conserved FoxO transcription factors are well-known to modulate the expression of genes involved in cell survival, stress response, metabolism and longevity (170). Mammals have four FoxO genes, FoxO1, 3, 4 and 6, which are expressed in the nervous system at different levels with high similarity in their function and regulation (171, 172). The expression of FoxO overall is increasing progressively in aging human and mouse brains. In mice, nervous system specific FoxO1/3/4 loss-of-function accelerates aging-related degeneration followed by motor dysfunction (173). By contrast, overexpression of a constitutively active FoxO3 has pro-apoptotic effects leading to neuronal loss, suggesting that fine-tuning FoxO level is of some importance to neurons. Intriguingly, inhibition of FoxO3 by expressing a dominant negative competitor is absolutely protective when a pathogenic α-syn is co-expressed to induce a disorder condition, highlighting FoxO as a potential target for ameliorating the cytotoxicity of misfolded pathogenic proteins of NDs (174).

4 Conclusions

Unlike other cells in an organism, mature neurons cannot divide and usually have large expanses of dendritic and axonal cytoplasm. They consequently face particular hurdles in preventing cellular waste and misfolded proteins from accumulating over a lifetime without the aid of cell division to dilute these burdens. Young neurons achieve this task by efficient stress response and clearing systems supported by robust cellular signaling transductions. In comparison, the stereotypic neuronal connections in the elderly allow transformation and accumulation of specific proteins, such as Aβ, tau, α-syn and prion, easily within the nervous system. In respect to this, aged brain quite often is the organ affected most severely under conditions of NDs with altered activities of pathways in proteostasis (**Figure 1**). Although it is still uncertain whether the abnormal pathway activities implicate a primary cause or secondary consequence, the current chicken-and-egg debates concerning this issue, as outlined above, definitely will provide in-depth understandings of NDs, as well as a fruitful source of knowledge for therapeutics to treat these brain symptoms in the future.

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Conflict of interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

High intensity focused ultrasound enhances anti-tumor immunity in melanoma through promoting CD4 Th1 effector T cell responses

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ABSTRACT

Background: Melanoma accounts for more than 80% of deaths from all dermatologic cancers, mainly due to its widespread metastasis. High intensity focused ultrasound (HIFU) is a promising technique for cancer therapy. Here, we investigated the efficacy of HIFU against melanoma and the underlying mechanisms.

Methods: A melanoma allograft mouse model was established to examine the tumor progression and survival rate. Anti-tumor immunity was determined by measuring cytokines, regulatory T cells (Tregs), Th17 cells and CD8+ effector T cells. Western blot, qPCR, RNAi and luciferase assay were performed to confirm the expression and regulation of microRNA (miR)-9-5p and transforming growth factor beta (TGF-β).

Results: HIFU exposure significantly suppressed melanoma growth and metastasis by activating interferon gamma (IFN-γ) secretion, inhibiting Tregs and Th17 cells, and stimulating CD8+ effector T cells. TGF-β was a direct target of miR-9-5p. The anti-tumor effect of HIFU might be mediated through the miR-9-5p/TGF-β pathway.

Conclusion: HIFU activates anti-tumor response and alters tumor microenvironment, which may serve as a potential therapeutic strategy for melanoma treatment.

Keywords: Melanoma · HIFU · Antitumor immunity · TGF-β · miR-9-5p

Introduction

Melanoma develops from the melanocytes and is the most malignant type of skin cancer. Although melanoma accounts for only 4% of skin cancers, it causes more than 80% of deaths from all dermatologic cancers (1, 2). In melanoma patients, the major cause of death is widespread metastasis (3). Melanoma can spread through the lymphatic and/or vascular system to the liver, brain, lung, bone, breast, colon and subcutaneous tissue even at the early stages (3, 4). Therefore, investigation and the development of effective therapy to inhibit the growth and metastasis have crucial importance in melanoma treatment.

High intensity focused ultrasound (HIFU), also known as focused ultrasound surgery, is a non-invasive therapeutic technique for localized treatment of tumors, which exhibits thermal and mechanical effects: the former one induces cancer cell destruction through coagulation necrosis, while the latter one generates radiation force, cavitation and micro-streaming on tumor tissues (5, 6). HIFU has been used to treat various tumors and improve the prognosis of cancer patients (6-11). Uchida *et al.* reported that the HIFU therapy improved prostate cancer outcomes, and the 10-year survival rate reached 89.6% among 918 patients (11).Breast cancer is an ideal target for HIFU treatment due to its superficial position.The

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complete ablation rate of HIFU therapy reached up to 71% in 173 breast cancer patients (10). HIFU treatment also improved the survival outcome of unresectable liver cancer patients, where out of 49 hepatocellular carcinoma (HCC) patients, 39 (79.5%) had primary effectiveness and only 4 (8.1%) had complications (9). In addition, HIFU exposure was also employed for treatment of kidney cancer, and applied as the palliative treatment for pancreatic cancer (7, 8). All these reports have indicated that HIFU is a promising strategy for the treatment of various cancers.

Although HIFU exposure has been used for the treatment of multiple cancers, the underlying molecular mechanisms are not yet well understood. Cumulative evidence has suggested that cell death and tissue damage during HIFU are closely linked to immune response, which is induced by the cell debris after cancer cell destruction, as well as tumor microenvironment alteration such as the changes of T cell subsets (12-14). Xia *et al*. reported the anti-tumor immune responses of HIFU exposure in HCC mouse model, the authors demonstrated that 14 days of treatment could significantly promote the secretion of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), enhance the cytotoxicity of cytotoxic T lymphocytes (CTL), and increase the number of CD8+ effector T cells (15). The dendritic cells (DC) were also found activated after HIFU exposure in a colorectal cancer mouse model: there was about 4-fold increase in CD11c+ cells and more than 5-fold CFSE+ DC accumulation in lymph nodes after HIFU exposure, accompanying with enhanced CTL activity and increased IFN-γ production (12). However, the therapeutic effect of HIFU exposure on melanoma treatment and the underlying mechanism is poorly defined.

In the current study, we demonstrated that HIFU exposure could significantly suppress melanoma growth and metastasis through activating the anti-tumor immunity and altering the tumor microenvironment in mouse and cell models. We further revealed that HIFU-induced decrease in regulatory T cells (Tregs) and Th17 cells was regulated by the microRNA (miR)-9-5p/transforming growth factor beta (TGF-β) pathway. Notably, HIFU exposure also increased the number of CD8+ effector T cells in melanoma tissues. This study may pave the way for the clinical application of HIFU in melanoma treatment.

Materials and Methods

Animals

6-8 weeks old C57BL/6J mice were employed in the current study, which were ordered from Shanghai Laboratory Animal Center (China). The mice were housed under standard breading conditions with 12 light/12 dark cycle, at 23ºC with 40-60% humidity, standard chow diet feeding, with water accessible at all times. All mouse experiments have been approved by the Shanghai Jiaotong University Affiliated Sixth People's Hospital East Campus.

Cell culture

The murine skin melanoma cell line B16-F10 (CRL-6475™) was acquired from American Type Culture Collection (Manassas, USA). B16-F10 cells were cultured in PRMI-1640 medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were placed in 5% CO2 cell culture incubator at 37 °C.

Melanoma xenograft model

200 μL single-cell suspension of B16-F10 melanoma cells (3×10^5) was implanted subcutaneously into the left flank of mice. Tumor size (width × length; mm^2) was determined with a caliper every 2 days. Tumor volume was calculated as previously described (16).

HIFU treatment

All experimental mice were randomly divided into sham-HIFU and HIFU groups when the diameter of larger tumor reached 7 to 8 mm. The HY2900 HIFU tumor therapy system (Haying Tech., Wuxi, China) was used for HIFU ablation in this study. The mice were anaesthetized using ketamine (2 mL/kg) through intravenous injection. After anesthesia, the skin on top of the tumor nodule area was shaved and layed with ultrasound transmission gel. The mice of HIFU group were treated with 4.5 W and 9.2 MHz ultrasound. Treatment was performed point by point, and started at the center of the nodule with 6 mm therapy depth. Each point was treated for 1 min, with 1 sec pulse duration, and 5 sec exposure separation. The procedures of sham-HIFU group were similar to HIFU group with no HIFU exposure.

Pulmonary metastasis assay

The mice bearing primary tumors were intravenously injected with B16-F10 cells at 7 days after HIFU treatment and monitored until death. The volume of melanoma tumor, number of pulmonary metastasis and cumulative survival rate were measured and recorded. The health mice without melanoma xenograft were used as the normal control group in this study.

Histological analysis

The tumor modules were excised from flank skin and fixed in 4% paraformaldehyde overnight and then paraffin embedding, sectioning, and hematoxylin eosin (H&E) staining were performed as previously described (17).

Enzyme-linked immunosorbent assay (ELISA)

The blood was collected from normal, sham-HIFU and HIFU mice after 14 days of treatment, centrifuged at 5000 g for 6 min. The plasma concentrations of IFN-γ, TNF-α, interleukin (IL)-6, and TGF-β were determined by using the Beyotime Biotechnology (Shanghai, China) specific ELISA kits: mouse IFNγ ELISA kit (PI508), mouse TNF-α ELISA kit (PT512), mouse IL-6 ELISA kit (PI326), and mouse/rat TGF-β ELISA kit (PT878), following the

PerkinElmer, Singapore). The cell viability of each well was normalized to that of the control. All cytotoxicity assays were conducted in triplicates (six wells per sample for each time point). Viability of the NSCs after treatments of various CM was determined using the MTT assay as well as LIVE/DEAD viability/cytotoxicity kit (Invitrogen, USA).

Flow cytometry analysis

The tumor nodules were placed individually in 0.1% collagenase type IV solution (Thermo Fisher Scientific, Waltham, USA), cut into small pieces and incubated at 37 °C for 30 min. The digested tumor tissue was filtered through a 40 μm cell strainer and then centrifuged at 500 g for 10 min at 4°C. The pellet was washed twice and then resuspended to acquire single melanoma tumor cell suspension. Tregs were identified by Foxp3+ (#56-5773-80, Invitrogen, Carlsbad, USA), CD4+ (#100405, BioLegend, San Diego, USA) and CD45+ (#147716, BioLegend, San Diego, USA). Th17 cells were identified by CD4+ and IL-17+ (#506903, BioLegend, San Diego, USA). CD8+ tumor infiltrating T lymphocytes were identified by CD45+ and CD8-APC (#100734, BioLegend, San Diego, USA).

The microRNA (miRNA, miR) prediction

The prediction of miRNAs which could bind to TGF-β was performed by using TargetScan (http://www.targetscan.org/vert_72/) and ENCORI (http://starbase.sysu.edu.cn/) online package and database, following the instructions.

Transfection and RNAi

B16-F10 cells (60% confluence) were transfected with 20 nM miR-9-5p, miR-9-5p negative control (NC), anti-miRNA oligonucleotide (AMO)-miR-9-5p or negative control miRNA using the Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, USA), following the manufacturer's instruction. The cells were harvested 48 h after transfection for mRNA and protein analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was prepared from melanoma tumor tissues or B16-F10 cells using the TRIzol kit (Invitrogen, Carlsbad, USA). 1 μg total RNA was transcribed reversely into cDNA using the M-MLV Reverse Transcriptase (Thermo Fisher Scientific). The qRT-PCR was performed using the CFX Real-Time PCR Detection System (Bio-Rad, Hercules, USA) with the SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, USA). Target gene expression level was normalized using the $2^{-\Delta\Delta CT}$ method with GAPDH as the internal control. Primers for the RT-qPCR were: TGF-β, forward 5'-ACT GGA GTT GTA CGG CAG TG-3', reverse 5'-GGC TGA TCC CGT TGA TTT CC-3'; GAPDH, forward 5'-CGC TCT CTG CTC CTC CTG TT-3', reverse 5'-CCA TGG TGT CTG AGC GAT GT-3'.

Immunoblotting analysis

B16-F10 cells or frozen melanoma tumor tissues were homogenized and lysed using the radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, USA). Samples were detected using Western blotting as described previously (18). The primary antibodies of TGF-β (ab92486, 1:1000 dilution) and GAPDH (EPR16891, 1:2000 dilution) were ordered from Abcam (Cambridge, MA, USA).

Luciferase reporter assay

B16-F10 cells were cultured in 12-well plates at the density of 6×10^4 cells/per well 24 h before transfection. The TGF-β 3'UTR-WT or TGFβ 3'UTR-mut reporter was co-transfected with pRLSV40 into the B16-F10 cells. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA). The results were normalized by the value of Renilla activity and presented as fold change to the control group.

Statistical analysis

Statistical analysis was performed using Prism 8 software. Student's t test, one-way or two-way analysis of variance (ANOVA) were used to analyze the differences between groups. Data were shown as mean ± standard deviation (SD).

Results

HIFU exposure suppresses melanoma tumor progression

The melanoma tumor bearing mice were administrated with sham-HIFU or HIFU, respectively, H&E staining result showed that the cell proliferation of melanoma in HIFU exposure mice was slowed down compared to that in the sham group mice (**Figure 1A**). Notably, HIFU exposure significantly inhibited the primary tumor growth. 22 days after B16-F10 cell subcutaneous injection, tumor volume in mice of the sham-HIFU group were 2-4-fold larger than that in the HIFU-treated mice (**Figure 1B**). We further investigated the pulmonary metastasis in mice with primary tumors after B16-F10 cell intravenous injection, as shown in **Figure 1C**, HIFU exposure markedly reduced the number of tumor nodules in the lung compared to the sham-HIFU treatment (19 vs 34, $P < 0.01$). Moreover, the cumulative survival rate showed that HIFU exposure mice had much longer survival rate than mice in the sham control group (**Figure 1D**, $P < 0.05$). These results indicated that HIFU exposure displayed promising anti-tumor effects on inhibiting tumor growth, attenuating pulmonary metastasis and improving host survival in mouse melanoma model.

HIFU exposure improves the anti-tumor immune response

Immune response was detected in HIFU and sham-HIFU treated mice by measuring serum concentrations of tumor

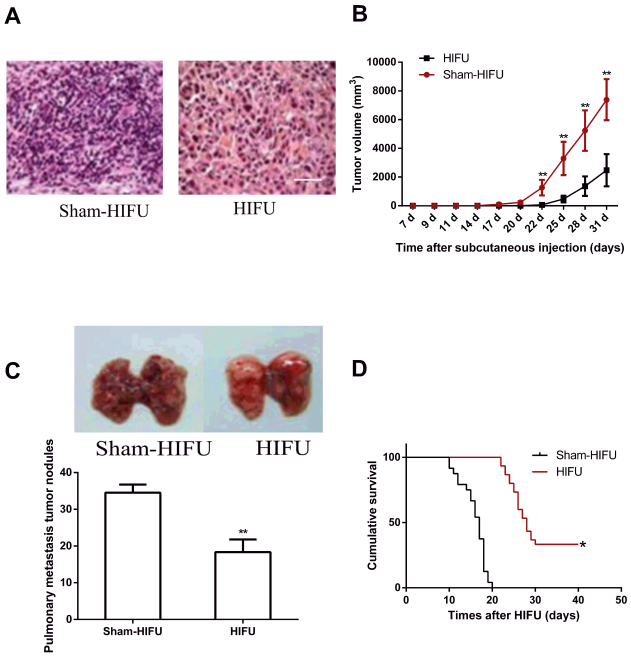

Figure 1. HIFU exposure inhibited melanoma growth and pulmonary metastasis. (A) H&E staining of tumor tissues 11 days after subcutaneous injection (immediately after HIFU treatment) Scale bar, 20 μm (n=6). (B) The average tumor volumes of HIFU and sham-HIFU mice were plotted (n=6). (C) Pulmonary metastasis tumor nodules were counted macroscopically after natural death (n=10). (D) The cumulative survival rate was analyzed by log rank test (n=30). *P < 0.05, **P < 0.01 compared to sham-HIFU mice.

microenvironment-associated cytokines, including IFN-γ, TNF-α, IL-6 and TGF-β. Pleiotropic molecule IFN-γ has been reported to possess the anti-proliferative, pro-apoptotic and anti-tumor abilities (19). As shown in **Figure 2A**, the concentration of IFN-γ in the serum of HIFU exposure mice was 2-fold higher than that in the sham-HIFU mice (68.3 pg/mL vs 32.7 pg/mL). Two pro-inflammatory cytokines, TNF-α and IL-6, were relatively higher in melanoma mice compared to normal mice. However, there was no difference between HIFU and sham-HIFU mice (**Figure 2B** and **2C**). The regulatory cytokine TGF-β is an important enforcer of immune tolerance, and tumors that secrete high levels of TGF-β may escape from immune surveillance (20). Here, we found that the TGF-β serum level was significantly decreased in HIFU treated mice compared to the sham control mice (**Figure 2D**). These data suggested the HIFU exposure might promote anti-cancer immunity via modulating cytokine secretion.

HIFU exposure inhibits Tregs and Th17 cells, and activates CD8+ tumor infiltrating lymphocytes (TILs)

To further investigate the anti-tumor immunity improvements after HIFU exposure, we detected the subset of CD4+ and CD8+ T cells using flow cytometric analysis. Tregs (Foxp3+CD4+/CD45+), the immuno-suppressive subset of CD4+ T cells, was significantly increased in melanoma mice compared to normal control (1.19% vs 3.23%). Noticeably, in comparison with mice with sham-HIFU treatment, Treg population in HIFU-treated mice was significantly decreased (**Figure 3**). Th17 cells also increase tumor progression by activating angiogenesis and immunosuppressive activities (21). In line with the results of Tregs, the Th17 cell population (CD4+/IL-17+) in HIFU exposure mice was 2.49%, which was markedly lower than that in sham-HIFU mice (4.47%, **Figure 4**). CD8+ TILs have critical tumor suppressive roles. As shown in **Figure 5**, there was almost 3-fold increase in the CD8+ (CD45+/CD8+) population in HIFU exposure mice compared to sham-HIFU mice (17.59% vs 6.18%). All these flow cytometric data indicated that HIFU exposure suppressed the immune tolerance and improved tumoricidal effector response in tumor tissues.

HIFU exposure promotes anti-tumor immunity through attenuating TGF-β expression in melanoma tissues

Since TGF-β plays a very important role in promoting the generation and differentiation of Tregs and Th17 subsets from naïve CD4+ T cells (22). We searched the candidate regulators of TGF-β in StarBase/ENCORI database and identified miR-9-5p, which was inhibited in melanoma tumor tissues and restored by HIFU treatment (**Figure 6A**). Then we tested the TGF-β expression in mouse melanoma samples with or without HIFU exposure. Both the mRNA and protein levels of TGF-β were significantly increased in melanoma tissues, notably, the up-regulated TGF-β expression was markedly inhibited by HIFU treatment (**Figure 6B-6D**). These results suggested that the anti-tumor effect of HIFU exposure might be mediated by the miR-9-5p/TGF-β pathway.

MiR-9-5p mediates HIFU-induced TGF-β down-regulation in melanoma cells

In order to investigate the mechanism of miR-9-5p-

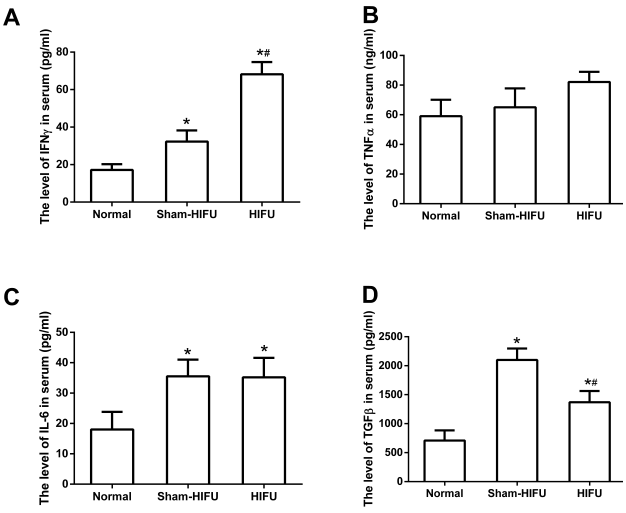

Figure 2. HIFU treatment enhanced antitumor immune response. IFN-γ (A), TNF-α (B), IL-6 (C) and TGF-β (D) in mice serum were analyzed by ELISA. *P < 0.05 as compared with normal, #P < 0.05 as compared with sham-HIFU (n=6).

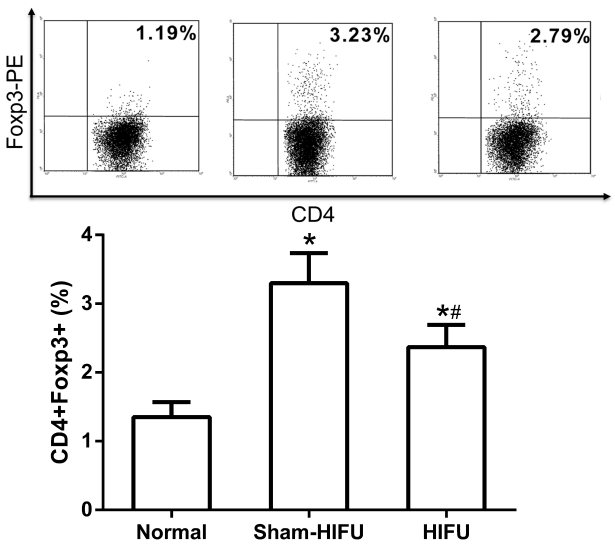

Figure 3. Flow cytometric analysis of Foxp3+CD4+/CD45+ Tregs in indicated groups (14 days after HIFU exposure). *P < 0.05 as compared with normal, #P < 0.05 as compared with sham-HIFU (n=6).

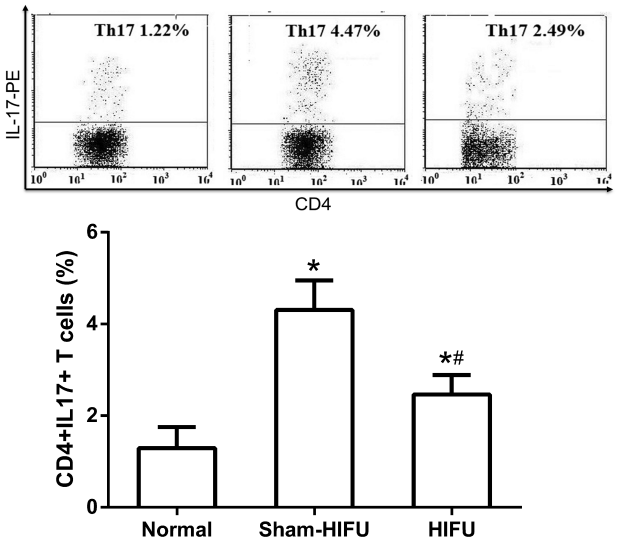

Figure 4. Flow cytometric analysis of CD4+/IL-17+ Th17 cells in indicated groups (14 days after HIFU exposure). *P < 0.05 as compared with normal, #P < 0.05 as compared with sham-HIFU (n=6).

mediated TGF-β regulation in melanoma, we performed miR-9-5p gain-of-function, loss-of-function, and promoter binding tests using RNAi and dual-luciferase reporter assay in B16-F10 cells. As shown in **Figure 7A**, in comparison with the control group, miR-9-5p overexpression could significantly reduce the mRNA level of TGF-β in B16-F10 cells. In contrast, the TGF-β expression level was dramatically increased when miR-9-5p was blocked by AMO-miR-9-5p (**Figure 7A**). The same regulatory pattern was observed at the translational level, where miR-9-5p gain-of-function decreased TGF-β protein level, while miR-9-5p loss-of-function significantly increased TGF-β protein level (**Figure 7B** and **7C**). The sequence analysis result showed that miR-9-5p was highly complementary with the TGF-β 3'UTR (**Figure 7D**). To further investigate whether miR-9-5p could directly bind to TGF-β promoter, we constructed the luciferase reporter using TGF-β 3'UTR-wt region and mutated 3'UTR sequence (TGF-β 3'UTR-mut). We found that miR-9-5p could significantly reduce the luciferase activity derived by TGF-β 3'UTR-wt instead of TGF-β 3'UTR-mut. These data indicated that TGF-β was a target of miR-9-5p that mediated the HIFU-induced TGF-β down-regulation, and then promoted anti-tumor immunity in melanoma.

Discussion

HIFU is a promising method for the non-invasive ablation of various tumors (6, 13). In this study, we applied the HIFU technique for melanoma treatment and demonstrated that HIFU exposure suppressed the proliferation and growth of the primary tumors, inhibited pulmonary metastasis, and improved the cumulative survival rate of melanoma mice. HIFU treatment not only activates immune response including IFN-γ up-regulation and TGF-β down-regulation but also changes T cell subsets in tumor microenvironments, such as suppression of Tregs and Th17 cells and activation of CD8+ effector T cells. In line with our findings, Liu *et al.* reported that HIFU treatment significantly stimulated DC maturation and tumor infiltration surrounding the thermal lesion compared to the control in the MC-38 and B16 tumor models (23). Moreover, the enhanced cytotoxic T lymphocyte activity and elevated IFN-γ secretion accompanying the expansion of DCs in lymph nodes were observed by another research group who performed HIFU

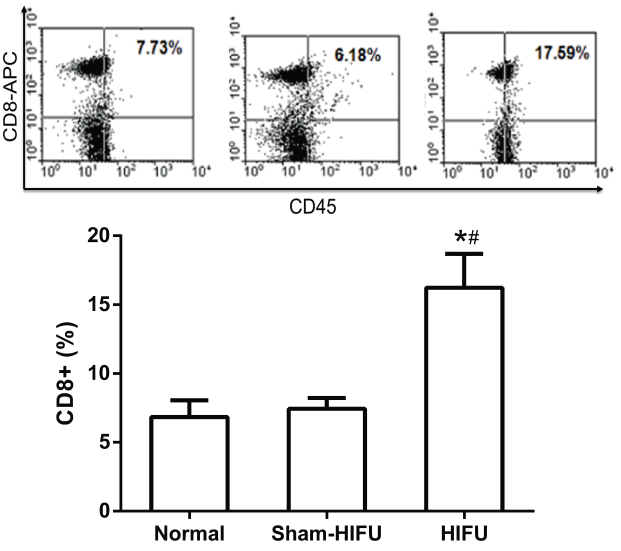

Figure 5. Flow cytometric analysis of CD8+ TILs in indicated groups (14 days after HIFU exposure). *P < 0.05 as compared with normal, #P < 0.05 as compared with sham-HIFU (n=6).

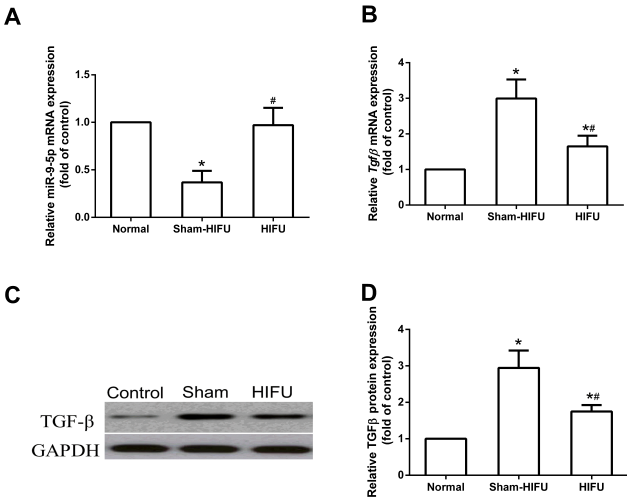

Figure 6. HIFU treatment caused the differential expressions of miR-9-5p and TGF-β. A-B. The levels of miR-9-5p (A) and TGF-β (B) in tumor tissues were detected by qPCR. (C-D) The levels of protein of TGF-β in tumor tissues were detected by Western blot. *P < 0.05 as compared with normal, #P < 0.05 as compared with sham-HIFU (n=6).

treatment on colon adenocarcinoma tumors (12). Based on the above findings, the activation of anti-tumor immunity might be one of the major advantages of HIFU treatment.

The T cell subset alteration after HIFU exposure is another important beneficial effect of HIFU treatment. CD8+ T cells are able to differentiate into cytotoxic T lymphocytes upon the activation of antigen-presenting cells, which then exert an efficient anti-tumor attack (24). However, Tregs play essential roles in maintaining the immune system self-tolerance and immunosuppression, thus promote tumor development and progression (25). In addition, Th17 cells are a double-edged sword in tumor immunity: on the one hand Th17 cells promote tumor progression through increasing angiogenesis and immunosuppression, while on the other hand Th17 cells induce the anti-tumor immune response by improving effector T cell filtration and IFN-γ secretion (22). In the current study, we found that HIFU exposure significantly reduced the population of Tregs and Th17 cells, while increased CD8+ effector T cells in melanoma tumor tissues. Meanwhile, alteration in other subsets of immune cells has been observed by previous studies. Fifteen pancreatic cancer patients were treated by HIFU exposure in a clinical trial, and the authors found the CD4+, CD4+/CD8+ and natural killer (NK) cells were increased in the serum of 10 patients, and NK cell activity was dramatically increased after HIFU treatment (26). Similarly, in a HCC cell vaccine study, the researchers found the cytotoxicity of cytotoxic T lymphocytes was highly increased after HIFU exposure, and these vaccines displayed stronger anti-tumor ability compared to non-HIFU ones (27). All these results suggest that HIFU treatment could induce systemic change in the tumor microenvironment, which consequently improves anti-tumor immunity.

MiRNAs play important roles in T lymphocyte differentiation and maturation, as well as immune response and immune tolerance (28). TGF-β promotes the CD4+ derived Treg differentiation, which mediates immunosuppression in various tumors (25, 29). Here, we have found that TGF-β is a target of miR-9-5p, the expression of which is significantly up-regulated by HIFU exposure in melanoma tissues. Luciferase reporter assay has confirmed that miR-9-5p is able to bind to TGF-β 3'UTR directly. Overexpression of miR-9-5p could inhibit TGF-β expression on both mRNA and protein levels in melanoma cells. A recent study showed that miR-134 targeted CD86, a T lymphocyte activation antigen, in mouse melanoma cells. HIFU exposure dramatically inhibited miR-134, then enhanced CD86 expression, which could promote the secretion of IFN-γ and TNF-α, and improve anti-tumor immunity in the melanoma allograft mouse model (16). In line with these results, Li *et al*. examined the therapeutic effect of HIFU on colorectal cancer metastasis and found that HIFU exposure increased the expression of miR-124, which targeted STAT3, a transcription factor overexpressed and activated in various cancer cells and tumor-associated immune cells, and then inhibited invasion and metastasis of colon cancer cells (30). The *in vitro* regulation of miR-9-5p/TGF-β pathway in melanoma cells has been demonstrated by this study, but whether they also play key regulatory roles in anti-tumor immunity *in vivo* is still unknown. Future work will further validate the *in vivo* regulation of HIFU-mediated miR-9-5p/TGF-β pathway in melanoma treatment.

In the current study, we have demonstrated that HIFU

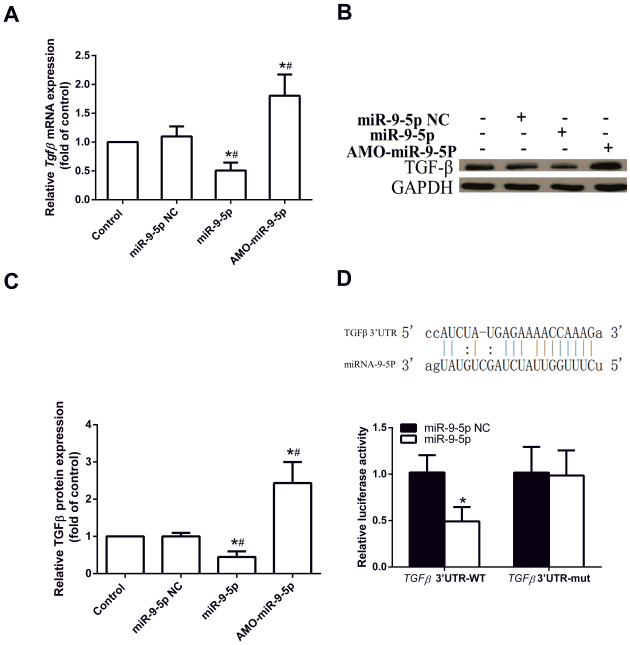

Figure 7. TGF-β was a target of miR-9-5p in melanoma cells. Delivery of miRNA-9-5p significantly decreased the TGF-β mRNA (A) and protein (B and C) expression levels in B16-F10 cells. (D) MiR-9-5p targeting TGF-β was validated by dual-luciferase reporter assay. *P<0.05 vs control, #P<0.05 vs miR-9-5p-NC (n=6).

exposure could suppress melanoma growth and metastasis in mouse and cell models. HIFU treatment activates anti-tumor immune response, inhibits Tregs and Th17 cells, and increases CD8+ effector T cells in melanoma. We also reveal that TGF-β is a direct target of miR-9-5p. Mechanistically, the anti-tumor effect of HIFU might be mediated by up-regulating miR-9-5p and then suppressing the expression of TGF-β in melanoma cells.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Funding

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